Factors Affecting the Disposition of Ivermectin in the Target Species

door

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Factors Affecting the Disposition of Ivermectin in the Target Species

Factoren die de lotgevallen van Ivermectine in doeldierspecies bepalen

(Met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het college voor promoties, in het openbaar te verdedigen op woensdag 4 december des namiddags te 2.30 uur

door

Céline Marielle Laffont

geboren op 4 May 1975 te Toulouse (Frankrijk)

Promotores: Prof. Dr. J. Fink-Gremmels
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Co-promotor: Dr. A. Bousquet-Mélou

The experiments described in this thesis were performed at the department of Veterinary Pharmacology, Pharmacy and Toxicology, Utrecht University, The Netherlands, and at the laboratory of Physiology and Therapeutics (UMR associée INRA), National Veterinary School of Toulouse, France.

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CONTENTS

Abbreviations

b.wt.	Body weight
i.a.	Intra-arterial
i.r.	Intraruminal
i.v.	Intravenous
p.o.	*Per os*
s.c.	Subcutaneous

CHAPTER 1

General Introduction

A) GENERAL PRESENTATION OF IVERMECTIN.

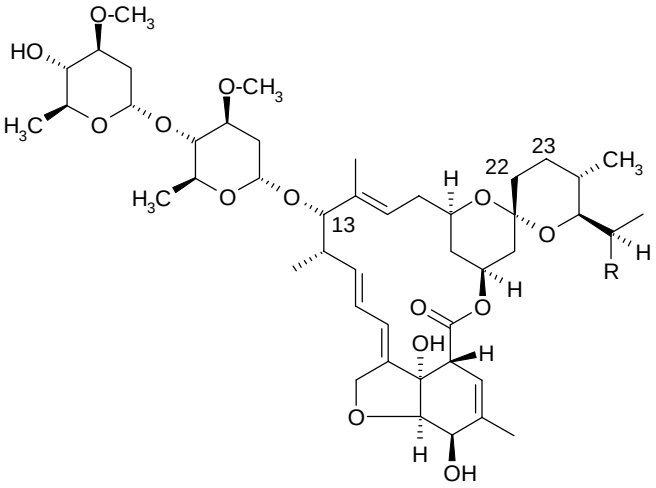
The discovery of avermectins, and to a lesser extent milbemycins, has revolutionised antiparasitic treatment in animal health and crop protection. These compounds are at least 25 times more potent than the preceding generation of anthelmintics (for example, oxfendazole: 5 mg/kg; ivermectin: 200 µg/kg), and present a broad spectrum of activity against nematode and arthropod parasites of domestic animals. It is this unique combined killing of endo- and ectoparasites which gave rise to the embracing name of "endectocides", under which they are now classified.

Discovered by Merck Sharp & Dohme laboratories, ivermectin was the first avermectin to be introduced onto the international animal health market in 1981. Within a period of 5 years, this drug had captured 16% of the worldwide sales for antiparasitic compounds in animal health (Bloomfield, 1988). Since then, several other endectocides (moxidectin, doramectin, eprinomectin, selamectin) have been commercialised, but ivermectin still remains the most widely used endectocide across animal species worldwide, with millions of cattle treated per year. Ivermectin is also currently used in human medicine to treat and control various parasitic conditions. It is the first-line treatment for onchocerciasis (river blindness), a severe public disease in Africa and tropical America, and has been approved for treatment of lymphatic filariasis, intestinal strongyloidiasis and scabies. More than 18 million people are estimated treated with this drug every year (Burkhart, 2000).

1) Chemistry.

Avermectins are closely related 16-membered macrocyclic lactones derived from the fermentation products of the actinomycete *Streptomyces avermitilis* (Burg and Stapley, 1989; Fisher and Mrozik, 1989). Although they share structural features with the macrolide antibiotics and antifungal macrocyclic polyenes, they are virtually devoid of any antibacterial or antifungal activity (Burg and Stapley, 1989). The milbemycins are structurally related to the avermectin group, but are lacking the disaccharide substituent at C-13 (see Fig. 1) (Fisher and Mrozik, 1989; Shoop et al., 1995a). Ivermectin is the generic name given to a mixture of 22,23-dihydroavermectin B1a (H_2B_{1a}; >80%) and 22,23-dihydroavermectin B1b (H_2B_{1b}; <20%), which are two equipotent semisynthetic derivatives of the avermectin family (Fig. 1) (Campbell, 1985; Fisher and Mrozik, 1989). Ivermectin is a large (874 MW) and neutral molecule, which is poorly soluble in water, dissolves in

a)



b)

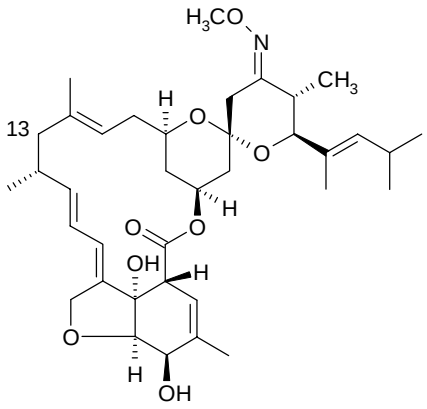


Figure 1. a) Chemical structure of ivermectin. R = CH_2CH_3 : 22,23-dihydroavermectin B_{1a} (>80%). R = CH_3 : 22,23-dihydroavermectin B_{1b} (<20%). **b)** Chemical structure of the milbemycin moxidectin.

most organic solvents and is highly soluble in lipids (Lo et al., 1985; Fisher and Mrozik, 1989). Its fluorescence properties are particularly useful for analytic determination by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) (Alvinerie et al., 1987; Fisher and Mrozik, 1989).

2) Existing pharmaceutical formulations.

Ivermectin is formulated for subcutaneous, oral or topical administration, depending on the species. A summary of the existing formulations (reviewed by McKellar and Benchaoui, 1996) is presented in Table 1. Most original formulations for cattle have been the intraruminal sustained-release device (SR-bolus) and the topical (pour-on) formulation. The SR-bolus formulation consists of a mini-osmotic pump, which is retained in the rumen after ingestion and releases the drug over 120 days at a constant rate of 40 µg/kg/day (Pope et al., 1985). It provides the advantage over other conventional formulations of long duration of activity and less frequent administration. The pour-on formulation is a high concentrated solution of drug, which is poured along the dorsal midline of the animal in a narrow strip extending from the withers to the tailhead. This application technique is very convenient, since the owner can apply the treatment himself with minimal risk of injury and little animal distress (Hennessy, 1997). The pour-on formulation has now largely displaced the conventional and less practical injectable formulation in farming practice.

3) Spectrum of activity and mode of action.

As for other avermectins and milbemycins, ivermectin has no useful activity against trematodes or cestodes (Shoop et al., 1995b) but is highly effective against the adult, developing and hypobiotic larvae of most gastrointestinal nematodes and lungworms (listed in Table 2) (Benz et al., 1989; McKellar and Benchaoui, 1996). It also presents a good systemic activity against several arthropod external parasites of domestic animals (Table 2) (Benz et al., 1989; McKellar and Benchaoui, 1996). It is noteworthy though that ivermectin is not equally active against all nematode species or all stages of a given species (Campbell, 1985; McKellar and Benchaoui, 1996). In cattle for instance, the dose-limiting gastrointestinal parasite species for ivermectin are *Cooperia* spp. and *Nematodirus* spp. (Shoop et al., 1995a; McKellar and Benchaoui, 1996). In addition, ivermectin was discovered to have excellent activity against the microfilariae of *Onchocerca volvulus* in man (Greene et al., 1989; Goa et al., 1991), *Onchocerca cervicalis* in horses (Campbell et al., 1989a), and *Dirofilaria immitis* in dogs (Campbell, 1989b).

It is difficult to assign a single mechanism of action for ivermectin in all parasite species. The main antiparasitic efficacy of ivermectin is now attributed to a high-affinity binding to glutamate-gated chloride ion channels in invertebrate nerve and muscle cells (Duce and Scott, 1985; Cully et al., 1994; Arena et al., 1995; Cheeseman et al., 2001). The opening of these channels results in a slow and

Table 1. *Formulations and dosages of ivermectin in current commercial use.*

Formulation	Species	Administration route	Dose rate (µg/kg b.wt.)
Oral tablets	Human	p.o.	150
	Human	p.o.	200
	Dog	p.o.	6
Oral chewable	Dog	p.o.	200
	Cat	p.o.	200
Oral drench	Cattle	p.o.	200
	Sheep, goat	p.o.	200
	Horse	p.o.	200
Oral paste	Cattle	p.o.	200
	Horse	p.o.	200
Feed premix	Pig	p.o.	100 µg/kg/day*
Sustained release bolus Osmotic pump	Cattle	Intraruminal	40 µg/kg/day for 120 days
Injectable	Cattle, sheep	s.c.	200
	Pig	s.c.	300
Pour-on	Cattle	topical	500

* recommended daily dosage for 7 days of treatment.

irreversible increase in membrane conductance, leading to the paralysis of the somatic musculature and particularly of the pharyngeal pump (Geary et al., 1993). An interaction with gamma-aminobutyric acid (GABA) -gated chloride channels has also been reported at higher drug concentrations (Turner and Schaeffer, 1989; McKellar and Benchaoui, 1996).

4) Pharmacokinetic properties.

Studies performed in humans (Fink and Porras, 1989) and in dogs (Daurio et al., 1992) suggest that the pharmacokinetics of ivermectin is dose-independent. The plasma disposition of ivermectin is characterised by a long persistence in the body due to a low plasma clearance and to an extensive distribution into fat consistent

Table 2. *Label efficacy claims for ivermectin in the different species.*

Cattle	Sheep
Gastrointestinal nematodes *Haemonchus placei* *Ostertagia* spp. *Trichostrongylus* spp. *Cooperia* spp. *Nematodirus* spp. *Strongyloides papillosus* *Oesophagostomum radiatum* *Bunostomum phlebotomum*	Gastrointestinal nematodes *Haemonchus contortus, H. placei* *Ostertagia* spp. *Trichostrongylus* spp. *Cooperia* spp. *Nematodirus* spp. *Strongyloides papillosus* *Oesophagostomum* spp. *Chabertia ovina* *Trichuris ovis* *Gaigeria pachyscelis*
Other nematodes *Dictyocaulus viviparus* *Parafilaria bovicola* *Thelazia* spp.	Lung nematodes *Dictyocaulus filaria* *Protostrongylus rufescens*
Warbles (*Hypoderma* spp. …)	Nasal bots *Oestrus ovis*
Sucking lice	
Mites (*Psoroptes* spp. *Sarcoptes* spp.)	Mites *Psoroptes ovis* *Psorergates ovis*
Aids in the control of Biting lice (*Damalinia bovis*) Mite (*Chorioptes* spp.) Horn flies (*Haematobia irritans)* Ticks (*Boophilus* spp.)	

(Campbell, 1985; McKellar and Benchaoui, 1996).

with the lipophilicity of the drug. The plasma elimination half-life after i.v. administration appears to be longer for polygastric species (2.8 days in cattle, 2.7 days in sheep) than for monogastric species (estimated to be 0.5 day and 1.8 days in pigs and dogs, respectively) (Lo et al., 1985; Wilkinson et al., 1985; Fink and Porras, 1989). Ivermectin total clearance appears to be low in most species. In cattle and sheep, the total clearance of ivermectin is 0.79 L/day/kg (Wilkinson et al., 1985) and 0.56 L/day/kg (Prichard et al., 1985), respectively, which corresponds to a body coefficient of extraction of <1% in both cases. Several

Table 2. *continued.*

Goat	Pig	Horse
Gastrointestinal nematodes *Haemonchus contortus* *Ostertagia circumcincta* *Trichostrongylus colubriformis* *Nematodirus spathiger* *Strongyloides papillosus* *Oesophagostomum columbianum* *Chabertia ovina*	Gastrointestinal nematodes *Ascaris suum* *Hyostrongylus rubidus* *Oesophagostomum* spp. *Strongyloides ransomi*	Gastrointestinal nematodes *Trichostrongylus axei* *Habronema muscae* *Strongyloides westeri* *Strongylus vulgaris* *Strongylus edentatus* *Strongylus equinus* *Cyathostomum* spp. *Oxyuris equi* *Parascaris equorum* *Triodontophorus* spp. *Cylicocyclus* spp *Cylicostephanus* spp. *Cylicodontophorus* spp. *Gyalocephalus* sp.
Lung nematodes *Dictyocaulus filaria*	Lung nematodes *Metastrongylus* spp.	
	Kidney worms *Stephanurus dentatus*	Lung nematodes *Dictyocaulus arnfieldi*
	Lice *Haematopinus suis*	Microfilaria *Onchocerca* sp.
	Mites *Sarcoptes scabiei*	Gastric bots *Gastrophilus* spp.

studies suggest that the plasma clearance of ivermectin is greater in goats > sheep > cattle (Lo et al., 1985; Scott et al., 1990). Given an extensive distribution in fat tissues, high volumes of distribution were observed in all species (4.6 L/kg in sheep, 1.9-2.2 L/kg in cattle, and 2.4 L/kg in dogs; Lo et al., 1985; Wilkinson et al., 1985; Fink and Porras, 1989). Binding studies in humans and dogs showed that ivermectin bound extensively to plasma proteins (93%; Okonkwo et al., 1993), including albumin and lipoproteins (HDL) (Rohrer and Evans, 1990).

Table 3. *Ivermectin plasma pharmacokinetic parameters obtained for the commercial formulations in the different species.*

Species	Dose (mg/kg) - route	N	C_{max} (ng/mL)	T_{max} (h)	AUC (ng.h/mL)	F(%)	Reference
Man	0.15 -p.o.	9	38.2	4.7	1545	—	Okonkwo et al. (1993)
Cattle	0.2-s.c.	20	31.7	95.5	8664	—	Toutain et al. (1997)
		5	54.6	34.8	10790	—	Toutain et al. (1988)
	0.5-topical	12	12.2	81.6	2772	15	Gayrard et al. (1999)
	SR bolus	6	28.5	364.8	59424		Alvinerie et al. (1998)
Sheep	0.2-s.c.	6	11.9	40.8	1536	—	Cerkvenik et al. (2002)
		5	30.8	60.0	5718	—	Marriner et al. (1987)
	0.2-p.o.	5	22.0	16.4	2039	36	Marriner at al. (1987)
	0.2-i.r.	4	17.6	23.5	2260	25*	Prichard et al. (1985)
Goat	0.2-s.c.	5	6.1	68.4	1440	—	Alvinerie et al. (1993)
	0.2-p.o.	6	16.0	< 24.0	516	—	Scott et al. (1990)
	0.5-topical	6	4.0	48.0	317	—	Scott et al. (1990)
Pig	0.3-s.c.	5	28.4	27.2	1714	—	Scott & McKellar (1992)
Horse	0.2-p.o.	6	16.4	15.0	—	—	Asquith et al. (1987)
		3	82.3	3.3	4822	37	Marriner et al. (1987)
	0.2-s.c.	3	60.7	80.0	13209	—	Marriner et al. (1987)
Dog	0.006-p.o.	16	2.97	5.3	107	—	Daurio et al. (1992)
	0.1-p.o.	16	44.3	4.2	1035	—	Daurio et al. (1992)

N, number of animals involved in the study; C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; AUC, area under the plasma concentration-time curve; F, bioavailability of ivermectin compared to the subcutaneous route (* to the intravenous route when available); SR bolus, sustained release bolus (40 µg/kg/day over 120 days).

The route of administration and the nature of the formulation influence markedly the systemic availability of ivermectin and its persistence in plasma at therapeutic concentrations (Table 3) (for review, see McKellar and Benchaoui, 1996). Studies from Lo et al. (1985) in cattle showed major differences in the pharmacokinetics of ivermectin after subcutaneous administration, whether the drug was injected as an aqueous micelle or non-aqueous solution. The use of the aqueous based-formulation resulted in a higher bioavailability of ivermectin, but in a shorter plasma elimination half-life similar to that obtained after i.v. administration. The longer plasma elimination half-life observed after administration of the non-aqueous solution (the commercialised formulation) has been attributed to a very slow absorption process (probably due to the precipitation of ivermectin at the injection site) which limits the decrease in plasma concentrations (so called flip-flop effect) (Lo et al., 1985). This prolonged persistence in plasma correlates with the sustained efficacy of the subcutaneous formulation against endo- and ectoparasites, which may last for up to 3-4 weeks for some nematode species (Campbell, 1985; Fink and Porras, 1989; Barth et al., 1997). No "flip-flop" effect has been reported for ivermectin oral formulations in sheep and cattle (Marriner et al., 1987; Steel, 1993; McKellar and Benchaoui, 1996).

Since ivermectin is barely administered by the i.v. route, the absolute bioavailability of ivermectin after oral or topical administration has not yet been determined. However, bioavailabilities relative to the subcutaneous route have been calculated. They indicate a low (15%) and erratic systemic availability for the topical formulation in cattle (Gayrard et al., 1999). Ivermectin bioavailability after oral or intraruminal dosing is also lower than for the subcutaneous route in many species. This has been shown in cattle (Pope et al., 1985; Chiu et al., 1990), sheep (Marriner et al., 1987; Bogan and McKellar, 1988), horses (Marriner et al., 1987) and pigs (Fink and Porras, 1989). Together with a lower persistence in plasma, a poor oral bioavailability explains why oral treatment is less effective than subcutaneous treatment against ectoparasites (mange mites, ticks, lice) (Campbell, 1985; McKellar and Benchaoui, 1996). In the case of the tablet formulations (humans), a low dissolution rate of tablets has been identified as a limiting factor of oral absorption (Fink and Porras, 1989). In ruminant species, the low oral bioavailability of ivermectin has been attributed to an extensive binding of the drug to the particulate phase of rumen digesta (>95% in sheep and cattle; Steel, 1993; Ali and Hennessy, 1996). The large binding of ivermectin to digesta would explain why in sheep the absolute bioavailability of ivermectin given intraruminally is 25%, while that after intra-abomasal administration is almost 100% (Prichard et al.,

1985). Changes in ivermectin pharmacokinetics with the diet (Taylor et al., 1992) and the level of feed intake (Ali and Hennessy, 1996) suggest that this ivermectin-digesta complex greatly influences the systemic availability of ivermectin after oral administration as well as the profile of faecal excretion (Steel, 1993).

Large interindividual variations in drug exposure have been observed following parenteral administration to cattle (Lo et al., 1985; Toutain et al., 1997), pigs (Scott and McKellar, 1992), sheep (Cerkvenik et al., 2002) and goats (Scott et al., 1990) taken under the same experimental conditions. These have been attributed to differences in body condition (fat content) and/or differences in absorption, metabolism and elimination of the parent drug in relation to the age, sex and physiological status of animals (Lo et al., 1985; Bogan and McKellar, 1988; Scott et al., 1990; Scott and McKellar, 1992; McKellar and Benchaoui, 1996; Toutain et al., 1997).

Metabolism and drug residue studies have been carried out in laboratory animals (rats) and target animal species (cattle, sheep) using radiolabelled ivermectin. Drug residues were higher in liver and fat than in other edible tissues, but were low in muscle (Chiu and Lu, 1989; Chiu et al., 1990). In sheep, cattle and rats, the major liver metabolites are the 24-hydroxymethyl-H_2B_{1a} and -H_2B_{1b} and the monosaccharide of 24-hydroxymethyl-H_2B_{1a} (Miwa et al., 1982; Chiu and Lu, 1989). In contrast, the main liver metabolites in pigs are the 3-O-desmethyl-H_2B_{1a} and -H_2B_{1b} (Chiu et al., 1984; Chiu and Lu, 1989). The formation of a 3-O-desmethyl metabolite has also been characterised in goats (Alvinerie et al., 1994). No glucuronide conjugates of metabolites have been identified so far. Metabolites that are less polar than the parent drug have been found in fat tissues from sheep and cattle (but not pig) (Chiu and Lu, 1989). It has been suggested that the polar metabolites produced in the liver are esterified as fatty acid esters and deposited in the fat as non-polar entities (Chiu and Lu, 1989). Finally, *in vitro* studies using liver microsomes have identified cytochrome P450 (CYP) 3A4 as the major enzyme for the metabolism of ivermectin in humans (CYP3A and CYP1A1 in rats) (Zeng et al., 1996, 1998).

Ivermectin is extensively eliminated into faeces, regardless of the species and administration route (Campbell, 1985), with less than 2% of the administered dose excreted in the urine (Chiu and Lu, 1989; Chiu et al, 1990). After subcutaneous administration to cattle, the parent drug accounts for approximately 40% of the drug faecal residues, the remainder consisting of metabolites (Halley et al., 1989). Because concentrations of ivermectin in bile are substantially higher than those in plasma (Bogan and McKellar, 1988; Chiu et al., 1990; Lifschiftz et al., 2000), it is currently believed that biliary secretion is the main elimination pathway of the

parent drug. Ivermectin is also excreted in milk in humans, dairy cows, ewes, goats and camels, which is consistent with its high lipid solubility (Bogan and McKellar, 1988; Toutain et al., 1988; Scott et al., 1990; Alvinerie et al., 1993; Ogbuokiri et al., 1994; Oukessou et al., 1999; Cerkvenik et al., 2002). Excretion in milk accounts for less than 1% of the dose in goats (Alvinerie et al., 1993) and sheep (Cerkvenik et al., 2002), and for approximately 5.5% of the dose in cattle (Toutain et al., 1988). It has been shown that lambs suckling ivermectin-treated ewes receive around 4% of a normal therapeutic dose *via* the milk (Bogan and McKellar, 1988).

5) Ivermectin toxicity.

The large-scale use of ivermectin in man and target animal species for 20 years has provided evidence for the safety of this drug at the applied dose rates (Burkhart, 2000). At very high doses however, neurointoxication may develop with symptoms such as ataxia, tremors, depression and death (Lankas and Gordon, 1989). This neurotoxicity is presumed to involve ivermectin binding to GABA-gated chloride channels, which are confined to the central nervous system in mammals (Turner and Schaeffer, 1989; Huang and Casida, 1997; Burkhart, 2000). No glutamate-gated chloride channels have been identified in mammals so far. It has been shown that ivermectin distributes poorly into the brain at the normal doses (Chiu and Lu, 1989; Chiu et al., 1990). The reason for this poor distribution has been attributed to the presence of a drug transporter at the blood-brain barrier called P-glycoprotein (described below), which pumps ivermectin back into blood (Schinkel et al., 1994; Didier and Loor, 1995; Lankas et al., 1997; Kwei et al., 1999; Nobmann et al., 2001). A deficiency in P-glycoprotein at the blood-brain barrier has been demonstrated in CF-1 mice (Lankas et al., 1997) and some breeds of Collie dogs (Mealey et al., 2001), which are unusually sensitive to the toxic effects of ivermectin at normal dosage (Pullian and Preston, 1989; Lankas et al., 1997). Furthermore, neonates have been shown to be more susceptible than adult rats to the neurotoxicity of ivermectin, probably due to an immature blood-brain barrier and a lack of functional P-glycoprotein (Lankas and Gordon, 1989; Burkhart, 2000). Finally, acute and chronic toxicity studies showed that ivermectin was not carcinogenic and was not selectively toxic to the developing embryo (Lankas and Gordon, 1989).

6) Environmental safety.

Fear has been expressed that the large-scale use of ivermectin in grazing cattle could have an overall negative impact onto the environment. Indeed, faecal

ivermectin remains biologically active long after its excretion by treated cattle and has been shown to be toxic to many non-target dung-breeding and dung-degrading insects (Wall and Strong; 1987; Sommer et al., 1993; Strong et al., 1996; McKellar, 1997). A few studies even suggested that the parent drug could affect the processes of dung colonisation and slow down the subsequent degradation of dung on the pasture (Wall and Strong, 1987; Strong et al., 1996). The ecotoxicity of ivermectin has been debated over 20 years, but it now seems that the risk of an overall environmental impact is low (for review, see McKellar, 1997). However, a possible local environmental effect of ivermectin cannot be excluded, depending on the local farm practices (number of animals treated in the herd, period and frequency of treatments) (McKellar, 1997).

It is noteworthy though that all formulations of ivermectin are not equivalent in terms of environmental exposure (Steel, 1993; Herd et al., 1996). The use of the pour-on formulation results in higher faecal ivermectin concentrations compared to the injectable formulation, which is quite surprising given the lower ivermectin plasma concentrations (Herd et al., 1996). An explanation for this apparent discrepancy has not yet been identified. The greatest ecological risk, however, has been associated with the use of SR bolus formulation in cattle. Because of a low oral bioavailability (40%, Pope et al., 1985), the constant delivery of drug in the rumen leads indeed to high and particularly persistent concentrations of parent ivermectin in the dung (Herd et al., 1996; Alvinerie et al., 1998).

7) Development of drug resistance.

Ivermectin was introduced at a time when benzimidazole and levamisole resistance in sheep was widespread. A similar situation is now emerging for ivermectin in sheep and goat, resistance involving *Haemonchus contortus*, *Ostertagia* spp. and *Trichostrongylus* spp. (McKellar and Benchaoui, 1996). Signs of resistance have been reported in cattle for *Cooperia* spp. (Coles et al., 1998).

The actual mechanisms of ivermectin resistance in nematodes are poorly understood (McKellar and Benchaoui, 1996; Gill and Lacey, 1998). Parasites resistant to avermectins, including ivermectin, are generally also resistant to milbemycins, and it has been suggested that the drug transporting P-glycoprotein could be one of the mechanisms involved in this cross-resistance (see below; Blackhall et al., 1998a; Xu et al., 1998; Molento and Prichard, 1999). Parasite resistance may also be associated with mutations of the gene encoding the α-subunit of glutamate-gated chloride channels (Blackhall et al., 1998b). The relative significance of these mechanisms is still under debate (Kwa et al., 1998). Animal

husbandry practices that contribute to the selection for resistance are frequent dosing and, to a lesser extent, underdosing depending on the frequency in the allele carrying resistance in the population (Smith et al., 1999).

B) P-GLYCOPROTEIN AND DRUG TRANSPORT.

P-glycoprotein (P-gp) is a drug efflux transporter associated with the plasma membrane (Fig. 2). This 170-kDa protein consists of two similar halves, each containing six putative transmembrane segments and an intracellular ATP binding site (Chen et al., 1986; Gottesman et al., 1996; Borst and Elferink, 2002). The well-conserved ATP binding regions and the presence of multiple transmembrane segments define P-gp as a member of the ATP-binding cassette (ABC) superfamily of transporters (Higgins, 1992). ABC transporters have been identified in organisms ranging from archebacteria, bacteria, yeast, plants, and insects to mammals (Higgins, 1992; Gottesman et al., 1996; Borst and Elferink, 2002). Up to 48 human ABC transporters have been identified to date, which are localised within intracellular membranes (endoplasmic reticulum, peroxisomes) or in the plasma membrane, depending on their physiological function (Borst and Elferink, 2002).

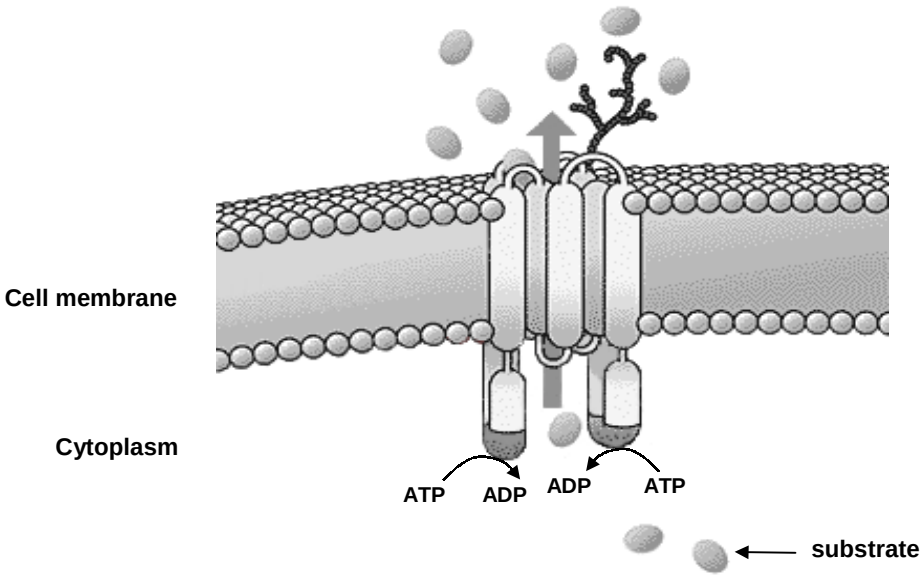


Figure 2. Representation of P-glycoprotein with its transmembrane segments and its two intracellular nucleotide (ATP) -binding sites.

Table 4. *Non-exhaustive list of compounds that are well-established P-gp substrates.*

Class of compounds	Molecules
Anticancer drugs	*Vinca* alkaloids (vinblastine, vincristine …) Anthracyclines (daunorubicin, doxorubicin …) Epipodophyllotoxins (etoposide, teniposide) Tubulin polymerising drugs (paclitaxel, docetaxel, colchicine)
Immunosuppressive agents	Cyclosporin A, FK506
Cardiac drugs	Digoxin, quinidine
Calcium channel blockers	Verapamil, diltiazem, nicardipine
HIV protease inhibitors	Indinavir, nelfinavir, ritonavir, saquinavir
Steroids	Aldosterone, cortisol, corticosterone, dexamethasone,
Antimicrobial agents	Sparfloxacin, grepafloxacin, erythromycin
Antihistamines	Terfenadine
Dopamine antagonists	Domperidone
β-adrenergic antagonists	Talinolol
Lipid lowering agents	Lovastatin
Opioids	Morphine, fentanyl
Antiemetics	Ondansetron, domperidone
Antidiarrheal agents	Loperamide
Anthelmintic agents	Ivermectin
Antimalarial agents	Quinine, chloroquine
Antituberculous agents	Rifampicin
Cytokines	Interleukins IL-2, IL-4, and gamma interferon (INF-γ)
Fluorescent dyes	Rhodamine 123, Fluo-3 AM (Acetoxymethy), calcein-AM, Hoechst 33342/33258

(Shapiro and Ling, 1998; Litman et al., 2001; Matheny et al., 2001)

P-gp was discovered in the mid 1970's through its ability to confer multidrug resistance (MDR) in mammalian tumour cells (Juliano and Ling, 1976). This observation has led to the finding that P-gp is an ATP-dependent efflux pump that expels a wide range of functionally and structurally unrelated compounds out of the cell, including anticancer agents (Germann, 1996; Gottesman et al., 1996; Shapiro and Ling, 1998; Litman et al., 2001; Borst and Elferink, 2002). In tumour cells, P-gp lowers the effective concentrations of drugs inside the cell, resulting thereby in therapeutics failure. The development of strategies that will successfully reverse multidrug resistance in cancer is now a major issue in chemotherapy.

An increasing number of compounds have been discovered to be substrates of P-gp, including clinically-important drugs such as immunosuppressive agents, HIV protease inhibitors, cardiovascular drugs, steroids, antimicrobial and antiparasitic agents (Shapiro and Ling, 1998; Litman et al., 2001; Matheny et al., 2001). An indicative list of P-gp substrates is presented in Table 4. Experiments performed with the National Cancer Institute drug screen suggest that this list could be extended by several hundreds (Litman et al., 2001). How P-gp recognises its substrates (mostly neutral or cationic lipophilic compounds) and transport them out of the cell is still not known in details. The most recent models for P-gp transport mechanisms are reviewed by Litman et al. (2001) and Borst and Elferink (2002).

1) Key role of P-gp in drug disposition.

The significance of P-gp extends far beyond resistance to chemotherapeutic agents. In addition to tumour cells, P-gp has been found to be physiologically expressed in various tissues, including the major organs of drug absorption, distribution and elimination. P-gp is present at high levels in the apical (lumenal) membrane of intestinal epithelial cells and kidney proximal tubule cells, in the biliary canalicular membrane of hepatocytes, the lumenal membrane of capillary endothelial cells at the blood-brain, blood-testis and blood-inner ear barriers, and in the apical membrane of placental trophoblasts (Thiebaut et al., 1987; Sugawara et al., 1988; Cordon-Cardo et al., 1989, 1990). As a result of this tissue distribution, P-gp has been shown to reduce the uptake of its substrates from the gastrointestinal tract (Van Asperen et al., 1998; Schellens et al., 2000; Benet and Cummins, 2001), to limit their penetration into critical tissues such as the brain, testis or foetus (Schinkel et al., 1994, 1995, 1996; Lankas et al., 1997, 1998; Smit et al., 1999), and enhance their elimination by biliary, intestinal or renal secretion (Schinkel et al., 1994, 1995, 1997; Hunter and Hirst, 1997; Smit et al., 1998; Van Asperen et al., 1998).

There are now accumulating *in vitro* and *in vivo* evidence that P-gp contributes significantly to the disposition of many drugs by mediating their elimination into the intestinal lumen, bile or urine (Schinkel et al., 1994, 1995, 1997; Hunter and Hirst; 1997; Smit et al., 1998; Van Asperen et al., 1998; Tanigawara, 2000; Matheny et al., 2001). Furthermore, preclinical and clinical studies have shown that intestinal P-gps could effectively limit the absorption of orally-administered drugs such as paclitaxel and docetaxel (Van Asperen et al., 1998; Aungst, 1999; Schellens et al., 2000; Matheny et al., 2001). There is a striking, albeit not complete, overlap between substrates of P-gp and those of the human cytochrome P450 3A4 (CYP3A4) (Wacher et al., 1995; Benet and Cummins, 2001). Thus, it has been suggested that the intestinal P-gps could act in combination with intestinal cytochrome P450 to reduce even more the systemic availability of orally-administered compounds (Wacher et al., 1995; Benet and Cummins, 2001). Briefly, the drug molecules that escape intestinal metabolism in the enterocyte would be pumped back into the intestinal lumen by P-gp, allowing thereby their re-exposure to intestinal metabolic enzymes following reabsorption. The P-gps located at the blood-brain barrier have been shown to limit access of xenobiotics to the central nervous system (CNS) and thus to serve a protective role against the neurotoxicity of several drugs such as ivermectin (described below) (Schinkel et al., 1994, 1995, 1996; Matheny et al., 2001). However, these P-gps can also limit the efficacy of drugs targeted to the CNS (Matheny et al., 2001; Borst and Elferink, 2002). In HIV therapy for instance, the CNS has been shown to serve as a reservoir for HIV infection due to the poor ability of HIV protease inhibitor to cross the blood brain barrier (Matheny et al., 2001).

P-gp expression is likely to vary among individuals, which significantly complicates prediction of drug disposition *in vivo.* Indeed, some natural polymorphisms in P-gp have been reported in humans (Borst and Elferink, 2002). Besides, P-gp can be induced *in vivo* by several drugs and food components (rifampicin, St John's wort, grapefruit juice) (Matheny et al., 2001; Borst and Elferink, 2002). Finally, as for cytochrome P450 isoenzymes, P-gp can play an important role in clinically-relevant drug-drug interactions (Dale, 1999; Matheny et al., 2001).

It is worthwhile to mention that other ABC transporters may play a role in drug disposition. A second major ABC transporter called MRP (multidrug resistance-associated protein, now MRP1) was identified by Cole et al. in 1992. Since then, eight homologues of MRP1 have been discovered, including MRP2 originally identified as the canalicular multispecific organic anion transporter cMOAT (for review, see Borst and Elferink, 2002). Like P-gp, MRP2 is located on the apical

membrane of epithelial cells (whereas MRP1 is basolateral) and is expressed in the liver, the intestines and the kidneys (König et al., 1999; Litman et al., 2001; Borst and Elferink, 2002). MRP2 has generally greater affinity for unconjugated amphiphilic anions or anionic conjugates of lipophilic compounds, whereas P-gp interacts predominantly with uncharged and cationic substances (König et al., 1999; Borst and Elferink, 2002). There is, however, some overlap in their substrate specificities with respect to neutral compounds (König et al., 1999; Litman et al., 2001; Borst and Elferink, 2002). MRP2 has been shown to play a major role in the transport of bilirubin glucuronide into bile, but mediates also the biliary, intestinal and renal secretion of several compounds (König et al., 1999; Gotoh et al., 2000; Suzuki and Sugiyama et al., 2000; Borst and Elferink, 2002).

2) Physiological role of P-gp.

One major physiological role of P-gp is certainly the detoxification of the organism and its protection against the potentially toxic compounds arising from the environment. P-gp is encoded by one gene in humans (MDR1) and two genes in rodents, mdr1a (or mdr3) and mdr1b (or mdr1), which probably fulfil the same function as the single human MDR1 gene (Borst and Elferink, 2002). It is noteworthy though that Mdr1a P-gp is the only expressed in the brain and in the intestines in mice (Borst and Elferink, 2002). Another MDR gene has been identified in humans (called MDR2 or MDR3) and in rodents (mdr2), which codes for a phospholipid transporter important in normal hepatobiliary function (Smit et al., 1993). It is generally considered that the MDR3 gene product is not involved in drug transport. However, a recent study showed that this protein was capable of transporting several drugs, including ivermectin (Smith et al., 2000).

The existence of additional physiological functions of the drug transporting P-gp is still under debate (reviewed by Borst and Elferink, 2002). This protein could play a possible role in the transport of lipids and peptides, and in the secretion of cytokines (interleukins 2 and 4, and gamma interferon) by certain subsets of lymphocytes expressing P-gp. Furthermore, P-gp is highly expressed in the human adrenal cortex (Sugawara et al., 1988), which has raised a possible role in cortical hormone transport (Ueda et al., 1992; Tanigawara, 2000). Interestingly, mdr1a/b double knockout mice are healthy and fertile in a protected environment and display no clear physiologic abnormalities or decreased lifespan (Schinkel et al., 1997). However, it is possible that other membrane pumps compensate the vital functions of these proteins in their absence.

3) Ivermectin, a P-gp substrate and inhibitor.

Studies using P-gp-transfected or overexpressing cell lines showed that ivermectin could be a P-gp substrate and a potent P-gp inhibitor (Schinkel et al., 1995; Didier and Loor, 1996; Pouliot et al., 1997; Smith et al., 2000; Eneroth et al., 2001). The implication of P-gp in the disposition of ivermectin was demonstrated *in vivo* with the use of P-gp-deficient mice (knockout or naturally deficient with mdr1a) or by co-administration of P-gp modulators in rat or mouse (Schinkel et al., 1994; Didier and Loor, 1995; Lankas et al., 1997, 1998; Alvinerie et al., 1999; Kwei et al., 1999). These studies have identified P-gp as a major barrier against the penetration of ivermectin into the brain and the foetus (Schinkel et al., 1994; Didier and Loor, 1995; Lankas et al., 1997, 1998; Kwei et al., 1999; Nobmann et al., 2001). Indeed, P-gp-deficient mice were shown to accumulate 30- to 90-fold more ivermectin in their brain than wild-type mice, and were also 100 times more sensitive to the neurotoxicity of ivermectin (Schinkel et al., 1994; Lankas et al., 1997; Kwei et al., 1999). Similarly, the absence of P-gp in the placenta resulted in an increased exposure and sensitivity of the fœtus to the ivermectin derivative abamectin (Lankas et al., 1998). However, the exact contribution of P-gp to the overall disposition of ivermectin is still not fully understood.

Little information is available regarding the involvement of P-gp in the disposition of other endectocides. It has been suggested that abamectin and moxidectin could be P-gp substrates as well (Lankas et al., 1997, 1998; Dupuy et al., 2001; Lifschitz et al., 2002).

Finally, homologues of P-gp have been identified in nematodes (*Haemonchus contortus*) (Xu et al., 1998), and it has been suggested that these P-gps could play a role in the cross-resistance of parasites to ivermectin and moxidectin (Blackhall et al., 1998a; Xu et al., 1998; Molento and Prichard, 1999). An overexpression of P-gp was indeed observed in resistant strains of nematodes compared with susceptible strains (Xu et al., 1998). Furthermore, resistant strains were more sensitive to ivermectin in the presence of P-gp inhibitors (Xu et al., 1998; Molento and Prichard, 1999). Recently, two putative P-gp genes have been identified in *Onchocerca volvulus* (Kwa et al., 1998; Huang and Prichard; 1999), which were expressed at low levels in ivermectin sensitive larval stages and at high levels in ivermectin tolerant adult worms (Huang and Prichard; 1999). The relevance of P-gp as a mechanism for drug resistance, however, is still under debate (Kwa et al., 1998).

C) SCOPE OF THE THESIS.

To understand and optimise the clinical efficacy of ivermectin, it has to be taken into account that many parasites do not feed on plasma. Thus, the compartmental distribution of ivermectin in secondary compartments such as bile and intestinal fluids needs to be documented. Ivermectin is extensively eliminated from blood into the digestive tract lumen as parent drug (40% of total faecal residues in cattle; Halley et al., 1989) and as less active metabolites. However, the routes by which unchanged ivermectin reaches the gut are not fully understood. As for other endectocides (Hennessy et al., 2000), biliary secretion has been assumed to be the major route of elimination of the parent drug. On the other hand, the possibility of a direct secretion of ivermectin through the gut wall has been raised (Bogan and McKellar, 1988). For some drugs such as ciprofloxacin, roxithromycin, and vinblastine, intestinal secretion is even more important than biliary secretion in the overall elimination process (Arimori et al., 1998; Dautrey et al., 1999; Van Asperen et al., 2000). Since ivermectin has been shown to be a P-gp substrate (Schinkel et al., 1994, 1995), intestinal P-gps could provide the mechanistic support for the secretion of parent ivermectin from blood into the intestinal lumen.

The existence of an intestinal secretion of ivermectin is examined in the first part of the thesis, using two validated *in vitro* and *in situ* models. The possible extrapolation of the results to the target animal species is further discussed, on the basis of the existence of functional P-gps in those species. The following issues were addressed:

- Investigation of an intestinal secretion of ivermectin by human epithelial Caco-2 cell monolayers, and examination of the role of P-gp and MRP2 in the secretion process (**chapter 2**).
- Comparative evaluation of the intestinal and biliary secretions of parent ivermectin in anaesthetised rats, using the intestinal closed-loop model. Ivermectin intestinal and biliary clearances were evaluated *in situ*, while ivermectin total (plasma) clearance was assessed from a parallel *in vivo* experiment. P-gp involvement in both intestinal and biliary secretions was investigated (**chapter 3**).
- Comparative evaluation of P-gp activity in sheep, cattle, horses, goats, pigs and rats, using a lymphocyte-based *ex vivo* model. The expression of P-gp in the intestines (jejunum) was also investigated (**chapter 4**).

Ivermectin elimination from blood, however, cannot always account for the total excretion of parent drug in faeces after parenteral administration. In cattle, higher faecal concentrations of ivermectin have been reported following topical administration than following subcutaneous administration, which is inconsistent with the lower plasma concentrations (Herd et al., 1996). One reason for this discrepancy could be that cattle are licking themselves, ingesting thereby a part of the topical dose. This hypothesis was examined in the second part of the thesis, as follows:

- Evaluation of the influence of animal licking behaviour on the absolute bioavailability, plasma disposition and faecal disposition of topical ivermectin in cattle, using 6 pairs of monozygotic twin cattle separated into two groups of licking and non-licking cattle (**chapter 5**).
- Quantification of the actual contribution of animal licking to the plasma and faecal disposition of topical ivermectin in cattle, by pharmacokinetic modeling of the i.v. and pour-on (plasma and faecal) data obtained in each pair of twins (**chapter 6**).

References

Ali DN and Hennessy DR (1996) The effect of level of feed intake on the pharmacokinetic disposition and efficacy of ivermectin in sheep. *J Vet Pharmacol Ther* **19:**89-94.

Alvinerie M, Sutra JF, Galtier P and Toutain PL (1987) Determination of ivermectin in milk by high performance liquid chromatography. *Ann Rech Vet* **18:**269-724.

Alvinerie M, Sutra JF and Galtier P (1993) Ivermectin in goat plasma and milk after subcutaneous injection. *Vet Res* **24:**417-421.

Alvinerie M, Tardieu D, Sutra JF, Bojensen G and Galtier P (1994) Metabolic profile of ivermectin in goats: an *in vivo* and *in vitro* evaluation in *European Association for Veterinary Pharmacology and Toxicology. Proceedings of the* 6^{th} *International Congress* (Lees P ed) pp 262, 7-11 August, Blackwell Scientific Publications. Edinburgh.

Alvinerie M, Sutra JF, Galtier P, Lifschitz A, Virkel G, Sallovitz J and Lanusse C (1998) Persistence of ivermectin in plasma and faeces following administration of a sustained-release bolus to cattle. *Res Vet Sci* **66:**57-61.

Alvinerie M, Dupuy J, Eeckhoutte C and Sutra JF (1999) Enhanced absorption of pour-on ivermectin formulation in rats by co-administration of the multidrug-resistant-reversing agent verapamil. *Parasitol Res* **85:**920-922.

Arena JP, Liu KK, Paress PS, Frazier EG, Cully DF, Mrozik H and Schaeffer JM (1995) The mechanism of action of avermectins in Caenorhabditis elegans: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. *J Parasitol* **81:**286-294.

Arimori K, Miyamoto S, Fukuda K, Nakamura C and Nakano M (1998) Characteristic difference in gastrointestinal excretion of clarithromycin and roxithromycin. *Biopharm Drug Dispos* **19:**433-438.

Asquith RL, Lane TJ, Plue RE, Seward RL and Kivipelto J (1987) The bioavailability of ivermectin in horses when administered in a liquid formulation by nasogastric intubation versus in an oral paste. *Equine Vet Sci* **7:**28-30.

Aungst BJ (1999) P-glycoprotein, secretory transport, and other barriers to the oral delivery of anti-HIV drugs. *Adv Drug Deliv Rev* **39:**105-116.

Barth D, Ericsson GF, Kunkle BN, Rehbein S, Ryan WG and Wallace DH (1997) Evaluation of the persistence of the effect of ivermectin and abamectin against gastrointestinal and pulmonary nematodes in cattle. *Vet Rec* **140:**278-279.

Benet LZ and Cummins CL (2001) The drug efflux-metabolism alliance: biochemical aspects. *Adv Drug Deliv Rev* **50:** S3-S11.

Benz GW, Roncalli RA and Gross SJ (1989) Use of ivermectin in cattle, sheep, goats, and swine in *Ivermectin and Abamectin* (Campbell WC ed) pp 215-229, Springer, New York.

Blackhall WJ, Liu HY, Xu M, Prichard RK and Beech RN (1998a) Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of Haemonchus contortus. *Mol Biochem Parasitol* **95:**193-201.

Blackhall WJ, Pouliot JF, Prichard RK and Beech RN (1998b) Haemonchus contortus: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. *Exp Parasitol* **90:**42-48.

Bloomfield G (1988) *Endoparasites – the Veterinary Market* pp 59-60, PJB Publications, Richmond, UK.

Bogan JA and McKellar QA (1988) The pharmacodynamics of ivermectin in sheep and cattle. *J Vet Pharmacol Ther* **11:**260-268.

Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71:**537-592.

Burg RW and Stapley EO (1989) Isolation and characterisation of the producing organism in *Ivermectin and Abamectin* (Campbell WC ed) pp 24-32, Springer, New York.

Burkhart CN (2000) Ivermectin: an assessment of its pharmacology, microbiology and safety. *Vet Hum Toxicol* **42:**30-35.

Campbell WC (1985) Ivermectin: an update. *Parasitol Today* **1:**10-16.

Campbell WC, Leaning WDH and Seward RL (1989a) Use of ivermectin in horses in *Ivermectin and Abamectin* (Campbell WC ed) pp 234-244, Springer, New York.

Campbell WC (1989b) Use of ivermectin in dogs and cats in *Ivermectin and Abamectin* (Campbell WC ed) pp 245-259, Springer, New York.

Cerkvenik V, Grabnar I, Skubic V, Doganoc DZ, Beek WM, Keukens HJ, Drobnic Kosorok M and Pogacnik M (2002) Ivermectin pharmacokinetics in lactating sheep. *Vet Parasitol* **104:**175-185.

Cheeseman CL, Delany NS, Woods DJ and Wolstenholme AJ (2001) High-affinity ivermectin binding to recombinant subunits of the Haemonchus contortus glutamate-gated chloride channel. *Mol Biochem Parasitol* **114:**161-168.

Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM and Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47:**381-389.

Chiu SL, Sestokas E, Taub R, Smith JL, Arison B and Lu AYH (1984) The metabolism of avermectin H_2B_{1a} and H_2B_{1b} by pig liver microsomes. *Drug Metab Dispos* **12**:464-469.

Chiu SL and Lu AYH (1989) Metabolism and tissue residues in *Ivermectin and Abamectin* (Campbell WC ed) pp 131-143, Springer, New York.

Chiu L, Green ML, Baylis FP, Eline D, Rosegay A, Meriwether H and Jacob TA (1990) Absorption, tissue distribution and excretion of tritium-labeled ivermectin in cattle, sheep and rat. *J Agric Food Chem* **38:**2072-2078.

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM and Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258:**1650-1654.

Coles GC, Stafford KA and MacKay PH (1998) Ivermectin resistance Cooperia species from calves on a farm in Somerset. *Vet Rec* **142:**255-256.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR

and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at the blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86:**695-698.

Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR and Melamed MR (1990) Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* **38:**1277-1287.

Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LHT, Schaeffer JM and Arena JP (1994) Cloning of an avermectin-sensitive glutamate-gated chloride channels from Caenorhabditis elegans. *Nature* **371:**707-711.

Dale KY (1999) The contribution of P-glycoprotein to pharmacokinetic drug-drug interactions. *J Clin Pharmacol* **39:**1203-1211.

Daurio CP, Cheung EN, Jeffcoat AR and Skelly BJ (1992) Bioavailability of ivermectin administered orally to dogs. *Vet Res Commun* **16:**125-130.

Dautrey S, Felice K, Petiet A, Lacour B, Carbon C and Farinotti R (1999) Active intestinal elimination of ciprofloxacin in rats : modulation by different substrates. *Br J Pharmacol* **127:**1728-1734.

Didier AD and Loor F (1995) Decreased biotolerability for ivermectin and cyclosporin A in mice exposed to potent P-glycoprotein inhibitors. *Int J Cancer* **63:**263-267.

Didier AD and Loor F (1996) The abamectin derivative ivermectin is a potent P-glycoprotein inhibitor. *Anticancer Drugs* **7:**745-751.

Duce IR and Scott RH (1985) Actions of dihydroavermectin B1a on insect muscle. *Br J Pharmacol* **85:**395-401.

Dupuy J, Larrieu G, Sutra JF, Eeckhoutte C and Alvinerie M (2001) Influence of verapamil on the efflux and metabolism of 14C moxidectin in cultured rat hepatocytes. *J Vet Pharmacol Ther* **24:**171-177.

Eneroth A, Astrom E, Hoogstraate J, Schrenk D, Conrad S, Kauffmann HM and Gjellan K (2001) Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. *Eur J Pharm Sci* **12:**205-214.

Fink DW and Porras AG (1989) Pharmacokinetics of ivermectin in animals and humans in *Ivermectin and Abamectin* (Campbell WC ed) pp 113-130, Springer, New York.

Fisher MH and Mrozik H (1989) Chemistry in *Ivermectin and Abamectin* (Campbell WC ed) pp 1-23, Springer, New York.

Gayrard V, Alvinerie M and Toutain PL (1999) Comparison of pharmacokinetic profiles of doramectin and ivermectin pour-on formulations in cattle. *Vet Parasitol* **81:**47-55.

Geary TG, Sims SM, Thomas EM, Vanover L, Davis JP, Winterrowd CA, Klein RD, Ho NF and Thompson DP (1993) Haemonchus contortus: ivermectin-induced paralysis of the pharynx. *Exp Parasitol* **77:**88-96.

Germann UA (1996) P-glycoprotein - a mediator of multidrug resistance in tumour cells. *Eur J Cancer* **32:** 927-944.

Gill JH and Lacey E (1998) Avermectin/milbemycin resistance in trichostrongyloids

nematodes. *Int J Parasitol* **28:**863-877.

Goa KL, McTavish D and Clissold SP (1991) Ivermectin. A review of its antifilarial activity, pharmacokinetic properties and clinical efficacy in onchocerciasis. *Drugs* **42:**640-658.

Gotoh Y, Suzuki H, Kinoshita S, Hirohashi T, Kato Y and Sugiyama Y (2000) Involvement of an organic anion transporter (canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2) in gastrointestinal secretion of glutathione conjugates in rats. *J Pharmacol Exp Ther* **292:**433-439.

Gottesman MM, Ambudkar SV, Cornwell MM, Pastan I and Germann U (1996) Multidrug resistance transporter in *Molecular biology of membrane transport disorders* pp 243-257 (Schultz SG et al. ed), Plenum Press, New York.

Greene BM, Brown KR and Taylor HR (1989) Use of ivermectin in humans in *Ivermectin and Abamectin* (Campbell WC ed) pp 311-323, Springer, New York.

Halley BA, Nessel RJ and Lu AYH (1989) Environmental aspects of ivermectin usage in livestock: general considerations in *Ivermectin and Abamectin* (Campbell WC ed) pp 162-172, Springer, New York.

Hennessy DR (1997) Modifying the formulation or delivery mechanism to increase the activity of anthelmintic compounds. *Vet Parasitol* **72:**367-382.

Hennessy DR, Page SW and Gottschall D (2000). The behaviour of doramectin in the gastrointestinal tract, its secretion in bile and pharmacokinetic disposition in the peripheral circulation after oral and intravenous administration to sheep. *J Vet Pharmacol Ther* **23:**203-213.

Herd RP, Sams RA and Ashcraft SM (1996) Persistence of ivermectin in plasma and faeces following treatment of cows with ivermectin sustained-release, pour-on or injectable formulations. *Int J Parasitol* **26:**1087-1093.

Higgins CF (1992) ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8:**67-113.

Huang J and Casida JE (1997) Avermectin B1a binds to high- and low-affinity sites with dual effects on the γ-aminobutyric acid-gated chloride channel of cultured cerebellar granule neurons. *J Pharm Exp Ther* **281:**261-266.

Huang YJ and Prichard RK (1999) Identification and stage-specific expression of two putative P-glycoprotein coding genes in Onchocerca volvulus. *Mol Biochem Parasitol* **102:**273-281.

Hunter J and Hirst BH (1997) Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv Drug Deliv Rev* **25:**129-157.

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455:**152-162.

König J, Nies AT, Cui Y, Leier I and Keppler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* **1461:**377-394.

Kwa MS, Okoli MN, Schulz-Key H, Okongkwo PO and Roos MH (1998) Use of P-glycoprotein gene probes to investigate anthelmintic resistance in Haemonchus contortus and comparison with Onchocerca volvulus. *Int J Parasitol* **28:**1235-1240.

Kwei GY, Alvaro RF, Chen Q, Jenkins HJ, Hop CE, Keohane CA, Ly VT, Strauss JR, Wang RW, Wang Z, Pippert TR and Umbenhauer DR (1999) Disposition of ivermectin and cyclosporin A in CF-1 mice deficient in mdr1a P-glycoprotein. *Drug Metab Dispos* **27:**581-587.

Lankas GR and Gordon LR (1989) Toxicology in *Ivermectin and Abamectin* (Campbell WC ed) pp 89-112, Springer, New York.

Lankas GR, Cartwright ME and Umbenhauer D (1997) P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicol Appl Pharmacol* **143:**357-365.

Lankas GR, Wise LD, Cartwright ME, Pippert T and Umbenhauer DR (1998) Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol* **12:**457-463.

Lifschitz A, Virkel G, Sallovitz J, Sutra JF, Galtier P, Alvinerie M and Lanusse C (2000) Comparative distribution of ivermectin and doramectin to parasite location tissues in cattle. *Vet Parasitol* **87:**327-338.

Lifschitz A, Virkel G, Sallovitz J, Imperiale F, Pis A and Lanusse C (2002) Loperamide-induced enhancement of moxidectin availability in cattle. *J Vet Pharmacol Ther* **25:**111-120.

Litman T, Druley TE, Stein WD and Bates SE (2001) From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* **58:**931-959.

Lo PK, Fink DW, Williams JB and Blodinger J (1985) Pharmacokinetic studies of ivermectin: effects of formulation. *Vet Res Commun* **9:**251-268.

Marriner SE, McKinnon I and Bogan JA (1987) The pharmacokinetics of ivermectin after oral and subcutaneous administration to sheep and horses. *J Vet Pharmacol Ther* **10:**175-179.

Matheny CJ, Lamb MW, Brouwer KL and Pollack GM (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy* **21:**778-796.

McKellar QA and Benchaoui HA (1996) Avermectins and milbemycins. *J Vet Pharmacol Ther* **19:**331-351.

McKellar QA (1997) Ecotoxicology and residues of anthelmintic compounds. *Vet Parasitol* **72:**413-435.

Mealey KL, Bentjen SA, Gay JM and Cantor GH (2001) Ivermectin sensitivity in collies is associated with a deletion mutation of the mdr1 gene. *Pharmacogenetics* **11:**727-733.

Miwa GT, Walsh JS, VandenHeuvel WJ, Arison B, Sestokas E, Buhs R, Rosegay A, Avermitilis S, Lu AY, Walsh MA, Walker RW, Taub R and Jacob TA (1982) The

metabolism of avermectins B1a, H2B1a, and H2B1b by liver microsomes. *Drug Metab Dispos* **10:**268-274.

Molento MB and Prichard RK (1999) Effects of the multidrug-resistance-reversing agents verapamil and CL 347,099 on the efficacy of ivermectin or moxidectin against unselected and drug-selected strains of Haemonchus contortus in jirds (Meriones unguiculatus). *Parasitol Res* **85:**1007-1011.

Nobmann S, Bauer B and Fricker G (2001) Ivermectin excretion by isolated functionally intact brain endothelial capillaries. *Br J Pharmacol* **132:**722-728.

Ogbuokiri JE, Ozumba BC and Okonkwo PO (1994) Ivermectin levels in human breast milk. *Eur J Clin Pharmacol* **46:**89-90.

Okonkwo PO, Ogbuokiri JE, Ofoegbu E and Klotz U (1993) Protein binding and ivermectin estimations in patients with onchocerciasis. *Clin Pharmacol Ther* **53:**426-430.

Oukessou M, Berrag B and Alvinerie MA (1999) A comparative kinetic study of ivermectin and moxidectin in lactating camels (Camelus dromedarius). *Vet Parasitol* **83:**151-159.

Pope DG, Wilkinson PK, Egerton JR and Conroy J (1985) Oral controlled-release delivery of ivermectin in cattle via an osmotic pump. *J Pharm Sci* **74:**1108-1110.

Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E *(*1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **53:**17-25.

Prichard RK, Steel JW, Lacey E and Hennessy DR (1985) Pharmacokinetics of ivermectin in sheep following intravenous, intra-abomasal or intraruminal administration. *J Vet Pharmacol Ther* **8:**88-94.

Pullian JD and Preston JM (1989) Safety of ivermectin in target animals in *Ivermectin and Abamectin* (Campbell WC ed) pp 149-161, Springer, New York.

Rohrer SP and Evans DV (1990) Binding characteristics of ivermectin in plasma from Collie dogs. *Vet Res Communications* **14:**157-165.

Schellens JH, Malingre MM, Kruijtzer CM, Bardelmeijer HA, van Tellingen O, Schinkel AH and Beijnen JH (2000) Modulation of oral bioavailability of anticancer drugs: from mouse to man. *Eur J Pharm Sci* **12:**103-110.

Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, Berns AJM and Borst P (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:**491-502.

Schinkel AH, Wagenaar E, van Deemter L, Mol CA and Borst P (1995) Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96:**1698-1705.

Schinkel AH, Wagenaar E, Mol CA and van Deemter L (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest* **97:**2517-2524.

Schinkel AH, Mayer U, Wagenaar E, Mol CA, van Deemter L, Smit JJ, van der Valk MA, Voordouw AC, Spits H, van Tellingen O, Zijlmans JM, Fibbe WE and Borst P (1997)

Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci U S A* **94:**4028-4033.

Scott EW, Kinabo LD and McKellar QA (1990) Pharmacokinetics of ivermectin after oral or percutaneous administration to adult milking goats. *J Vet Pharmacol Ther* **13:**432-435.

Scott EW and McKellar QA (1992) The distribution and some pharmacokinetic parameters of ivermectin in pigs. *Vet Res Commun* **16:**139-146.

Shapiro AB and Ling V (1998) The mechanism of ATP-dependent multidrug transport by P-glycoprotein. *Acta Physiol Scand* **163:**227-234.

Shoop WL, Mrozik H and Fisher MH (1995a) Structure and activity of avermectins and milbemycins in animal health. *Vet Parasitol* **59:**139-156.

Shoop WL, Ostlind DA, Rohrer SP, Mickle G, Haines HW, Michael BF, Mrozik H and Fisher MH (1995b) Avermectins and milbemycins against Fasciola hepatica: in vivo drug efficacy and in vitro receptor binding. *Int J Parasitol* **25:**923-927.

Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA, et al. (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75:**451-462.

Smit JW, Schinkel AH, Weert B and Meijer DK (1998) Hepatobiliary and intestinal clearance of amphiphilic cationic drugs in mice in which both *mdr1a* and *mdr1b* genes have been disrupted. *Br J Pharmacol* **124:**416-424.

Smit JW, Huisman MT, Van Tellingen O, Wiltshire HR and Schinkel AH (1999) Absence of pharmacological blocking of placental P-glycoprotein profoundly increases fetal drug exposure. *J Clin Invest* **104:**1441-1447.

Smith G, Grenfell BT, Isham V and Cornell S (1999) Anthelmintic resistance revisited: underdosing, chemoprophylactic strategies, and mating probabilities. *Int J Parasitol* **29:**77-91.

Smith AJ, van Helvoort A, van Meer G, Szabo K, Welker E, Szakacs G, Varadi A, Sarkadi B and Borst P (2000) MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. *J Biol Chem* **275:**23530-23539.

Sommer C, Gronvold J, Holter P and Nansen P (1993) Effects of ivermectin on two afrotropical dung beetles, Onthophagus gazella and Diastellopalpus quinquedens (Coleoptera: Scarabaeidae). *Vet Parasitol* **48:**171-179.

Steel JW (1993) Pharmacokinetics and metabolism of avermectins in livestock. *Vet Parasitol* **48:**45-57.

Strong L, Wall R, Woolford A and Djeddour D (1996) The effects of faecally excreted ivermectin and fenbendazole on the insect colonisation of cattle dung following the oral administration of sustained-release boluses. *Vet Parasitol* **62:**253-266.

Sugawara I, Kataoka I, Morishita Y, Hamada H, Tsuruo T, Itoyama S and Mori S (1988) Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as

revealed by a monoclonal antibody, MRK 16. *Cancer Res* **48:**1926-1929.

Suzuki H and Sugiyama (2000) Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur J Pharm Sci* **12:**3-12.

Tanigawara Y (2000) Role of P-glycoprotein in drug disposition. *Therapeutic drug monitoring* **22:**137-140.

Taylor SM, Mallon TR and Blanchflower WJ, Kennedy DG and Green WP (1992) Effects of diet on plasma concentrations of oral anthelmintics for cattle and sheep. *Vet Rec* **130:**264-268.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84:**7735-7738.

Toutain PL, Campan M, Galtier P and Alvinerie M (1988) Kinetic and insecticidal properties of ivermectin residues in the milk of dairy cows. *J Vet Pharmacol Ther* **11:**288-291.

Toutain PL, Upson DW, Terhune TN and McKenzie ME (1997) Comparative pharmacokinetics of doramectin and ivermectin in cattle. *Vet Parasitol* **72:**3-8.

Turner MJ and Schaeffer JM (1989) Mode of action of ivermectin in *Ivermectin and Abamectin* (Campbell WC ed) pp 73-88, Springer, New York.

Ueda K, Okamura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T and Hori R (1992) Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J Biol Chem* **267:**24248-24252.

Van Asperen J, Van Tellingen O and Beijnen JH (1998) The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacol Res* **37:**429-435.

Van Asperen J, Van Tellingen O and Beijnen JH (2000) The role of mdr1a P-glycoprotein in the biliary and intestinal secretion of doxorubicin and vinblastine in mice. *Drug Metab Dispos* **28:**264-267.

Wacher VJ, Wu CY and Benet LZ (1995) Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol Carcinog* **13:**129-134.

Wall R and Strong L (1987) Environmental consequences of treating cattle with the antiparasitic drug ivermectin. *Nature* **327:**418-421.

Wilkinson PK, Pope DG and Baylis FP (1985) Pharmacokinetics of ivermectin administered intravenously to cattle. *J Pharm Sci* **74:**1105-1107.

Xu M, Molento M, Blackhall W, Ribeiro P, Beech R and Prichard R (1998) Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homologue. *Mol Biochem Parasitol* **91:**327-335.

Zeng Z, Andrew NW, Woda JM, Halley BA, Crouch LS and Wang RW (1996) Role of cytochrome P450 isoforms in the metabolism of abamectin and ivermectin in rats. *J Agric Food Chem* **44:**3374-3378.

Zeng Z, Andrew NW, Arison BH, Luffer-Atlas D and Wang RW (1998) Identification of cytochrome P4503A4 as the major enzyme responsible for the metabolism of ivermectin

by human liver microsomes. *Xenobiotica* **28:**313-321.

CHAPTER 2

Active Secretion of the Anthelmintic Ivermectin across Human Intestinal Epithelial Caco-2 Cell Monolayers

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Abstract

In a recent study using rat intestinal loops, we suggested that intestinal secretion could be a major route for the elimination of parent ivermectin from blood. In order to confirm an intestinal secretion process and delineate its underlying mechanisms, we examined the bilateral transport of [^{3}H]ivermectin across human intestinal epithelial Caco-2 cell monolayers. Directional transport of [^{3}H]ivermectin in the secretory direction was demonstrated, with a basolateral-to-apical flux exceeding up to 10 times that in the opposite direction, depending on the donor concentration. The net secretion of [^{3}H]ivermectin was totally abolished at 4°C and in the presence of the P-gp inhibitor cyclosporin A (50 µM). Furthermore, basolateral-to-apical transport was partly inhibited by the P-gp modulators verapamil (100 µM), testosterone (100 µM) but not digoxin (100 µM). Altogether, these results suggest that P-glycoprotein plays an important role in the intestinal secretion of ivermectin. The organic anion *p*-aminohippurate (100 µM) and LTC4 (0.5 µM), a modulator of MRP2 (multidrug resistance associated-protein 2), had no significant effect on [^{3}H]ivermectin transport. However, MK571 (100 µM), another MRP2 modulator, significantly reduced [^{3}H]ivermectin secretory flux, suggesting the involvement of MRP2 in the secretion process. Expression of P-glycoprotein and MRP2 in Caco-2 cells was confirmed by Western immunoblotting. In conclusion, our results provide further evidence for an active secretion of ivermectin from blood into the intestinal lumen, which is of clinical significance since many gastrointestinal parasites do not feed on plasma. The relevance of ivermectin secretory transport is discussed in terms of oral bioavailability.

Introduction

Ivermectin is an extremely potent antiparasitic drug with a broad spectrum of activity against ecto- and endoparasites and a wide margin of safety. It is currently used for control and treatment of various parasitic conditions in human and veterinary medicine, and has become the first-line treatment for human onchocerciasis, a major public disease (Burkhart, 2000).

Ivermectin is almost exclusively excreted into faeces, irrespective of the species and administration route, with less than 2% of the dose excreted in the urine (Chiu et al., 1990; McKellar and Benchaoui 1996). A large amount of ivermectin is eliminated unchanged from blood (40% of a i.v. dose in cattle; Laffont et al., 2001), but the routes by which parent ivermectin reaches the digestive tract are not fully understood. Since high concentrations of ivermectin have been reported in bile (Chiu et al., 1990), it is currently believed that biliary secretion is the main elimination pathway of the parent drug. However, in a recent study using rat intestinal loops, we showed that ivermectin could be secreted from blood through the gut wall (Laffont et al., 2002). All segments of the rat small intestine excreted the parent drug after systemic administration, and ivermectin small intestinal clearance was approximately 5-fold higher than ivermectin biliary clearance.

The purpose of the present study was to confirm the existence of an intestinal secretion process using the human colonic adenocarcinoma cell line Caco-2. This cell line is a well-established *in vitro* model of the intestinal epithelium and is widely used for prediction of intestinal absorption or secretion of drug molecules. Caco-2 cells form monolayers of well-differentiated cells, which display many morphological and functional characteristics of the normal enterocytes (reviewed by Boulenc, 1997). They express many transporters normally found in the small intestine, including the drug transporting P-glycoprotein (P-gp) and multidrug resistance-associated protein MRP2 (Hosoya et al., 1996; Boulenc, 1997; Hunter and Hirst, 1997; Hirohashi et al., 2000; Walgren et al., 2000). P-gp and MRP2 are two drug efflux pumps localised at the apical membrane and have been shown to mediate the intestinal secretion of many compounds (Hunter and Hirst, 1997; Van Asperen et al., 1998; König et al., 1999; Walgren et al., 2000). Since ivermectin has been shown to be a substrate of P-gp (Schinkel et al., 1994, 1995; Pouliot et al., 1997), the implication of P-gp in the secretion process was examined. The possible involvement of MRP2 was also investigated, as MRP2 has the capacity to transport neutral compounds (König et al., 1999).

Materials and Methods

Chemicals

Ivermectin, cyclosporin A, verapamil hydrochloride, testosterone, digoxin, *p*-aminohippurate, leukotriene C4 (LTC_4) were purchased from Sigma (St. Louis, MO, USA). MK571 was from Alexis Biochemicals (Grünberg, Germany). LTC_4 was commercially available as a solution in 70% (v/v) ethanol. For ivermectin and other compounds, which were poorly soluble in transport medium, stock solutions were prepared in dimethyl sulfoxide (DMSO). [^{3}H]ivermectin was kindly provided by Merial (NJ, USA). Cell culture media and reagents were from Gibco BRL (Breda, the Netherlands). P-gp and MRP2 monoclonal antibodies, C219 and M2III-6, were purchased from Fujirebio Diagnostics, Inc. (Malvern, PA, USA) and Monosan (Uden, the Netherlands), respectively. Biotinylated goat anti-mouse IgG and streptavidin-AP conjugate were from Zymed (CA, USA) and Boehringer (Mannheim, Germany), respectively.

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC). Cells were routinely cultured at 37°C in a humidified 5% CO_2 atmosphere, using Dulbecco's modified Eagle's medium (DMEM) supplied with phenol red and 4.5 g/L glucose, and supplemented with 10% heat inactive foetal calf serum (FCS), 1% non-essential amino acids, 2 mM glutamine, and antibiotics (100 µg/mL streptomycin and 100 IU/mL penicillin). Cells were grown in 75-cm^2 tissue culture flasks and subcultured every week by trypsinisation, replacing medium every two or three days. For transport experiments, cells were harvested with trypsin-EDTA and seeded onto inserts (Falcon, 0.4 µm pore size, 0.3 cm^2, high density PEPT) at 100,000 cells/cm^2 in 24-well culture plates. The cells used for transport studies were between passage 89 and 96, after which cell culture was restarted from the original frozen stock. Cells were grown as epithelial monolayers for 19 days, refreshing medium 3 times per week for two weeks and daily thereafter.

Transepithelial transport studies

The MTT test was used to assess the potential cytotoxicity of ivermectin and of P-gp or MRP2 modulators at the applied concentrations. The MTT test, which measures the reduction of dimethylthiazol diphenyl tetrazolium bromide (MTT) to formazan by the mitochondrial enzyme succinate dehydrogenase was based on Denizot and Lang (1986). No detectable cytotoxicity was observed in Caco-2 cells

after 3 h of exposure (data not shown). Although previous *in vitro* transport studies on ivermectin did not address the problem, ivermectin was found to bind very significantly to glassware and plastics of cell culture material (at 0.1-1 µM, the recovery of radiolabelled drug in cells plus compartments did not exceed 30%). Aspecific binding could be prevented during preparation of loading solutions of ivermectin by sonication, but could not be avoided during transport experiments and thus had to be taken into account. As aspecific binding was saturated at high concentrations (25 µM), quantitative investigations were limited.

Transport experiments were performed at 37°C or 4°C using phenol red-free, serum-free (to avoid ivermectin binding to serum) DMEM as transport medium. Loading solutions of [^{3}H]ivermectin (0.1-25 µM; 0.15 µCi/mL) were prepared in transport medium, and lucifer yellow was added at the final concentration of 20 µg/mL. Aliquots (200 µL) were taken from the loading solution of [^{3}H]ivermectin, after and before addition of lucifer yellow, to evaluate quenching. The linearity of [^{3}H]ivermectin transport over time was checked at various donor concentration levels (1, 5, 10, 25 µM) in both absorptive and secretory directions. Caco-2 cells were washed with phosphate buffered saline and were pre-equilibrated in transport medium for 1 h at 37°C in a humidified 5% CO_2 atmosphere. Apical medium was removed, and the inserts were transferred to a fresh 24-well plate. [^{3}H]ivermectin loading solution was added to the apical (apical-to-basolateral transport, AP > BL) or basolateral (basolateral-to-apical transport, BL > AP) side of the inserts, with the corresponding volume of transport medium added on the opposite side. After incubation for 30 min, 1, 2 or 3 h at 37°C in a humidified 5% CO_2 atmosphere, aliquots of 200 µL were sampled from the receiver compartment and when possible from the donor compartment. Three inserts per time point were used in BL > AP experiments, while in AP > BL experiments, inserts (n=3) were moved at intermediate times to other wells containing fresh transport medium. The amounts of ivermectin recovered in the samples were evaluated by liquid scintillation counting taking into account quenching.

Once the linearity of [^{3}H]ivermectin transport was checked over 3 h, only one time point (t = 3h) was investigated. Bilateral transport of [^{3}H]ivermectin was then examined over a broader range of concentrations (0.1, 0.5, 1, 2, 5, 10, 25 µM). This was done in a single experiment to avoid possible changes in P-gp expression (Anderle et al., 1998). After a 3-h incubation at 37°C in a humidified 5% CO_2 atmosphere, aliquots of 200 µL were sampled from the receiver and donor compartments, and radioactivity was measured. Three inserts per condition were used. Bilateral transport of [^{3}H]ivermectin was also investigated at 4°C at the donor concentration of 1 µM.

Inhibition studies

The P-gp substrates and/or inhibitors verapamil (100 µM), cyclosporin A (50 µM), testosterone (100 µM) and digoxin (100 µM) were applied to block the P-gp function (Ford and Hait, 1990; Dale, 1999). MK571 (100 µM), leukotriene LTC_4 (0.5 µM), and the organic anion *p*-aminohippurate (100 µM) were used as MRP2 modulators (König et al., 1999; Litman et al., 2001). Transport studies were performed as described above, with a single time-point after 3 h of incubation at 37°C. P-gp or MRP2 modulators were added to both sides of the inserts, and the bilateral transport of [^{3}H]ivermectin was measured at the donor concentration of 1 µM. The final concentration of DMSO or ethanol (in the case of LTC_4) were 0.65% (v/v) and 0.4% (v/v), respectively. Control wells consisting of 0.65% DMSO or 0.4% ethanol were thus included in each experiment. The time course of transport of [^{3}H]ivermectin (1 µM) in the presence of cyclosporin A (50 µM) was additionally measured over 3 h.

Monolayer integrity

The integrity of the cell monolayers was routinely checked by measuring the transepithelial electrical resistance (TEER) and the permeability to lucifer yellow, a marker for the paracellular route. The TEER was measured at the start and at the end of the transport experiments using the Millicell® -ERS voltohmmeter, and monolayers with a TEER below 200 ohm.cm^2 were discarded. The paracellular flow was monitored by the appearance of lucifer yellow on the receiver side for up to 3 h. The concentrations of lucifer yellow were measured by fluorometry (Cytofluor 2300 Fluorescence Measurement System, Millipore, Corp. Bedford, MA, USA) at the emission and excitation wavelengths of 409 nm and 525 nm, respectively.

Analysis of P-gp and MRP2 expression by Western blotting

Caco-2 cells grown on inserts for 19 days and used for transport studies were scraped in 200 µL of PBS and the samples were homogenised. Brush-border-enriched proteins were also prepared from Caco-2 cells cultured in tissue culture flasks by a previously described method (Pinto et al., 1983). Primary cultures of rat hepatocytes were used as a positive control for P-gp: after one day of culture on 10-cm dishes, hepatocytes were washed twice and scraped in PBS. After centrifugation, the cells were resuspended in 500 µL of PBS and the samples were homogenised. Protein concentrations were determined using the Pierce BCA protein assay reagent (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as a standard. Seven µg of protein were loaded in each lane and

electrophoresed on SDS-polyacrylamide gels (4% stacking and 8% running gel) in a Mini-Protean III Cell (Biorad, Veenendaal, The Netherlands), as described by Laemmli (1970). Electroblotting was performed onto a polyvinylidene difluoride membrane according to the method of Towbin et al. (1979). For the detection of P-gp (170 kDa), the monoclonal antibody C219 specific for P-glycoprotein was used in a dilution of 1:200 in TBS (Tris-buffered saline) containing 0.3% (v/v) Tween 20 and 1% (w/v) BSA. MRP2 (190 kDa) was detected with the monoclonal antibody M2III-6 diluted 1:20 in TBS-Tween-BSA. Biotinylated goat anti-mouse IgG and streptavidin-AP conjugate were used for detection of the primary antibody.

Data analysis

Data analysis was carried out using the SYSTAT® 8.0 software (SPSS Inc., Chicago, IL). Secretion-to-absorption ratios were calculated by dividing the rate of [^{3}H]ivermectin transport in the BL > AP direction by that in the AP > BL direction. Transport rates were estimated by linear regression analysis on the time course plot of the amount of [^{3}H]ivermectin transported (in kinetics studies) or were obtained as the total amount of [^{3}H]ivermectin transported to the receiver side over 3 h divided by the time of incubation. Data are presented as means ± SD. Statistical analysis was performed using the Student's *t*-test. A $p < 0.05$ was regarded as significant.

Results

The linearity of transport of [^{3}H]ivermectin was confirmed over 3 h at different concentration levels, in both AP > BL and BL > AP directions. The results of the kinetic studies are presented in Fig. 1. Transport in the BL > AP direction was greater than that in the AP > BL direction at each sampling point, indicating a net secretion of [^{3}H]ivermectin from basolateral to apical surfaces of the Caco-2 cell monolayers. The bilateral transport of [^{3}H]ivermectin was measured for a broader range of donor concentrations (0.1, 0.5, 1, 2, 5, 10 and 25 µM) after a 3-h incubation period. Under the same experimental conditions, the secretion-to-absorption ratio of [^{3}H]ivermectin decreased from 10 to 2 with donor concentrations increasing from 0.1 µM to 25 µM.

The net secretion of [^{3}H]ivermectin (1 µM) was abolished at 4°C, with no significant difference between absorptive (0.48 ± 0.11 pmol/3h) and secretory fluxes (0.66 ± 0.07 pmol/3h), supporting the involvement of energy-dependent active transport mechanisms. By comparison, the secretion-to-absorption ratio for 1

µM [^{3}H]ivermectin at 37°C was 4.2 - 4.6.

Inhibition studies showed a significant reduction of the secretory flux of [^{3}H]ivermectin in the presence of all P-gp modulators except digoxin. BL > AP transport of [^{3}H]ivermectin was inhibited by 71%, 40% and 35% by cyclosporin A, verapamil and testosterone, respectively (see Fig. 2A). Cyclosporin A was the most effective modulator, producing a complete inhibition of the net secretion of [^{3}H]ivermectin in all individual experiments (4 independent experiments performed

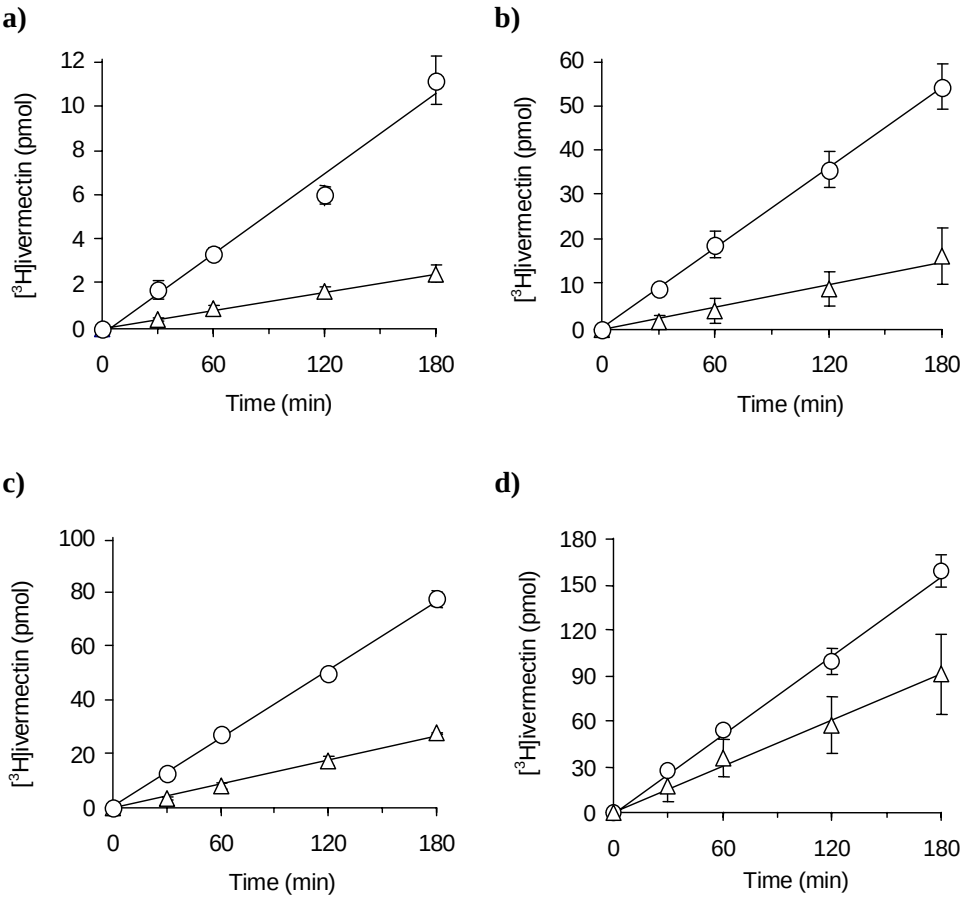

Figure 1. Vectorial transport of [^{3}H]ivermectin across Caco-2 cell monolayers. The monolayers were incubated at 37°C with 1 µM **(a)**, 5 µM **(b)**, 10 µM **(c)** or 25 µM **(d)** [^{3}H]ivermectin added to either the basolateral side (○) or apical (△) side of the inserts. The radioactivity on the opposite side was measured at indicated time points. Each point is the mean ± SD of three monolayers.

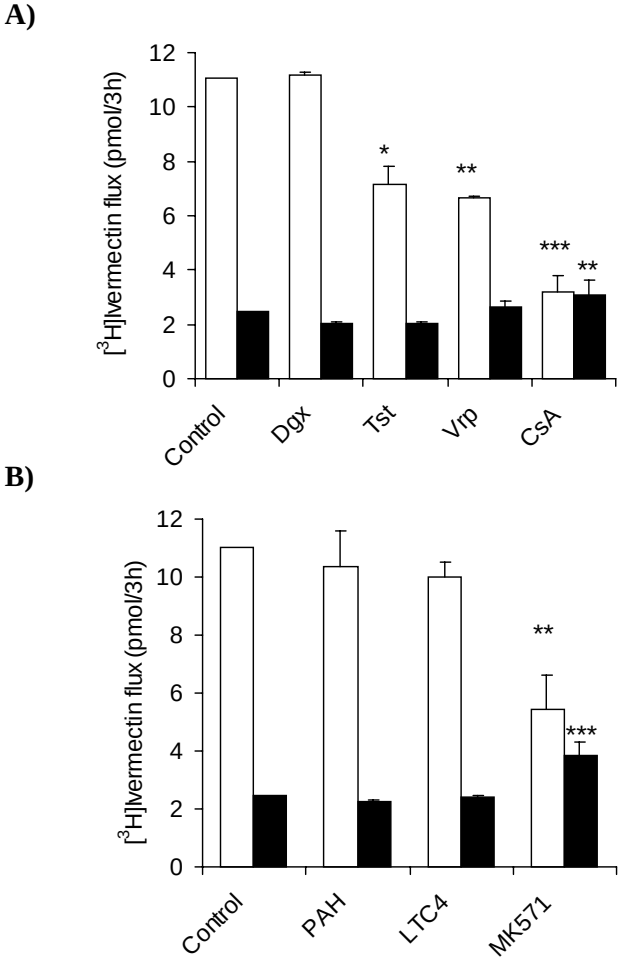

Figure 2. Secretory flux (open columns) or absorptive flux (filled columns) of [^{3}H]ivermectin across Caco-2 cell monolayers in the presence or absence of inhibitors.
A) Digoxin (Dgx, 100 µM), testosterone (Tst, 100 µM), verapamil (Vrp, 100 µM) and cyclosporin A (CsA, 50 µM) were applied as P-gp modulators. **B)** *P*-aminohippurate (PAH, 100 µM), LTC_4 (0.5 µM) and MK571 (100 µM) were applied as MRP2 modulators. [^{3}H]ivermectin (1 µM) was added either to the basolateral or apical compartment, with or without inhibitors added to both sides of the inserts. After a 3-h incubation at 37°C, the radioactivity on the receiver side was measured. Each column represents the mean ± SD of 3 to 9 monolayers. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

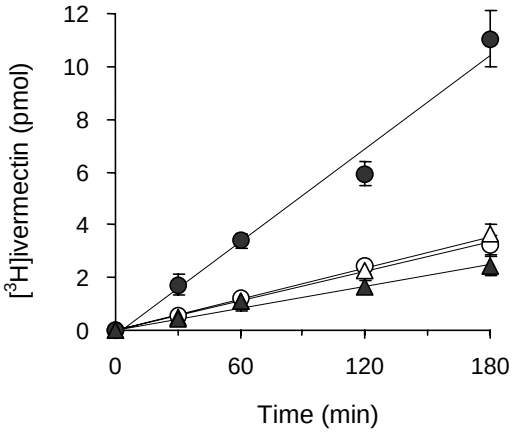

Figure 3. Vectorial transport of [^{3}H]ivermectin across Caco-2 cell monolayers in the absence (filled symbols) or presence (open symbols) of cyclosporin A (50 µM). [^{3}H]ivermectin (1 µM) was added to either the basolateral (●, ○) or apical (▲, △) side of the inserts. The radioactivity on the opposite side was measured at indicated time points. Each point is the mean ± SD of three monolayers.

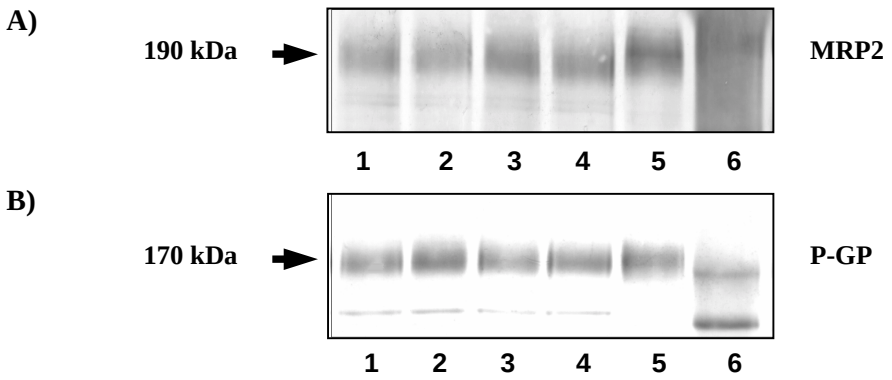

Figure 4. Western immunoblots of MRP2 (**A**) and P-gp (**B**) expression in Caco-2 cells. **Lines 1-4:** cell lysate of 19-day old Caco-2 cells grown on inserts (4 different monolayers); **line 5:** brush-border enriched preparation from Caco-2 cells grown in tissue culture flasks; **line 6:** cell lysate of primary rat hepatocytes as a positive control for P-gp and negative control for MRP2.

in triplicate) (see Figs. 2A and 3). *P*-aminohippurate and LTC_4 had no significant effect on the bilateral transport of [^{3}H]ivermectin at the applied concentrations (Fig. 2B). In contrast, MK571 significantly reduced the secretory flux of [^{3}H]ivermectin by approximately 51% (Fig. 2B shows the mean ± SD of 3 independent experiments performed in triplicate). A significant increase in AP > BL transport was observed in the presence of cyclosporin A (+26%) and MK571 (+57%). The expression of P-gp and MRP2 in Caco-2 cells was confirmed under our experimental conditions by Western immunoblotting (Fig. 4).

Discussion

The present study provides convincing evidence for a polarised transport of ivermectin across Caco-2 cell monolayers in the secretory direction. These findings are in agreement with our previous study in the rat (Laffont et al., 2002) and reinforce the hypothesis of an intestinal elimination pathway for ivermectin from blood into the intestinal lumen.

In the presented experiments, only radioactivity was measured. However, it can be assumed that no substantial amounts of metabolites were produced during the 3-h experiments and that radioactivity was indicative for the amount of parent compound. In general, no appreciable levels of cytochrome P450 3A4 (CYP3A4) activity are found in Caco-2 cells (Boulenc, 1997; Schmiedlin-Ren et al., 1997). This was confirmed under our experimental conditions by measuring the rate of testosterone 6ß-hydroxylation (data not shown). CYP3A4 has been shown to be responsible for the metabolism of ivermectin by human liver microsomes, and no ivermectin metabolites could be detected in its absence (Zeng et al., 1998).

As has been previously reported (Andrew and Halley, 1996), we found that ivermectin could bind to plastic material and glassware. The problem of aspecific binding did not impair the use of the Caco-2 cell model for bilateral transport studies or mechanistic insights. However, no permeability coefficients could be calculated. Furthermore, as aspecific binding was saturated at high concentrations (25 µM), no conclusions could be made on the apparent saturation of BL > AP transport with increased donor concentrations. These considerations question the relevance of previous quantitative studies on ivermectin transport by P-gp, which did not address this problem and nevertheless calculated transport and binding parameters.

The net secretion of ivermectin was totally abolished at 4°C, which is consistent with the involvement of active transport mechanisms. Furthermore, BL > AP transport was significantly reduced by the P-gp inhibitors cyclosporin A, verapamil

and testosterone, indicating that P-gp played a role in the secretion process. The lack of effect of digoxin might be explained by different binding sites on P-gp or by a lower affinity of digoxin for P-gp compared to that of ivermectin which is high (Pouliot et al., 1997). It is noteworthy that the directional transport of ivermectin across Caco-2 cells is less efficient than for other P-gp substrates. By comparison, under the same experimental conditions, we measured a secretion-to-absorption ratio of 23 for rhodamine 123, a prototypic P-gp substrate (data not shown). A less efficient transport of ivermectin is probably due to a higher affinity of ivermectin for P-gp and hence a lower dissociation rate constant (Pouliot et al., 1997), and is consistent with the low plasma clearance of ivermectin encountered *in vivo* (Fink and Porras, 1989; McKellar and Benchaoui 1996).

Our findings are in agreement with our previous study in the rat showing a 50% inhibition of the jejunal secretion of ivermectin after co-administration of verapamil (Laffont et al., 2002). It is noteworthy though that verapamil had no significant effect on ivermectin secretion by the duodenum and ileum in the same animals. Although the use of verapamil *in vivo* is not exclusive in demonstrating a P-gp-mediated process, these results pointed towards the existence of other transport mechanisms. In the present study, the MRP modulator LTC_4 and the organic anion *p*-aminohippurate failed to inhibit ivermectin BL > AP transport, which is in line with the results of previous studies using kidney proximal tubules of killifish or porcine brain endothelial capillaries (Fricker et al., 1999; Nobmann et al., 2001). However, addition of the MRP-selective inhibitor MK571 resulted in a significant inhibition (51%) of the BL > AP flux of ivermectin, suggesting that MRP2 could be involved as well in the secretion process. It can seem surprising then, that cyclosporin A, known as a potent inhibitor of P-gp, totally abolished the net secretion of ivermectin across Caco-2 cells. However, cyclosporin A has been reported to also inhibit MRP2 at high concentrations (Legrand et al., 1998; König et al., 1999). This is the first time an interaction of ivermectin with MRP2 is reported. Such an interaction remains to be confirmed *in vitro* by using MRP2-transfected vesicles as well as *in vivo*. It has been shown that MRP2 is highly expressed in the proximal small intestine, with a gradual decrease in expression from jejunum to distal ileum (Mottino et al., 2000). Therefore, it could significantly contribute to the overall intestinal elimination process.

We identified P-gp as an important mechanism for the intestinal secretion of ivermectin, which is in agreement with the literature. Previous *in vitro* studies using P-gp transfected or overexpressing tumour cell lines demonstrated indeed that ivermectin could be a P-gp substrate (Schinkel et al., 1995; Pouliot et al., 1997). *In vivo*, P-gp is expressed in various tissues other than the intestines,

including the liver, the kidneys, the blood-brain and blood testis barriers and the placenta (Thiebaut et al., 1987; Cordon-Cardo et al., 1989). P-gp has been shown to play a major role in the protection of the brain against the neurotoxicity of ivermectin by restricting its entry in this organ (Schinkel et al., 1994; Kwei et al., 1999). However, its contribution to the elimination of ivermectin had never been clearly investigated. A possible role of P-gp in ivermectin biliary secretion remains to be demonstrated.

From the present *in vitro* studies, it can be expected that the secretory transport of ivermectin by Caco-2 cells could antagonise the absorption of orally-administered ivermectin. *In vivo* however, despite a low oral bioavailability of ivermectin in many target species (cattle, sheep, pig, horse; McKellar and Benchaoui, 1996), this secretory transport may not be of significant relevance. In sheep, ivermectin is 25% bioavailable after intraruminal administration but is 100% bioavailable after intra-abomasal administration (Prichard et al., 1985). It appears that in ruminant species, the major factor limiting oral bioavailability is an extensive binding of the drug to the particulate phase of the rumen digesta (>95% in cattle and sheep; Ali and Hennessy, 1996). A good intestinal absorption of ivermectin could be explained by the saturation of counter-active transport at the local concentrations, high rates of passive diffusion, or the existence of gastrointestinal sites with a lower expression of efflux transporters where drugs can be easily absorbed (Aungst, 1999; Doppenschmit et al., 1999). Furthermore, it must be noted that all these species have a low ivermectin plasma clearance, corresponding to a body extraction ratio of <1%. Even considering the possibility of a synergistic action of intestinal CYP3A isoenzymes and P-gp in reducing even more systemic availability (Benet and Cummins, 2001), it is unlikely that such a low extraction ratio (transport and metabolic processes) could significantly affect the oral bioavailability of this drug. The hypothesis that P-gp could act as a barrier against oral absorption arised in fact from the observation that P-gp-deficient mice showed 3-times higher ivermectin plasma concentrations than wild-type mice after oral administration (Schinkel et al., 1994; Kwei et al., 1999). Ivermectin, however, is relatively well absorbed in mice following oral administration (over 60% of the dose; Kwei et al., 1999), and an improved oral bioavailability cannot explain such an increase in plasma concentrations. Thus, a reduced elimination of ivermectin has to be considered as well.

Finally, our study suggests that ivermectin could interact at the intestinal level with other drugs that are also P-gp substrates (antifungal and antimicrobial agents such as fluoroquinolones and erythromycin, anti-malarial agents, steroids, cardiac drugs … ; Dale, 1999; Matheny et al., 2001). Indeed, P-gp has been shown to be a

major source of pharmacological drug-drug interactions (Dale, 1999). In addition, ivermectin is very persistent in plasma after parenteral or oral administration, irrespective of the species (Fink and Porras, 1989, McKellar and Benchaoui, 1996). Since ivermectin binds with a high affinity to P-gp compared to other P-gp substrates and is a potent modulator of the P-gp function (Pouliot et al., 1997; Eneroth et al., 2001), it can be expected that ivermectin alters the disposition of concomitantly administered drugs rather than the contrary.

In conclusion, our study provides further evidence for the existence of an intestinal secretion of parent ivermectin from blood into the intestinal lumen, and suggests that P-gp, and to a lesser extent MRP2, are involved in the secretion process. These findings are of significant clinical relevance, as they contribute to a better understanding of the efficacy of ivermectin against gastrointestinal parasites in humans and target animal species.

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References

Ali DN and Hennessy DR (1996) The effect of level of feed intake on the pharmacokinetic disposition and efficacy of ivermectin in sheep. *J Vet Pharmacol Ther* **19:**89-94.

Anderle P, Niederer E, Rubas W, Hilgendorf C, Spahn-Langguth H, Wunderli-Allenspach H, Merkle H and Langguth P (1998) P-glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels. *J Pharm Sci* **87:** 757-762.

Andrew NW and Halley BA (1996) Stability of ivermectin in rumen fluids. *J Vet Pharmacol Ther* **19:**295-299.

Aungst BJ (1999) P-glycoprotein, secretory transport, and other barriers to the oral delivery of anti-HIV drugs. *Adv Drug Deliv Rev* **39:**105-116.

Benet LZ and Cummins CL (2001) The drug efflux-metabolism alliance: biochemical aspects. *Adv Drug Deliv Rev* **50:** S3-S11.

Boulenc X (1997) Intestinal cell models: their use in evaluating the metabolism and absorption of xenobiotics. *S.T.P Pharma Sciences* **7:**259-269.

Burkhart CN (2000) Ivermectin: an assessment of its pharmacology, microbiology and safety. *Vet Hum Toxicol* **42:**30-35.

Chiu L, Green ML, Baylis FP, Eline D, Rosegay A, Meriwether H and Jacob TA (1990) Absorption, tissue distribution and excretion of tritium-labeled ivermectin in cattle, sheep and rat. *J Agric Food Chem* **38:**2072-2078.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at the blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86:**695-698.

Dale KY (1999) The contribution of P-glycoprotein to pharmacokinetic drug-drug interactions. *J Clin Pharmacol* **39:**1203-1211.

Denizot F and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89:**271-277.

Doppenschmitt S, Spahn-Langguth H, Gunnar Regardh C and Langguuth P (1999) Role of P-glycoprotein-mediated secretion in absorptive drug permeability: An approach using passive membrane permeability and affinity to P-glycoprotein. *J Pharm Sci* **88:**1067-1072.

Eneroth A, Astrom E, Hoogstraate J, Schrenk D, Conrad S, Kauffmann HM and Gjellan K (2001) Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. *Eur J Pharm Sci* **12:**205-214.

Fink DW and Porras AG (1989) Pharmacokinetics of ivermectin in animals and humans in *Ivermectin and Abamectin* (Campbell WC ed) pp 113-130, Springer, New York.

Ford JM and Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:**155-199.

Fricker G, Gutmann H, Droulle A, Drewe J and Miller DS (1999) Epithelial transport

of anthelmintic ivermectin in a novel model of isolated proximal kidney tubules. *Pharm Res* **16:**1570-1575.

Hirohashi T, Suzuki H, Chu XY, Tamai I, Tsuji A and Sugiyama Y (2000) Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). *J Pharmacol Exp Ther* **292:**265-270.

Hosoya KI, Kim KJ and Lee VH (1996) Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharm Res* **13:**885-890.

Hunter J and Hirst BH (1997) Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv Drug Deliv Rev* **25:**129-157.

König J, Nies AT, Cui Y, Leier I and Keppler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* **1461:**377-394.

Kwei GY, Alvaro RF, Chen Q, Jenkins HJ, Hop CE, Keohane CA, Ly VT, Strauss JR, Wang RW, Wang Z, Pippert TR and Umbenhauer DR (1999) Disposition of ivermectin and cyclosporin A in CF-1 mice deficient in mdr1a P-glycoprotein. *Drug Metab Dispos* **27:**581-587.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680-685.

Laffont CM, Alvinerie M, Bousquet-Melou A and Toutain PL (2001) Licking behaviour and environmental contamination arising from pour-on ivermectin for cattle. *Int J Parasitol* **31:**1687-1692.

Laffont CM, Toutain PL, Alvinerie M and Bousquet-Melou A (2002) Intestinal secretion is a major route for parent ivermectin elimination in the rat. *Drug Metab Dispos* **30:**626-630.

Legrand O, Simonin G, Perrot JY, Zittoun R and Marie JP (1998) P-gp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. *Blood* **12:**4480-4488.

Litman T, Druley TE, Stein WD and Bates SE (2001) From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* **58:**931-959.

Matheny CJ, Lamb MW, Brouwer KL and Pollack GM (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy* **21:**778-796.

McKellar QA and Benchaoui HA (1996) Avermectins and milbemycins. *J Vet Pharmacol Ther* **19:**331-351.

Mottino AD, Hoffman T, Jennes L and Vore M (2000) Expression and localization of multidrug resistant protein mrp2 in rat small intestine. *J Pharmacol Exp Ther* **293:**717-723.

Nobmann S, Bauer B and Fricker G (2001) Ivermectin excretion by isolated functionally intact brain endothelial capillaries. *Br J Pharmacol* **132:**722-728.

Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussault E, Lacroix B,

Simon-Asman P, Haffen K, Fogh J and Zweibaum A (1983) Enterocyte-like differentiation and polarisation of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* **47:**323-330.

Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E *(*1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **53:**17-25.

Prichard RK, Steel JW, Lacey E and Hennessy DR (1985) Pharmacokinetics of ivermectin in sheep following intravenous, intra-abomasal or intraruminal administration. *J Vet Pharmacol Ther* **8:**88-94.

Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, Berns AJM and Borst P (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:**491-502.

Schinkel AH, Wagenaar E, van Deemter L, Mol CA and Borst P (1995) Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96:**1698-1705.

Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Lown KS and Watkins PB (1997) Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1alpha,25-dihydroxyvitamin D3. *Mol Pharmacol* **51:**741-754.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84:**7735-7738.

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350-4354.

Van Asperen J, Van Tellingen O and Beijnen JH (1998) The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacol Res* **37:**429-435.

Walgren RA, Lin JT, Kinne RK and Walle T (2000) Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. *J Pharmacol Exp Ther* **294:**837-843.

Zeng Z, Andrew NW, Arison BH, Luffer-Atlas D and Wang RW (1998) Identification of cytochrome P4503A4 as the major enzyme responsible for the metabolism of ivermectin by human liver microsomes. *Xenobiotica* **28:**313-321.

CHAPTER 3

Intestinal Secretion is a Major Route for Parent Ivermectin Elimination in the Rat

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Abstract

The transepithelial intestinal elimination of ivermectin was studied using the intestinal closed-loop model in the rat. The common bile duct was cannulated, and duodenum, jejunum, and ileum were isolated *in situ* with their intact blood supplies. Following administration of 100, 200 or 400 µg/kg b.wt. ivermectin *via* the carotid artery, the elimination of parent ivermectin into the small intestinal lumen over 90 min was approximately 5-fold higher than in bile. The major amount of secreted ivermectin was recovered in the jejunum, but the duodenum showed a higher intestinal elimination capacity than the other intestinal segments with respect to the intestinal length. Systemic co-administration of the P-glycoprotein blocker verapamil significantly reduced the elimination capacity of jejunum by 50%, which resulted in a 30% decrease of ivermectin overall elimination by the small intestine. In contrast, verapamil did not significantly affect ivermectin secretion in duodenum, ileum, or bile in the same animals. Ivermectin small intestinal and biliary clearances were estimated to account for 27 and 5.5% of the total drug clearance, which was evaluated from a parallel *in vivo* experiment in which rats were given 200 µg/kg b.wt. ivermectin intra-arterially. In conclusion, intestinal secretion plays a greater role than biliary secretion in the overall elimination of ivermectin in the rat, providing major amounts of active drug to the intestinal lumen and to faeces. This is discussed in terms of therapeutic efficacy against intestinal parasites in humans and in animals and of ecotoxicity resulting from the contamination of livestock dung with parent drug.

Introduction

Ivermectin is an anthelmintic of unprecedented efficacy, currently used worldwide to treat and control various parasitic diseases in human and veterinary medicine. It has become the drug of choice in the treatment of a major public disease, human onchocerciasis, and is administered to more than 18 million people each year (Burkhart, 2000). Ivermectin is extensively eliminated by the faecal route as parent drug and metabolites, regardless of the species and of the route of administration with less than 2% excreted in the urine (Campbell, 1985). As the biliary concentration of ivermectin is substantially higher than that in plasma (Bogan and McKellar, 1988; Lifschizt et al., 2000), it has been assumed that biliary secretion was the major pathway of elimination of the parent drug. The study of Hennessy et al. (2000), performed in sheep with the structural analogue doramectin, supported this hypothesis. However, absolute amounts of biliary secreted ivermectin have never been determined in human or animal species. In addition, recent findings have shown that ivermectin was a substrate for some intestinal efflux transporters, which points towards the possible existence of another elimination pathway *e.g.* the intestinal secretion of drug from blood into the intestinal lumen.

P-glycoprotein (P-gp) is a plasma membrane protein, which is able to pump a broad range of structurally and functionally unrelated compounds out of the cell in an energy-dependent manner (reviewed by Sharom, 1997). First identified as a factor for multidrug resistance in mammalian tumour cells (Juliano and Ling, 1976), P-gp has been discovered to be physiologically expressed in a number of tissues including the liver, the intestines, and the blood-brain barrier (Thiebaut et al., 1987; Cordon-Cardo et al., 1989). The strategic distribution of P-gp on the biliary canalicular membrane of hepatocytes and on the apical side of enterocytes provides a mechanistic support for both the biliary and the intestinal secretion of xenobiotics (Hunter and Hirst, 1997; Smit et al., 1998; Van Asperen et al., 1998).

Ivermectin was shown to be actively excreted *in vitro* by multidrug resistant tumour cells (Pouliot et al., 1997) and by cells transfected by the gene coding for P-gp in human or in mouse, *MDR1* and *mdr1a*, respectively (Schinkel et al., 1995; Smith et al., 2000). *In vivo,* the kinetic disposition of ivermectin was modified in mice lacking their P-gp I function (Schinkel et al., 1994; Lankas et al., 1997; Kwei et al., 1999), with a markedly increased accumulation in brain tissue and signs of neurotoxicity. In the intestines, P-gps constitute a barrier against the absorption of orally-administered ivermectin (Kwei et al., 1999), but their role in ivermectin elimination was never documented.

The purpose of the current study was to investigate *in vivo* the existence, and the extent, of a non-biliary intestinal elimination pathway with direct secretion of ivermectin from systemic blood through the gut wall. The involvement of P-gp in ivermectin elimination was assessed by co-administration of verapamil, a prototypic P-gp substrate and inhibitor (Ford and Hait, 1990), which was already shown to affect the *in vivo* disposition of ivermectin in the rat following topical application (Alvinerie et al., 1999).

Materials and Methods

Materials

Ivermectin (IVOMEC INJECTABLE OVIN®) was purchased from Merial (Lyon, France). Verapamil hydrochloride was from Sigma-Aldrich (St. Quentin Fallavier, France), and [^{14}C]-inulin (62.5 µCi/mL) was obtained from PerkinElmer Life Sciences (Boston, MA, USA). All other chemicals used were of the highest grade available.

Animals

Male Wistar rats (Iffa Credo, L'Arbresle, France) weighing 323 ± 30 g were used in the experiments. All the investigations were performed in accordance with the European Regulations for the use of laboratory animals.

Pilot experiments (open-perfusion model)

Two rats were prepared as described below for the closed-loop model (Trial 1), but the intestinal elimination was investigated from a 20-cm jejunal loop only, following systemic administration with 200 or 400 µg/kg b.wt. ivermectin. The jejunal segment was isolated 2 cm distal to the ligament of Treizt, and perfused continuously with thermostated saline at the constant flow rate of 0.6 mL/min determined as optimal by Savina et al. (1981). A catheter was placed at the distal end of the segment, allowing serial recuperation of intestinal perfusates into tubes at 8-min intervals, up to 184 min. The rats were sacrificed at the end of the experiments. The 20-cm segment was excised, opened lengthwise, and the residual content was scraped. Acetonitrile was added to the perfusate samples in order to achieve an acetonitrile:water 4:5 v/v mixture, and all samples were stored at –20°C until analysis.

Trial 1 (intestinal closed-loop model)

After an overnight fast with free access to water, animals were anaesthetised by

i.p. administration of 1.5 g/kg b.wt. urethane and 40 mg/kg b.wt. α-chloralose. Rats were tracheotomised for a facilitated respiration under anaesthesia and placed under a heating lamp. The left carotid artery was cannulated with a polyethylene tube (i.d. 0.58 mm) and perfused using an electric syringe with thermostated (37°C) saline at the constant flow rate of 1 mL/h. A midline abdominal incision was made, and a polyethylene tube (i.d. 0.3 mm) was inserted into the common bile duct, near the hilum of the liver, for bile collection. The segments of the small intestine were exposed *in situ* with their blood supply intact. A polyethylene catheter (25 × 0.9 mm, BD Biosciences, Meylan, France) was placed at the proximal end of the duodeno-jejunum (starting from the pylorus and ending 30 cm above the ileocaecal junction), and the intestinal contents were washed out by perfusing thermostated (37°C) saline for 20 min at the constant rate of 0.6 mL/min. A small incision was made at the distal end of the duodeno-jejunum to allow collection of intestinal contents and effluents. Five minutes before the end of intestinal perfusion, the segment was gently ligated at its distal end and filled with perfusion liquid. The proximal end of the segment was ligated, and one additional ligature was placed 1 cm distal to the ligament of Treitz to separate the duodenum from the jejunum. The two segments were carefully returned to the abdominal cavity. The ileal segment (30 cm) was prepared following the same procedure. Care was taken not to interrupt the normal blood supply of the segments during their preparation. The abdominal cavity was then covered with a compress soaked with saline to prevent heat loss and evaporation. The perfusion of the carotid artery with saline was interrupted and ivermectin was injected rapidly (1min) *via* the catheter at the dose of 100, 200 or 400 µg/kg b.wt. (ivermectin was diluted in polyethylene glycol 400 to fit low dose rates). Saline was perfused again during 90 min.

In the competition experiments, the i.a. perfusion of saline was stopped 5 min prior to ivermectin administration, and verapamil was given at a loading rate of 24 mg/kg b.wt. per hour during 5 min *via* the carotid artery. Ivermectin was then injected i.a. at the standard dose of 200 µg/kg b.wt., and verapamil was perfused again at 4.8 mg/kg b.wt. per hour and during 90 min.

Bile was collected throughout the experiment. Animals were sacrificed at 90 min after ivermectin administration. Blood was previously collected by cardiac puncture in some treated and non-treated rats. The intestinal segments were excised, their length measured, and they were opened lengthwise. The whole intestinal content was carefully collected using a cell scraper, and 3 mL of acetonitrile was added to the samples. The scraping procedure was very gentle to remove the mucous layer with minimal disruption of the intestinal cells. Blood

samples were centrifuged, and plasma was collected. Biliary, plasma and intestinal samples were stored at –20°C until analysis. Five to six rats were used to test each dose level of ivermectin in the non-competition experiments, and seven rats were used in the competition experiments.

Another group of animals (n = 8) was taken for an accurate estimation of the area under the plasma concentration-time curve between 0 and 90 min post-administration. Rats were anaesthetised as described above, but no other surgical procedure was performed. Blood was sampled at 2, 5, 15, 30, 60 and 90 min after i.a. administration of 200 µg/kg b.wt. ivermectin.

Validation of the study model of intestinal elimination

To determine the extent of non-specific paracellular transport, three rats were prepared as described for the closed-loop model. They were injected with 9 µCi of [^{14}C]-inulin *via* the carotid artery. At 90 min post-administration, the intestinal content was collected from duodenum, jejunum, and ileum, and radioactivity was measured in a beta scintillation counter (Kontron Beta V, Montigny Le Bretonneux, France).

Trial 2: determination of ivermectin total (plasma) clearance

Forty-two rats were used to evaluate ivermectin total clearance. Rapid anaesthesia was achieved by i.p. injection of 87 mg/kg b.wt. xylazine and 13 mg/kg b.wt. ketamine. The left carotid artery was cannulated, and ivermectin was injected *via* the catheter at the dose of 200 µg/kg b.wt. The rats began to recover 30 min after the beginning of anaesthesia. At 2, 5, 15, 30, 60, 90 min, 4, 8, 24 h, and 2, 3, 4, 5, 7, 9, 12, 15, 18 days post-administration, two to three rats were taken for terminal blood sampling performed by cardiac puncture under anaesthesia (xylazine and ketamine, i.p.).

Analytical assay

Ivermectin was analysed using a high-performance liquid chromatography method with automated solid phase extraction and fluorescence detection, as previously described by Alvinerie et al. (1987). Minor modifications were made to fit analysis of low plasma and bile volumes (extraction of 100 µL of plasma or bile).

The intestinal samples were sonicated twice for 20 min. After 2 min of centrifugation at 2000 *g*, the clean supernatant was transferred into a first tube, and the pellet was resuspended in 3 mL of acetonitrile, sonicated for 20 min, and centrifuged (2000 *g*, 2 min). The resulting clean supernatant was removed into a

second tube. Deionised water was added to the tubes to achieve an acetonitrile/water 4:5 v/v mixture. Solid phase extraction was then performed as described by Alvinerie et al. (1987).

Ivermectin H_2B1a (22,23 dihydroavermectin B1a) was designated as analyt. The limit of quantification of the high-performance liquid chromatography method was 0.05 ng/mL. Accuracy and precision (intra-assay variation) expressed as relative standard deviation were less than 8 and 6%, respectively.

Pharmacokinetic and statistical analysis

The plasma concentrations were fitted using non-linear regression analysis (SYSTAT ® 8.0, SPSS Inc., Chicago, IL). The areas under the mean plasma concentration-time curve, AUC (0-t_{last}) (from 0 to the last quantifiable sample) and AUC (0-90) (from 0 to 90 min), were calculated using the trapezoidal rule. Ivermectin total clearance was obtained by dividing the administered dose (200 µg/kg) by AUC (0-t_{last}). Biliary and intestinal clearances were computed as the ratio of the total amount of drug (H_2B1a) eliminated within 90 min into bile and in the small intestinal lumen, respectively, divided by the corresponding plasma AUC (0-90) obtained in Trial 1. The mean residence time (MRT), the plasma terminal half-life ($t_{1/2\lambda z}$), and the steady-state volume of distribution (V_{ss}) were calculated according to the classical pharmacokinetic equations associated with non-compartmental analysis (Gibaldi and Perrier, 1982).

The results were expressed as means ± SD. Analysis of variance was used to test the significance of differences between the three intestinal segments and the three dose levels (SYSTAT ® 8.0). Post hoc comparisons were performed using the Bonferroni test. A *t* test was done to compare verapamil-treated and non-treated rats A $p < 0.05$ was regarded as significant.

Results

Validation of the study model

Less than 0.1% of the administered radioactivity was detected in any of the intestinal segments of rats receiving labelled inulin, indicating that the integrity of intestinal epithelium was preserved under the described experimental conditions.

Pilot experiments (open-perfusion model)

The perfusion of the 20-cm jejunal segment with saline resulted in low basal concentrations of parent ivermectin in most perfusate samples. However, very high concentrations of parent ivermectin were observed in intestinal effluents each time

some intestinal mucous had leaked accidentally *via* the distal catheter into the sample. These results indicate that ivermectin was largely associated with mucous. Following systemic administration of 200 and 400 µg/kg ivermectin, 111 and 163 ng ivermectin were eliminated unchanged from the 20-cm loop, respectively. About 65 and 74% of these amounts were obtained from perfusate samples, and 35 and 26% by collection of residual mucous in the lumen of the loop.

Trial 1 (closed-loop model)

At the end of the 90-min experiments, the contents of the closed intestinal loops consisted almost exclusively of mucous containing high amounts of ivermectin as parent drug. The total amount of ivermectin recovered in the small intestinal lumen exceeded systematically (from 3.2 to 8.8 times) that eliminated in bile over the same 90-min period, in the same animal (Table 1). The ratio of intestinal-to-biliary elimination did not show any significant difference between the tested doses (Table 1). The cumulative amount of ivermectin excreted into bile and in the small

Table 1. *Total amounts (ng/kg b.wt.) of ivermectin eliminated into bile and in the small intestinal lumen over 90 min post-administration in rat, and biliary and intestinal clearances.*

	Dose (µg/kg)		
	100	**200**	**400**
Bile (ng/kg)	170 ± 71 [a]	389 ± 111 [b]	667 ± 152 [c]
Biliary clearance (mL/day/kg)		55.7	
Small intestine (ng/kg)	862 ± 368 [a]	1936 ± 204 [b]	3526 ± 669 [c]
Intestinal clearance (mL/day/kg)		277.2	
- Duodenum (ng/kg)	146 ± 34 [a]	338 ± 124 [b]	511 ± 161 [b]
- Jejunum (ng/kg)	578 ± 318 [a]	1320 ± 134 [b]	2239 ± 541 [b]
- Ileum (ng/kg)	138 ± 32 [a]	277 ± 62 [b]	776 ± 172 [c]
Ratio $_{\text{small intestine/bile}}$	5.2 ± 1.5 [a]	5.4 ± 1.8 [a]	5.5 ± 2.0 [a]

Ivermectin was administered intra-arterially at the doses of 100 (n = 6), 200 (n = 5) and 400 µg/kg b.wt. ivermectin (n = 5). The data are presented as means ± SD. Comparisons were performed between the dose levels for each parameter. Values followed by the same superscript ([a, b] or [c]) are not statistically different ($p > 0.05$).

intestinal lumen within 90 min following i.a. administration of ivermectin accounted for approximately 1% of the administered dose, independently of the dose level. The bile flow rate was homogenous among the rats and equal to 0.84 ± 0.13 mL/h.

Over the dose range tested, the intestinal elimination of ivermectin was greater in the jejunum than in the other intestinal segments (Table 1). However, when normalised by the length of the segment, ivermectin elimination was statistically higher in the duodenum at the doses of 100 and 200 µg/kg and statistically lower in the ileum at the dose of 100 µg/kg, in the same animal (Fig. 1).

Co-administration of the P-gp inhibitor, verapamil, significantly reduced the overall small intestinal elimination of ivermectin by 28% (1397 ± 378 versus 1936 ± 204 ng/kg in controls, $p < 0.05$) but did not statistically affect ivermectin secretion into bile (346 ± 129 versus 389 ± 111 ng/kg in controls). The elimination capacity of the jejunum was markedly reduced by 50% in the treated rats compared with the controls, whereas no statistical difference could be observed with respect to the rate of elimination in the duodenum or in the ileum (Fig. 2). Plasma

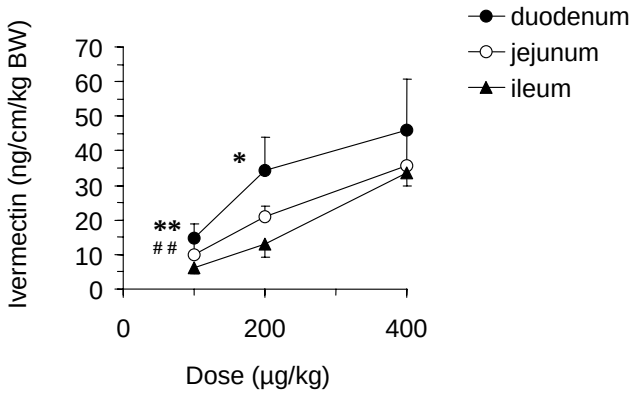

Figure 1. Segmental elimination of ivermectin through the gut wall following intra-arterial administration to rats at the doses of 100 (n = 6), 200 (n = 5) and 400 µg/kg b.wt. ivermectin (n = 5). The data (mean ± SD) correspond to the elimination capacities of the intestinal segments, which were obtained by dividing the total amount of parent drug eliminated in duodenum, jejunum, or ileum over 90 min by the length of the corresponding segment. * ($p < 0.05$) and ** ($p < 0.01$) indicate a significant difference between duodenum and jejunum in the same animal, whereas $^{\#\#}$ ($p < 0.01$) indicates a significant difference between jejunum and ileum.

concentrations of ivermectin at 90 min post-administration were not significantly different between treated rat (82 ± 9.6 ng/mL) and non-treated rats (71 ± 9.6 ng/mL). No signs of toxicity were detected in any of the seven rats treated with verapamil under our experimental conditions.

The second group of rats taken for an accurate estimation of AUC (0-90) showed plasma levels of ivermectin very similar to those obtained in the rats given 200 µg/kg ivermectin in the closed-loop study. The AUC (0-90) was equal to 168 ng.h/mL. Small intestinal and biliary clearances *in situ* were 0.277 and 0.056 L/day/kg, respectively.

Trial 2

The plasma data were best fitted with a triexponential concentration-time curve (Fig. 3). The values of AUC (0-t_{last}) and AUC (0-90) were 2233 ng.h/mL and 355 ng.h/mL, respectively. The MRT, $t_{1/2\ \lambda z}$ and V_{ss} were 1.09 days, 2.51 days and 2.34 L/kg, respectively. Ivermectin total clearance was found to be equal to 2.15 L/day/kg. Trial 2 was referred to as an *in vivo* situation, considering that xylazine and ketamine produced a short-duration surgical anaesthesia with moderate side effects.

The difference between the AUCs (0-90) in Trial 2 and that in Trial 1 (twice

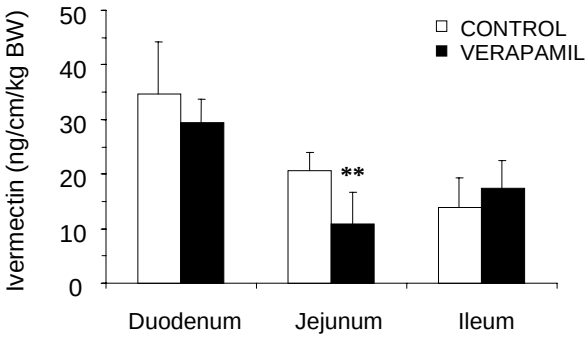

Figure 2. Influence of verapamil on the elimination capacities of the intestinal segments in rats given 200 µg/kg b.wt. i.a. ivermectin. The data (mean ± SD) correspond to the total amounts of parent drug eliminated in each intestinal segment over 90 min post-administration in treated (n = 7) and non-treated rats (n = 5), divided by the length of the segment. ** ($p < 0.01$).

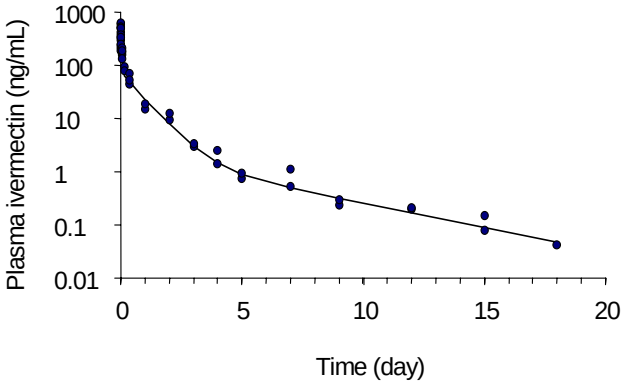

Figure 3. Ivermectin plasma concentration-time profile following intra-arterial administration of rats (n = 42) with 200 µg/kg ivermectin. Each point corresponds to one rat.

lower) shows that anaesthesia with urethane and α-chloralose, albeit required by the model, most likely influenced the plasma disposition of ivermectin. This was taken into account for the assessment of ivermectin total (plasma) clearance in the rats used in Trial 1. Assuming the homogeneity of the rats used in Trials 1 and 2, the total plasma clearance of ivermectin in Trial 1 (*in situ*) was estimated from that obtained in Trial 2 (*in vivo*) corrected by the ratio of the AUCs (0-90) *e.g.* 1.02 L/day/kg. It results that the small intestinal clearance of ivermectin accounted for 27% of the corrected total (plasma) clearance versus 5.5% for the biliary clearance.

Discussion

At all three different ivermectin dose rates, the amount of parent ivermectin eliminated in the small intestinal lumen was 5 times higher than in bile. Since conjugates of parent ivermectin have never been detected in bile, this study clearly demonstrates that the major route for the elimination of parent ivermectin in the rat intestine is not biliary but intestinal secretion. Such a difference between intestinal and biliary elimination has been already observed for other drugs such as roxithromycin (Arimori et al., 1998), ciprofloxacin (Dautrey et al., 1999) and vinblastine (Van Asperen et al., 2000).

The open-perfusion model is commonly used to study the intestinal secretion of

drugs, as it enables measurement of an overall intestinal elimination by keeping the reabsorption phenomenon negligible (Dautrey et al., 1999). The use of this model was, however, not desirable in the present study given the extensive binding of ivermectin to intestinal mucous. Indeed, the random leakage of mucous in perfusate samples observed in pilot experiments impairs a reliable and reproducible quantification of the drug intestinal elimination. Consequently, we used the alternative closed-loop model, of which the advantage was to extend the investigation from 20-cm loops (open-perfusion model) to the totality of the small intestine, allowing thereby a more reliable prediction of the *in vivo* state. The resulting drawback was, however, a possible reabsorption into blood of the ivermectin secreted in the intestinal lumen and thus the measurement of the net intestinal elimination of drug compared with the overall biliary secretion. It is therefore possible that the intestine-to-bile elimination ratio might be slightly underestimated.

On the other hand, it cannot be excluded that the gentle scraping procedure for collection of intestinal contents resulted in a slight overestimation of ivermectin intestinal elimination. This procedure was necessary to ensure collection of the totality of the mucous present in the lumen as it is known to be partly bound to the glycocalyx layer of enterocytes. However, this technique implies that some intestinal mucosa tissue might be scraped as well. In pilot experiments (open-perfusion model), up to 74% of the drug eliminated by a 20-cm loop was already obtained from perfusate samples and not by scraping. Thus, even if the contribution of intestinal mucosa tissue to the total collection of ivermectin cannot be excluded, this would not affect the conclusions of the study. The experiments were terminated 90 min after ivermectin administration, before a deterioration of animal condition and depletion of bile salts could have been able to affect the bile flow, and thus the extent of ivermectin elimination in bile.

Compared with the other intestinal segments, the jejunum provided the major source of parent ivermectin regardless of the administered dose. However, the intestinal elimination capacity (expressed per unit of intestinal length) appeared to decrease gradually from proximal-to-distal small intestine, with maximal values in the duodenum. Regional differences could possibly be attributed to differences in passive diffusion, expression levels of efflux transporters, or in carrier affinity (Makhey et al., 1998), but other factors should be considered as well in the interpretation of data such as a different proportion of villi in the intestinal segments (Olivier et al., 1998). Data were expressed per unit of intestinal length and not of intestinal area, since the loop diameter along the segments was too variable to be properly assessed.

In duodenum and jejunum, increasing the dose from 200 to 400 µg/kg did not result in a significantly higher absolute elimination of ivermectin, which is consistent with the involvement of an active (saturable) transport mechanism. As the P-gp substrate and inhibitor verapamil significantly inhibited the elimination of ivermectin into the jejunum by 50%, the involvement of a P-gp carrier-mediated process in the jejunum can be assumed. In contrast, neither the i.a. perfusion of verapamil nor a i.a. bolus administration of 5 mg/kg verapamil (data not shown) could significantly affect ivermectin elimination in bile, duodenum, or ileum in the same animals. It is likely that, at the applied low (non-toxic) dosages, verapamil did not completely block the P-gp function. However, these results also suggest that other efflux mechanisms might play a significant role in the secretion process. This would be in agreement with the observations of Kwei et al. (1999) who systemically administered 200 µg/kg b.wt. ivermectin to mdr1a-P-gp-deficient mice with a non-cannulated gallbladder and reported only a partial 30% reduction (from 3.4 to 2.4% of the administered dose) of the cumulative ivermectin excreted in bile plus intestinal contents, compared with wild-type mice. Additional *in vitro* or *ex vivo* studies should clarify the potential role of P-gp and/or other carriers without the drawbacks related to the use of P-gp inhibitors *in vivo* and highlight the differences between intestinal segments.

Finally, qualitative analysis of chromatograms indicated the ability of the intestinal epithelium to excrete ivermectin-derived metabolites (data not shown). As for the parent drug, this should be further investigated *in vitro*.

Biliary and small intestinal clearances of ivermectin were found to account for 5.5 and 27% of ivermectin total (plasma) clearance, respectively. Intestinal clearance was calculated solely from the small intestine, but other regions of the digestive tract may participate as well in the elimination process. Ivermectin elimination by distal parts of the digestive tract has never been investigated thus far, but colon has been reported to display high levels of P-gp expression (Thiebaut et al., 1987) and therefore could provide a significant contribution to the absolute intestinal elimination of ivermectin as for other xenobiotics (Mayer et al., 1996; Ramon et al., 1996; Makhey et al., 1998; Van Asperen et al., 2000). Besides these considerations, our findings suggest an important metabolism of ivermectin in the rat. The proportion of metabolites in faecal drug residues has never been documented in rat following systemic administration, but is known to represent approximately 60 and 70% in cattle and swine, respectively (Halley et al., 1989), which would be consistent with our results.

Our study suggests the existence of an intestinal route of elimination of ivermectin in human and target animal species. The observations reported in cattle

by Bogan and McKellar (1988) support this hypothesis. Following subcutaneous administration, ivermectin was found at rather high concentrations in small intestinal mucous with no significant difference between mucous distal and proximal to the bile duct opening. Furthermore, ileal fluid showed three times higher concentrations of ivermectin than in intestinal fluids sampled proximal to the bile duct. In contrast, following systemic administration with the structural analogue doramectin to sheep, Hennessy et al. (2000) recovered 130% of the administered dose in bile. This would imply that bile was the major elimination pathway, which is difficult to conciliate with our results apart from interspecies differences.

The characterisation of an intestinal elimination pathway in human and target animal species would be of therapeutic significance. Not all parasites feed on plasma, and the compartmental distribution of drugs out of the plasma in secondary compartments (bile, intestinal secretions) needs to be taken into consideration to discuss and optimise the efficacy of antiparasitic drugs. It has been suggested that ivermectin could be available to parasites in intestinal mucous (Bogan and McKellar, 1988). Consequently, an extensive elimination of ivermectin along the digestive tract following systemic absorption or parenteral administration would provide high concentrations of drug at the site of action. It is also possible that the mucous functions as a reservoir for exchange of drug into intestinal fluid.

Besides clinical considerations, it is noteworthy that a large part of the ivermectin excreted in cattle dung (38% of the dose after systemic administration; Laffont et al., 2001) may arise from intestinal secretion. The presence of parent (active) ivermectin in faeces can have deleterious effects on non-target organisms such as some dung-degrading and dung-breeding insects (Wall and Strong, 1987; Sommer et al., 1993). Although the issue of environmental impact of ivermectin used in large scale in cattle is still under debate, our study provides new directions into the pharmacokinetic behaviour of endectocides to reduce the faecal excretion of parent drug. The therapeutically desirable intestinal secretion should be then evaluated against the undesirable exposure of the environment with faeces containing ivermectin.

In conclusion, we have provided evidence that the major route for the elimination of parent ivermectin in the rat intestine is not biliary but intestinal secretion, which enforces the role of the intestines in the elimination of xenobiotics. *In vitro* studies are now underway to clarify the mechanisms involved in ivermectin intestinal elimination. A better understanding of these mechanisms is of interest for the further development of endectocides to optimise their safety and efficacy.

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References

Alvinerie M, Sutra JF, Galtier P and Toutain PL (1987) Determination of ivermectin in milk by high performance liquid chromatography. *Ann Rech Vet* **18:**269-724.

Alvinerie M, Dupuy J, Eeckhoutte C and Sutra JF (1999) Enhanced absorption of pour-on ivermectin formulation in rats by co-administration of the multidrug-resistant-reversing agent verapamil. *Parasitol Res* **85:**920-922.

Arimori K, Miyamoto S, Fukuda K, Nakamura C and Nakano M (1998) Characteristic difference in gastrointestinal excretion of clarithromycin and roxithromycin. *Biopharm Drug Dispos* **19:**433-438.

Bogan JA and Mc Kellar QA (1988) The pharmacodynamics of ivermectin in sheep and cattle. *J Vet Pharmacol Ther* **11:**260-268.

Burkhart CN (2000) Ivermectin: an assessment of its pharmacology, microbiology and safety. *Vet Hum Toxicol* **42:**30-35.

Campbell WC (1985) Ivermectin: an update. *Parasitol Today* **1:**10-16.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at the blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86:**695-698.

Dautrey S, Felice K, Petiet A, Lacour B, Carbon C and Farinotti R (1999) Active intestinal elimination of ciprofloxacin in rats : modulation by different substrates. *Br J Pharmacol* **127:**1728-1734.

Ford JM and Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:**155-199.

Gibaldi M and Perrier D (1982) *Pharmacokinetics* (Swarbrick J ed; 2nd edn). Dekker M, New York.

Halley BA, Nessel RJ and Lu AYH (1989) Environmental aspects of ivermectin usage in livestock: general considerations, in *Ivermectin and Abamectin* (Campbell WC ed) pp 162-172, Springer, New York.

Hennessy DR, Page SW and Gottschall D (2000) The behaviour of doramectin in the gastrointestinal tract, its secretion in bile and pharmacokinetic disposition in the peripheral circulation after oral and intravenous administration to sheep. *J Vet Pharmacol Ther* **23:**203-213.

Hunter J and Hirst BH (1997) Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv Drug Deliv Rev* **25:**129-157.

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455:**152-162.

Kwei GY, Alvaro RF, Chen Q, Jenkins HJ, Hop CEAC, Keohane CA, Ly VT, Strauss JR, Wang RW, Wang Z, Pippert TR and Umbenhauer DR (1999) Disposition of ivermectin and cyclosporin A in CF-1 mice deficient in mdr1a P-glycoprotein. *Drug Metab Dispos* **27:**581-587.

Laffont CM, Alvinerie M, Bousquet-Mélou A and Toutain PL (2001) Licking behaviour and environmental contamination arising from pour-on ivermectin for cattle. *Int J Parasitol* **31:**1687-1692.

Lankas GR, Cartwright ME and Umbenhauer D (1997) P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicol Appl Pharmacol* **143:**357-365.

Lifschitz A, Virkel G, Sallovitz J, Sutra JF, Galtier P, Alvinerie M and Lanusse C (2000) Comparative distribution of ivermectin and doramectin to parasite location tissues in cattle. *Vet Parasitol* **87:**327-338.

Makhey VD, Guo A, Norris DA, Hu P, Yan J and Sinko PJ (1998) Characterisation of the regional intestinal kinetics of drug efflux in rat and in human intestine and Caco-2 cells. *Pharm Res* **15:**1160-1167.

Mayer U, Wagenaar E, Beijnen JH, Smit JW, Meijer DKF, Van Asperen J, Borst P and Schinkel AH (1996) Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr1a P-glycoprotein. *Br J Pharmacol* **119:**1038-1044.

Olivier RE, Jones AF and Rowland M (1998) What surface of the intestinal epithelium is effectively available to permeating drugs? *J Pharm Sci* **87:**634-639.

Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E *(*1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **53:**17-25.

Ramon J, Dautrey S, Farinoti R, Carbon C and Rubinstein E (1996) Excretion of ciprofloxacin into the large bowel of the rabbit. *Antimicrob Agents Chemother* **40:**11-13.

Savina PM, Staubus AE, Gaginella TS and Smith DF (1981) Optimal perfusion rate determined for in situ intestinal absorption studies in rats. *J Pharm Sci* **70:**239-243.

Schinkel AH, Smit JJM, Van Tellingen O, Beijnen JH, Wagenaar E, Van Deemter L, Mol CAAM, Van der Valk MA, Robanus-Maandag EC, Te Riele HPJ, Berns AJM and Borst P (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell **77:**491-502.

Schinkel AH, Wagenaar E, Van Deemter L, Mol CAAM and Borst P (1995) Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96:**1698-1705.

Sharom FJ (1997) The P-glycoprotein efflux pump: how does it transport drugs? *J Membr Biol* **160:**161-175.

Smit JW, Schinkel AH, Weert B and Meijer DKF (1998) Hepatobiliary and intestinal clearance of amphiphilic cationic drugs in mice in which both *mdr1a* and *mdr1b* genes have been disrupted. *Br J Pharmacol* **124:**416-424.

Smith AJ, Van Helvoort A, Van Meer G, Szabó K, Welker E, Szakács G, Váradi A, Sarkadi B and Borst P (2000) MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. *J Biol Chem* **275:**23530-23539.

Sommer C, Gronvold J, Holter P and Nansen P (1993) Effects of ivermectin on two afrotropical dung beetles, *Onthophagus gazella* and *Diastellopalpus quinquedens* (Coleoptera: Scarabaeidae). *Vet Parasitol* **48:**171-179.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localisation of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84:**7735-7738.

Van Asperen J, Van Tellingen O and Beijnen JH (1998) The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacol Res* **37:**429-435.

Van Asperen J, Van Tellingen O and Beijnen JH (2000) The role of mdr1a P-glycoprotein in the biliary and intestinal secretion of doxorubicin and vinblastine in mice. *Drug Metab Dispos* **28:**264-267.

Wall R and Strong L (1987) Environmental consequences of treating cattle with the antiparasitic drug ivermectin. *Nature* **327:**418-421.

CHAPTER 4

Comparative Evaluation of P-gp Activity in Cattle, Sheep, Goats, Pigs, Horses and Rats using a Lymphocyte-based *Ex Vivo* Model

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Abstract

Peripheral blood lymphocytes (PBL) were used to evaluate the expression of functional P-gp in cattle, sheep, goat, horse, pig and rat. Flow cytometric analysis of rhodamine-123 (Rh-123) dye efflux and its inhibition by prototypic P-gp inhibitors were used to quantify P-gp activity. Human PBL were included in the study as a reference. The efflux of Rh-123 could be demonstrated in lymphocytes from all species, but considerable interspecies and interindividual variations were observed. The average efflux of Rh-123 over 2.5 h at 37°C was 78 ± 18.1% for goats, 55 ± 11.2% for cattle, 49 ± 12.0% for sheep, 38 ± 6.8% for pigs, 30 ± 3.9% for horses and 17 ±11.7% for rats *vs* 55 ± 4.0% for men. Verapamil (40 µM), cyclosporin A (20 µM) and ivermectin (1 µM) clearly ($p < 0.001$) inhibited the efflux of Rh-123 in PBL of goats, cattle, sheep, pigs and men. These inhibitors had an overall significant effect ($p<0.05$) on Rh-123 efflux in PBL from rats and horses, but the inhibition was not obvious in all animals tested. In human PBL, similar inhibition of Rh-123 efflux by P-gp modulators and the human-specific P-gp antibody MRK-16 suggests that this inhibition reflects P-gp activity. Using the monoclonal antibody C219, Western-blots confirmed the expression of P-gp in PBL from goats, sheep, cattle and pigs but not from rats and horses, and in intestinal tissue (jejunum) of all animal species. In conclusion, this study demonstrates the expression of functional P-gps in lymphocytes of all animal species studied. However, the large interindividual variations in P-gp activity suggest that additional wide-scale studies are performed. This PBL-based model offers new perspectives to study the P-gp function in the different animal species at a population level.

Introduction

The drug transporting P-glycoproteins (P-gps) are now recognised as major determinants of disposition for numerous pharmacologic agents (Tanigawara, 2000; Matheny et al., 2001; Borst and Elferink, 2002). P-gp is an efflux trasnporter localised in the plasma membrane, which uses ATP to extrude a broad range of structurally and functionally unrelated substances from the cell (Borst and Elferink, 2002). Among the P-gp substrates are antibiotics (erythromycin, fluoroquinolones), antiparasitic drugs (ivermectin and probably moxidectin), steroids (cortisol, dexamethasone), anti-diarrheal agents (loperamide), immunosuppressive agents (cyclosporin A) and anticancer drugs (*Vinca* alkaloids, epipodophyllotoxines, taxanes, anthracyclines) (Matheny et al., 2001; Borst and Eferink, 2002; Lifschitz et al., 2002). P-gp was originally identified in mammalian tumour cells as overexpressed proteins conferring multidrug resistance (Juliano and Ling, 1976). It has been shown to be also expressed in various normal tissues, including the liver, the kidneys, the intestines, the blood-brain and blood-testis barriers, and the placenta (Thiebaut et al., 1987; Cordon-Cardo et al., 1989). It has become clear that P-gp plays an important role in the protection of the body against the potentially toxic compounds arising from the environment. P-gp limits the uptake of drugs and xenotoxins from the gastrointestinal tract, protects critical organs such as the brain and the fœtus against an undesirable drug exposure, and enhances drug elimination by biliary, renal or intestinal secretion (Tanigawara, 2000; Matheny et al., 2001; Borst and Elferink, 2002).

P-gp is encoded by the two genes in humans (MDR1 and MDR3) and three genes in rodents (mdr1a, mdr1b and mdr2), but only the human MDR1 P-gp and the murine mdr1a and mdr1b isoforms appear to be involved in drug transport (Borst and Elferink, 2002). Five P-gp genes have been identified in pigs, four of which show similarity with the MDR1, mdr1a and mdr1b homologues (Childs and Ling, 1996). Recently, the first ruminant P-gp gene was cloned and sequenced from sheep (Longley et al., 1999). In cattle, the presence of three P-gp genes is expected based on genomic DNA analysis (Childs and Ling, 1996). However, although the physiological role of P-gp has been extensively documented in humans and laboratory animals, little information is available about the P-gp function in other animal species. In pigs and cattle, expression of functional P-gps has been shown in freshly isolated brain capillaries or primary cultures of brain capillary endothelial cells (Fontaine et al., 1996; Miller et al., 2000; Nobmann et al., 2001). In contrast, none or very low P-gp activity was detected in the porcine kidney proximal tubule cell line (LLC-PK1) (Schinkel et al., 1995). It is difficult

though to appreciate the extent of the P-gp function *in vivo* from immortalised cell lines or primary cells, since protein expression is often modified during cell culture, varying from total loss of expression to overexpression.

The purpose of this study was to measure the P-gp function directly in the animals. Peripheral blood lymphocytes have been shown to express functional P-gps in humans (Drach et al., 1992; Klimecki et al., 1994; Ludescher et al., 1998) and are widely used to study and monitor the evolution of multidrug resistance in leukaemic patients. Therefore, we isolated peripheral blood lymphocytes from various animal species (cattle, sheep, goat, pig, horse and rat) and evaluated the P-gp function by flow cytometric analysis. The fluorescent dye Rhodamine (Rh-123) has been extensively used to measure P-gp activity in human lymphocytes (Beck et al., 1996; Canitrot et al., 1996; Huet et al., 1998; Broxterman, 1999; Parasrampuria et al., 2001). In the present study, this activity was evaluated upon the extent of Rh-123 efflux from the cells and the ability of P-gp blockers to reduce dye efflux. Verapamil, cyclosporin A and ivermectin were selected as efficient modulators of the P-gp function (Ford and Hait, 1990; Pouliot et al., 1997; Eneroth et al., 2001). Furthermore, expression of P-gp was examined by Western-blot analysis in lymphocytes and intestinal tissue (jejunum) of all animal species studied.

Materials and Methods

Materials

Rhodamine 123 (Rh-123), verapamil hydrochloride, cyclosporin A, ivermectin and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture media and reagents were from Gibco BRL (Breda, the Netherlands). Ficoll Hypaque (Ficoll Paque®) was purchased from Pharmacia Biotech (Uppsala, Sweden). Monoclonal P-gp antibodies, MRK-16 and C219, were obtained from Kamiya Biomedical Co. (Seattle, WA, USA) and Fujirebio Diagnostics, Inc. (Malvern, PA, USA), respectively. Biotinylated goat anti-mouse IgG and streptavidin-AP conjugate were from Zymed (CA, USA) and Boehringer (Mannheim, Germany), respectively. All other chemicals used were of the highest grade available. Stock solutions of Rh-123 (40 mM), verapamil (40 mM), cyclosporin A (20 mM) and ivermectin (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until analysis.

Animals

All the investigations were performed in accordance with the ethical rules for animal experimentation.

Blood samples were taken from healthy animals belonging to the Veterinary Faculty of Utrecht University. A total of 7 to 10 animals per species were used for functional studies: 7 pigs (3 boars 120-250 kg; 4 young castrated pigs, 30-40 kg), 7 cows (350-550 kg), 10 ewes (40-50 kg), 10 female goats (30-35 kg), 7 horses (3 mares, 500-550 kg; 2 stallions, 450 and 490 kg, 2 castrated males, 540 and 560 kg), 7 rats (Wistar males, 300-450 g). Four healthy human volunteers (men, 30-40 years old) registered as blood donors at the Academic Hospital Utrecht (UMC) were included in the studies after obtaining informed consent. In the case of rats, blood was collected by terminal cardiac puncture following anaesthesia with a pentobarbital solution containing heparin, and intestinal tissue (jejunum) was collected for Western-blot analysis. For other species, intestinal tissues were taken from animals of various origin at public slaughter houses.

Isolation of peripheral blood mononuclear cells

Peripheral blood (20-25 mL in all species except rat: 8-10 mL) was obtained from each individual and collected into heparinised tubes. Peripheral blood mononuclear cells were isolated on a Ficoll density gradient by centrifugation for 20 min at 2,000 rpm at room temperature (no brake). Interphase cells were collected, washed twice, and resuspended in phenol red-free RPMI-1640 medium buffered with 20 mmol/L HEPES, pH 7.4, and supplemented with 10% fetal calf serum (FCS). Samples were analysed for the P-gp function on the same day within 2 to 3 hours. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere until the beginning of transport studies.

Measurement of Rh-123 efflux

This functional assay measures the ability of cells to extrude the fluorescent dye Rh-123 and determines whether or not this efflux, if any, is blocked by P-gp inhibitors. After centrifugation, peripheral blood mononuclear cells (approximately 5.0 x 10^6 cells/mL) were stained with 4 µM of Rh-123 for 30 min at 37°C in serum-free RPMI-1640 medium (without phenol red). The cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in ice-cold, dye-free, serum-free RPMI 1640 in the presence or absence of P-gp inhibitors to allow Rh-123 efflux. Final concentrations for the inhibitors were 20 µM for cyclosporin A, 40 µM for verapamil and 1 µM for ivermectin. These concentrations were selected from previous published studies (cyclosporin A and verapamil; Wang et al., 2000; Parasrampuria et al., 2001) or following preliminary titration studies (ivermectin, data not shown). The final concentration of DMSO in the samples was adjusted at 0.1%. Cells were incubated for 2.5 h at 37°C, protected from light by aluminium

foil, and cell-associated Rh-123 fluorescence was measured by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) at time 0 and every 30 min thereafter up to 2.5 h. Dead cells were excluded based on propidium iodide uptake. Fluorescence channel-1 (530 nm) was used for evaluating Rh-123-associated cellular fluorescence, fluorescence channel-3 (610 nm) for propidium iodide signal. The samples were gated on forward scatter *vs* size scatter to allow selection of the lymphocyte population and exclude clumps and debris. After gating the propidium iodide-negative population of cells, it was possible to measure the intracellular fluorescence of Rh-123 only in living lymphocytes. At least 5000 suitable events per sample were collected. Data acquisition was performed using the computer program CELLQUEST. Cells that had not been exposed to Rh-123 were used as a negative control. The percentage of efflux of Rh-123 was calculated as follows:

$$\text{Efflux}_{(t)}\,(\%) = 100 \times [mFI_0 - mFI_{(t)}] / mFI_0 \qquad \text{Eq. 1}$$

where $mFI_{(t)}$ and mFI_0 are the median fluorescence intensity of Rh-123 at time *t* and time 0, respectively. The percentage of Rh-123 efflux in the presence and absence of inhibitors was plotted as a function of time between 0 and 2.5 h, and the corresponding areas under the curve ($AUC_{Inhibitor}$ and $AUC_{Control}$, respectively) were calculated. Percentages of inhibition were determined for each individual, using the following equation:

$$\text{Inhibition}\,(\%) = 100 \times (1 - AUC_{Inhibitor} / AUC_{Control}) \qquad \text{Eq. 2}$$

Human peripheral blood lymphocytes were used as a positive control. Efflux studies were performed as described above using the peripheral blood mononuclear cells obtained from 4 healthy volunteers. The monoclonal P-gp antibody MRK-16 was used to evaluate the efficacy of verapamil, cyclosporin A and ivermectin in the blockade of P-gp at the applied concentrations. MRK-16 recognises a human-specific epitope of the extracellular domain of P-gp, and its binding blocks the P-gp function. To ensure that the antibody had correctly bound to P-gp, the decrease in Rh-123 cellular fluorescence was analysed 30 min after resuspending the stained cells in dye-free medium. $AUC_{Inhibitor}$ and $AUC_{Control}$ were thus calculated from t = 30 min to t = 2.5 h.

Immunoblot analysis of P-gp expression in lymphocytes and intestinal tissues

Lymphocytes were isolated from blood following lysis of erythrocytes. Polynuclear cells and monocytes were excluded from the cell suspension by

adhesion to plastics following serial incubation in tissue culture flasks. This isolation procedure was preferred above the Ficoll gradient method, albeit more appropriate for functional studies, as the latter does allow complete removal of erythrocytes. Briefly, 40 mL of ice-cold lysis buffer [155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM Na_2EDTA (pH 7.4)] were added to 10-15 mL of chilled blood. After 10 min of incubation and gentle mixing on ice, samples were centrifuged (600 *g*, 5 min, 4°C). Cells were resuspended in 5 mL of ice-cold lysis buffer and were incubated on ice for another 10 min. After adding 45 mL of ice-cold PBS, cells were collected by centrifugation (267 *g*, 5 min, 4°C). After two washing steps at 4°C with RPMI 1640 containing 10% FCS (centrifugation at 167 *g*, 10 min, 4°C), cells were resuspended in 10 mL of RPMI 1640 containing 10% FCS. Serial incubations (1 h) in two tissue culture flasks allowed removal of polynuclear cells and monocytes, which had attached to the plastics. The remaining lymphocyte suspension was collected, counted, and washed with PBS. After the last centrifugation step, the pellet was resuspended in 100-150 µL of RIPA-buffer and was stored at –20°C until use. Brush-border-enriched proteins were prepared from intestinal tissues accordingly to a previously described method (Pinto et al., 1983). The human colon adenocarcinoma cell line Caco-2 was used as a positive control for P-gp expression. Caco-2 cells (purchased from the ATCC, passage 90) were routinely cultured in supplemented Dulbecco's modified Eagle's medium and maintained at 37°C in a humidified 5% CO_2 atmosphere. The cells were grown in tissue culture flasks for 19 days, replacing medium three times per week, after which they were scraped in PBS, washed, and lysed in RIPA buffer. Samples were stored at –20°C until use.

Protein concentrations were determined using the Pierce BCA protein assay reagent (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as a standard. Forty µg of protein of each sample were loaded in each lane and electrophoresed on SDS-polyacrylamide gels (4% stacking and 8% running gel) in a Mini-Protean III Cell (Biorad, Veenendaal, The Netherlands), as described by Laemmli (1970). Four µg of proteins from intact Caco-2 cells or brush-border-enriched preparations of Caco-2 cells were also loaded. Electroblotting was performed onto a polyvinylidene difluoride membrane according to the method of Towbin et al. (1979). The transfer membrane was then washed and incubated overnight at 4°C with the P-gp monoclonal antibody C219 used in a dilution of 1:200 in TBS (Tris-buffered saline) containing 0.3% (v/v) Tween 20 and 1% (w/v) BSA to prevent aspecific binding of the antibody. Biotinylated goat anti-mouse IgG and streptavidin-AP conjugate were used for detection of the primary antibody.

Statistical analysis

All results are presented as means ± SD. Statistical analysis was carried out by using a one-way ANOVA to compare the efflux of Rh-123 (without inhibitor) between species, and a two-way ANOVA (treatment, individual) to assess the effect of treatment by P-gp inhibitors in a given species. Multiple comparisons were performed using the Bonferroni test. A $p < 0.05$ was regarded as significant.

Results

Functional studies with Rh-123

The results of Rh-123 efflux studies in the different species are summarised in Fig. 1 (Fig. 2A for men) and Table 1. Lymphocytes of all species effluxed the fluorescent dye Rh-123 over 2.5 h, however wide interspecies variations could be observed. It is noteworthy that in all species, the efflux of Rh-123 was particularly important over the first 30 min of incubation. This is probably due to a high passive diffusion of the dye resulting from a high concentration gradient between intracellular and extracellular compartments.

The species could be classified into three groups depending on a low, medium and high Rh-123 efflux. The highest decrease in Rh-123 intracellular levels was obtained in goat lymphocytes (78 ± 18.1 % after 2.5 h), with a complete efflux of the dye in three of the ten animals (93-97%). A rather high but significantly lower efflux of Rh-123 was observed in lymphocytes from horses, pigs, sheep, cattle and men (30 ± 3.9 %, 38 ± 6.8%, 49 ± 12.0%, 55 ± 11.2% and 55 ± 4.0% after 2.5 h, respectively). In contrast, lymphocytes from rats showed a poor efflux of Rh-123 of 17 ± 11.7% after 2.5 h. This efflux was significantly lower than for other animal species except horse. However, wide interindividual variations could be observed among rats: in 4 rats, lymphocytes effluxed 16 - 39% of the dye, while in the 3 other rats, lymphocytes retained almost completely Rh-123 (4 - 10% of efflux).

Figure 1 (next page). Time-dependent efflux of Rh-123 by peripheral blood lymphocytes of **a)** rats (n = 7), **b)** horses (n = 7), **c)** pigs (n = 7), **d)** sheep (n = 10), **e)** cattle (n = 7), and **f)** goats (n = 10). After staining with 4 µM Rh-123 at 37°C for 30 min, freshly isolated lymphocytes were resuspended in dye-free medium in the absence (●) or presence of the P-gp inhibitors verapamil (40 µM; ○), cyclosporin A (20 µM, △) and ivermectin (1 µM, □). Efflux of Rh-123 was measured over 2.5 h of incubation at 37°C. Data are presented as means ± SD.

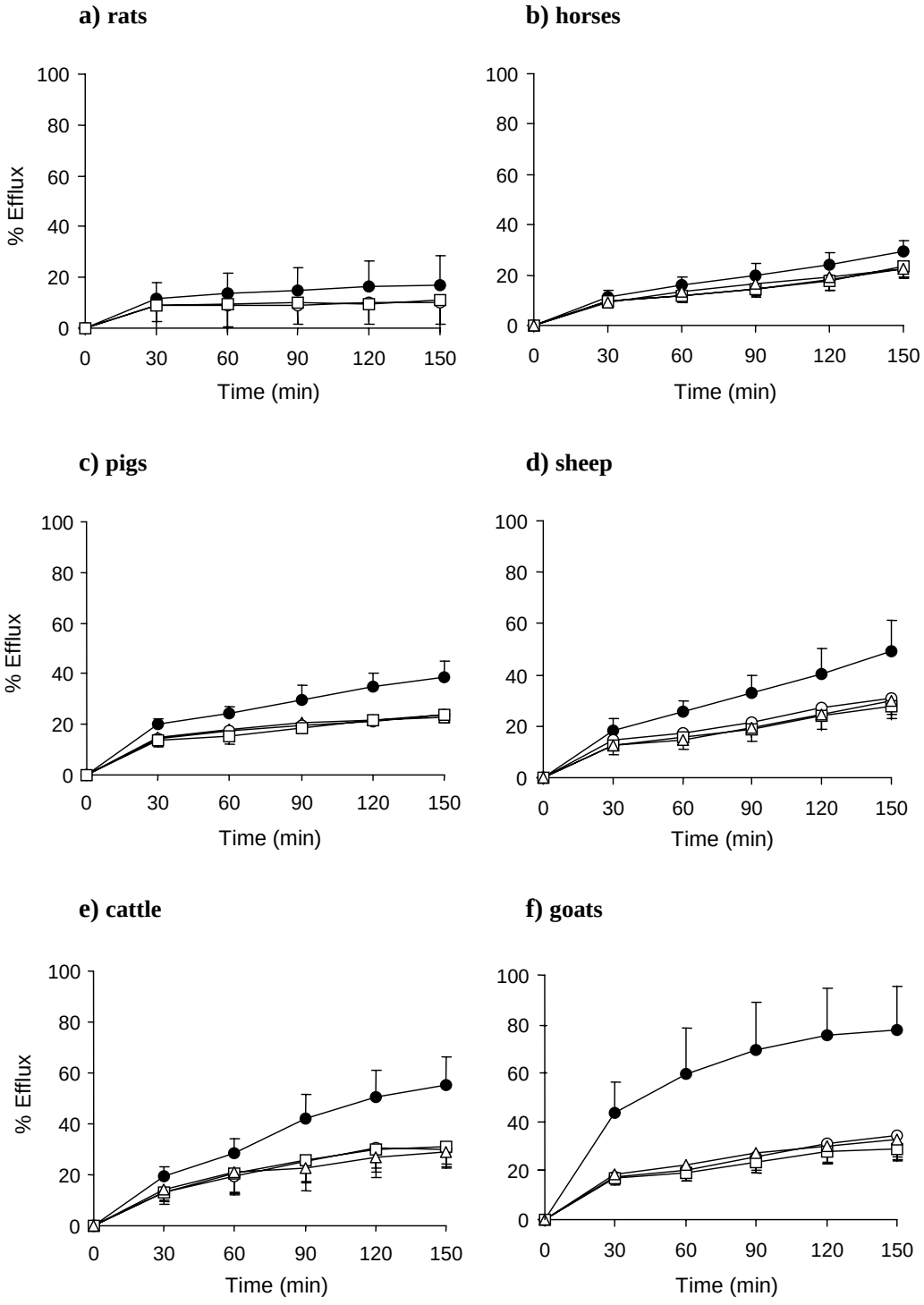

a) rats
b) horses
c) pigs
d) sheep
e) cattle
f) goats
% Efflux
Time (min)
0
20
40
60
80
100
0
30
60
90
120
150

Table 1. *Efflux of the fluorescent dye Rh-123 by peripheral blood lymphocytes of goats (n=10), cattle (n=7), sheep (n=10), pigs (n=7), horses (n=7) and rats (n=7). Men (n=4) were included in the study as a reference.*

Species	Efflux of Rh-123 after 2.5 h (%)	Range (%)	Inhibition (%)		
			Verapamil	Cyclosporin A	Ivermectin
Goat	78 ± 18.1	46 – 97	66 ± 11.8	64 ± 9.7	66 ± 9.7
Man	55 ± 4.0	50 – 58	52 ± 9.7	52 ± 7.7	54 ± 6.9
Cattle	55 ± 11.2	35 – 68	39 ± 10.2	42 ± 12.3	38 ± 10.3
Sheep	49 ± 12.0	33 – 72	31 ± 12.2	39 ± 11.4	38 ± 10.6
Pig	38 ± 6.8	31 – 48	32 ± 14.8	29 ± 16.8	34 ± 15.2
Horse	30 ± 3.9	22 – 34	23 ± 13.7	19 ± 12.4	23 ± 14.4
Rat	17 ±11.7	4 – 39	50 ± 14.8	—	41 ± 31.3

After staining with 4 µM Rh-123 at 37°C, freshly isolated lymphocytes were resuspended in dye-free medium in the absence or presence of the P-gp inhibitors verapamil (40 µM), cyclosporin A (20 µM) and ivermectin (1 µM) to allow dye efflux. Efflux of Rh-123 was measured over 2.5 h of incubation at 37°C. Data are presented as means ± SD.

To determine whether P-gp was responsible for the efflux of Rh-123, stained lymphocytes were incubated in the presence of the P-gp modulators verapamil (40µM), cyclosporin (20 µM) and ivermectin (1 µM). P-gp modulators had an overall significant effect on Rh-123 efflux in all species (albeit at the limit of significance in rats). A clear inhibition ($p < 0.001$) of dye efflux was observed in lymphocytes of goats (approximately 65%), cattle (40%), sheep (36%), pigs (32%) and men (53%) (Figs. 1 and 2A; Table 1). The inhibition of Rh-123 efflux was also statistically significant in lymphocytes of rats and horses ($p < 0.05$), but was obvious in only 4 of the 7 horses (2 males and 2 mares) and 3 of the 7 rats. On the basis of these considerations, we concluded to only low levels of P-gp activity in lymphocytes of rats and horses.

Verapamil (40 µM), cyclosporin A (20 µM) and ivermectin (1 µM) inhibited Rh-123 efflux to a similar extent. Indeed, no statistical difference in inhibition could be observed between the different modulators, irrespective of the species. One exception, however, was found in rats for cyclosporin A, which resulted in a higher dye efflux than for control lymphocytes. As the use of propidium iodide

supports the integrity of the plasma membrane, no convincing explanation of this leakage has been found and the corresponding data were not included in the analysis.

The relevance of the inhibition of Rh-123 efflux by the three P-gp modulators was examined in human lymphocytes, by using the human-specific monoclonal antibody MRK-16, which blocks the P-gp function. To ensure that the antibody had correctly bound to P-gp, the analysis of Rh-123 efflux was performed not immediately after resuspending stained cells in dye-free medium but after 30 min of incubation. The resulting profiles of inhibition are presented in Fig. 2B. They show no significant difference in the decrease of Rh-123 intracellular fluorescence between samples treated with MRK-16, verapamil, cyclosporin A and ivermectin.

Wide interindividual variations were generally observed within a given species (see the range of Rh-123 efflux in Table 1). These were particularly obvious in goats, cattle, sheep, pigs and rats, in which the efflux of Rh-123 could vary by a factor 2 among individuals (a factor 10 in rats). In pigs, a striking and significant difference in P-gp activity was observed between young castrated animals (30-35

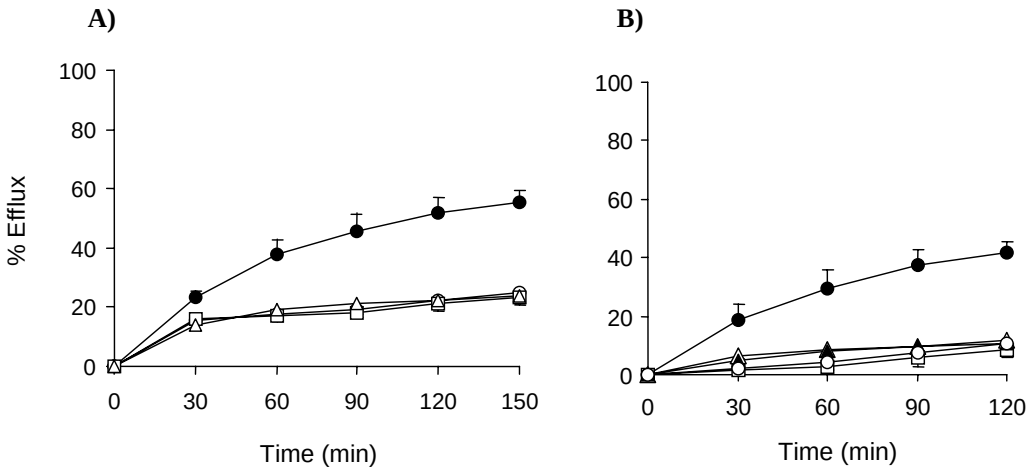


Figure 2. Efflux of Rh-123 by human peripheral blood lymphocytes (4 healthy volunteers). **A)** Rh-123 efflux in the absence (●) or presence of the P-gp inhibitors verapamil (40 µM; ○), cyclosporin A (20 µM, △) and ivermectin (1 µM, □) after staining with 4 µM Rh-123. **B)** Comparison of the inhibition of Rh-123 efflux by the human-specific monoclonal antibody against P-gp MRK-16 (▲), verapamil (40 µM; ○), cyclosporin A (20 µM, △) and ivermectin (1 µM, □) in human lymphocytes. Data are presented as means ± SD.

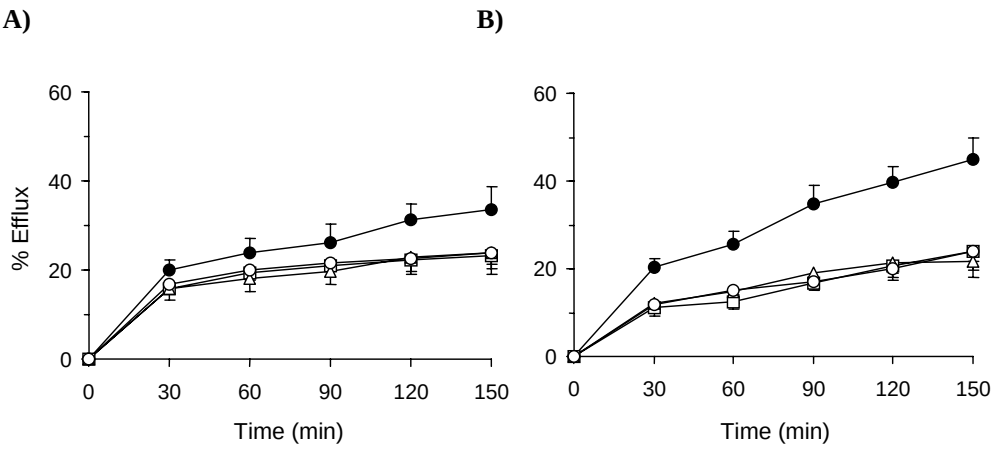
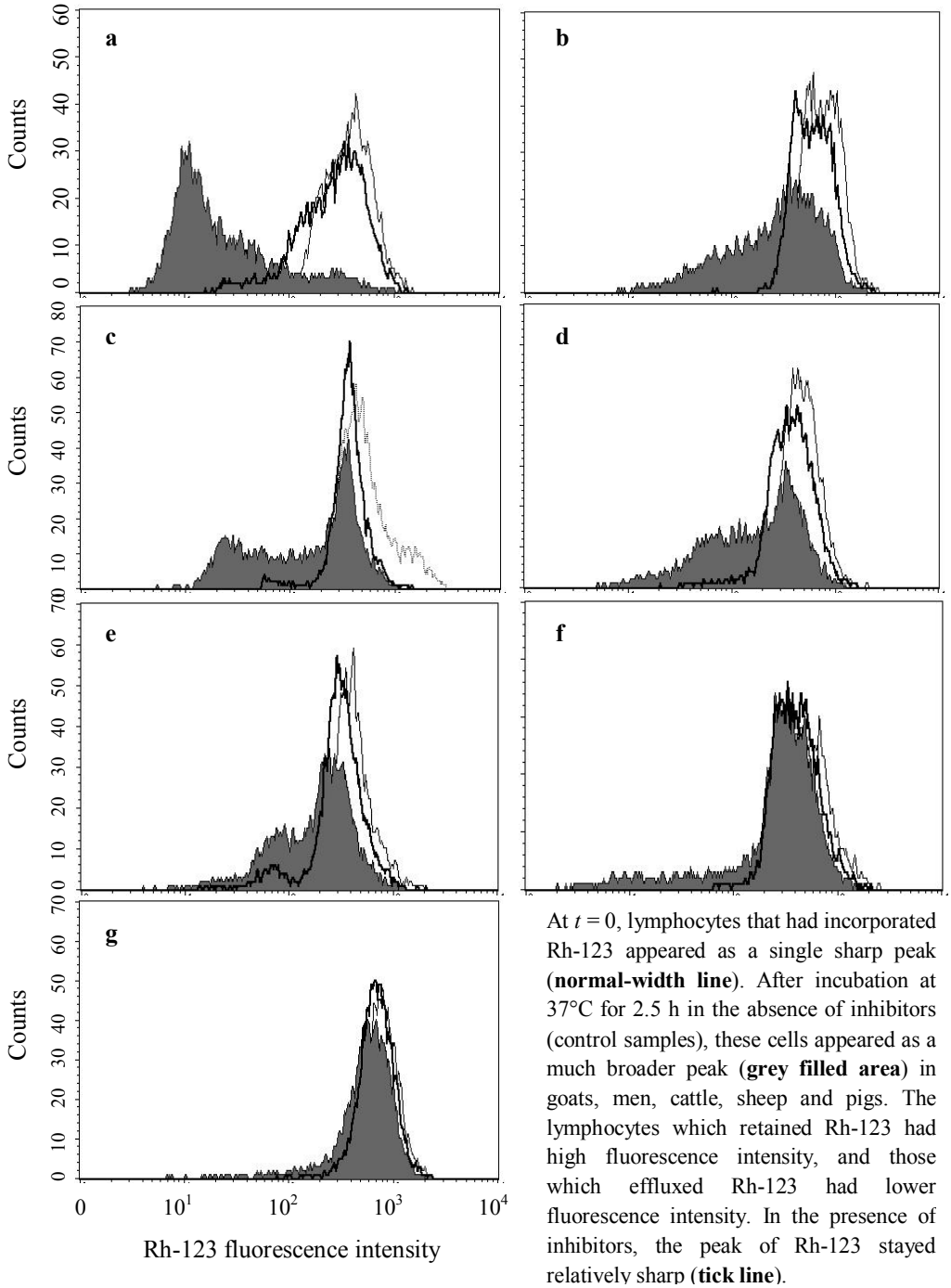


Figure 3. Comparison of Rh-123 efflux by lymphocytes derived from 4 young castrated pigs (**A**) and 3 older boars (**B**). After staining with 4 µM Rh-123 at 37°C, freshly isolated lymphocytes were resuspended in dye-free medium in the absence (●) or presence of the P-gp inhibitors verapamil (40 µM; ○), cyclosporin A (20 µM, △) and ivermectin (1 µM, □). Efflux of Rh-123 was measured over 2.5 h of incubation at 37°C. Data are presented as means ± SD.

kg) and older boars (200-300 kg) under our experimental conditions (Fig. 3). The boars (n=3) showed a higher efflux of Rh-123 (45 ± 5.0% *vs* 34 ± 2.1% for the young castrated pigs; $p < 0.05$), which was also more sensitive to P-gp inhibitors (46% *vs* 23% of inhibition, respectively; $p < 0.05$).

The histograms obtained by flow cytometric analysis for a representative animal of each species are presented in Fig. 4. At $t = 0$, lymphocytes that have incorporated Rh-123 appear as a single sharp peak. After incubation at 37°C for 2.5 h in the absence of inhibitors (control samples), these cells appear as a much broader peak, indicating an heterogeneous distribution of Rh-123 fluorescence in the cell population. These results suggest a differential efflux of Rh-123 in subsets of lymphocytes due to a different P-gp activity.

Figure 4 (next page). Representative sample histograms obtained after flow cytometric analysis of Rh-123 retention in lymphocytes of **a)** goats, **b)** men, **c)** cattle, **d)** sheep, **e)** pigs, **f)** horses and **g)** rats. *X axis:* cellular-associated fluorescence intensity of Rh-123 (logarithmic scale). *Y axis:* counted events (arithmetic scale).



At $t = 0$, lymphocytes that had incorporated Rh-123 appeared as a single sharp peak (**normal-width line**). After incubation at 37°C for 2.5 h in the absence of inhibitors (control samples), these cells appeared as a much broader peak (**grey filled area**) in goats, men, cattle, sheep and pigs. The lymphocytes which retained Rh-123 had high fluorescence intensity, and those which effluxed Rh-123 had lower fluorescence intensity. In the presence of inhibitors, the peak of Rh-123 stayed relatively sharp (**tick line**).

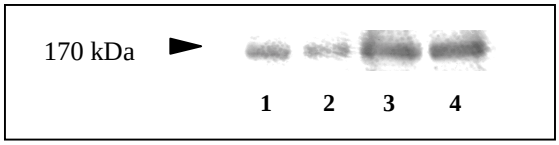


Figure 5. Western-blot analysis of P-gp expression in peripheral blood lymphocytes from goats using the monoclonal antibody C219. Four individual animals are represented.

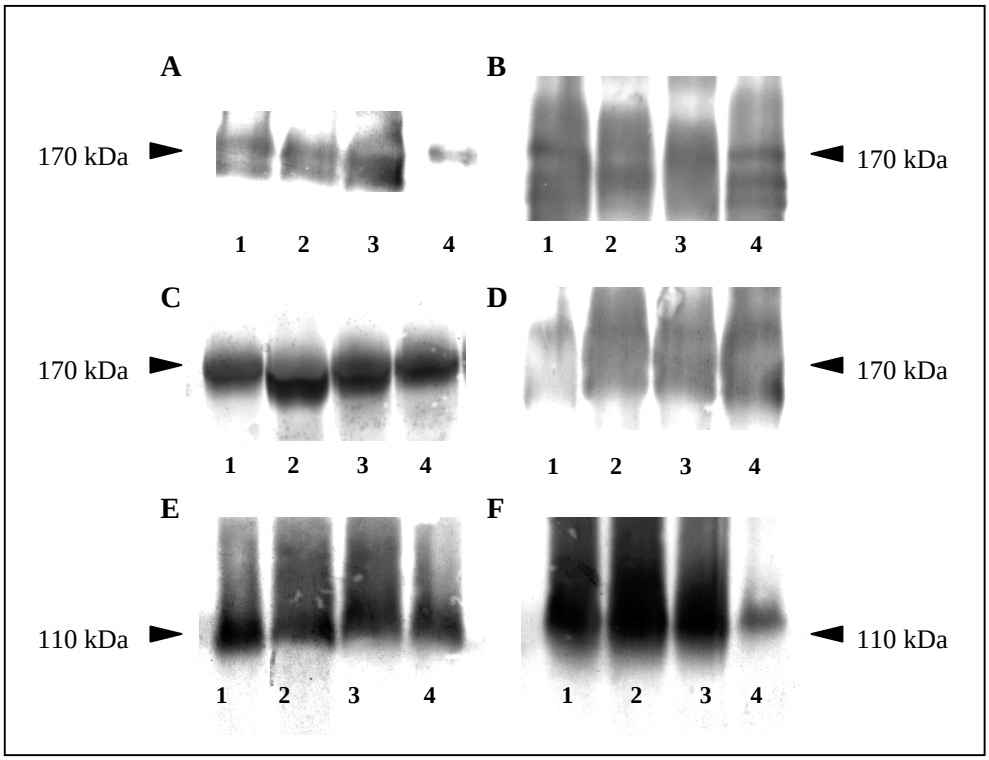


Figure 6. Western-blot analysis of P-gp expression in small intestinal tissue (jejunum) from **A)** horses, **B)** pigs, **C)** goats, **D)** sheep, **E)** rats, and **F)** cattle, using the monoclonal antibody C219. Four individual animals are represented for each species.

P-gp expression by Western-blot analysis

The expression of P-gp in lymphocytes and intestinal tissue (jejunum) was examined using the C219 human monoclonal antibody to P-gp. Western-blot analysis clearly demonstrated the expression of P-gp in goat lymphocytes (Fig. 5). P-gp expression could also be detected in lymphocytes from sheep, cattle and pigs, but the 170-kDa band obtained was weaker than for goats (data not shown). In contrast, no P-gp expression could be detected in lymphocytes from horses and rats (data not shown). In intestinal tissues of goats, pigs, sheep and horses, C219 interacted with a band of protein of approximately 170 kDa molecular weight (Fig. 6). In intestinal tissues of rats and cattle, however, C219 interacted with a band of protein of 90-110 kDa molecular weight (Fig. 6). No aspecific binding was found.

Discussion

The purpose of the study was to assess the expression of functional P-gps in freshly isolated peripheral blood lymphocytes from cattle, sheep, goats, horses, pigs and rats. The mitochondria-binding fluorescent dye Rh-123 was shown to be a reliable marker of the P-gp function in human lymphocytes, on the basis of both the efflux of Rh-123 and its inhibition by P-gp modulators (Beck et al., 1996; Broxterman, 1999). According to these criteria, three groups of species with high, medium or low P-gp activity could be identified. Goat lymphocytes clearly showed higher P-gp activity than other species, with an average efflux of Rh-123 of 78% after 2.5 h of incubation at 37°C. Under the same experimental conditions, lymphocytes from pigs, sheep, cattle and men displayed a lower but marked efflux of Rh-123 of 38-55%. In all these species, P-gp activity was confirmed by clear inhibition of Rh-123 efflux by the P-gp modulators verapamil, cyclosporin A, and ivermectin from 32% to 65%. A less obvious but still significant P-gp activity could be detected in lymphocytes from horses and rats. Approximately 30% of the intracellular Rh-123 was effluxed by horse lymphocytes after 2.5 h of incubation at 37°C *vs* 17.7% for rat lymphocytes. However, although P-gp modulators had an overall significant effect in reducing dye efflux in these species, the inhibition of Rh-123 efflux was observed only in 4 of the 7 horses and 3 of the 7 rats. It must be stressed that the low activity of P-gp in rat and horse lymphocytes was detected because AUCs from $t = 0$ to $t = 2.5$ h were measured. By comparison, Schluesener et al. (1992) did not find any significant efflux of Rh-123 in rat lymphocytes but used a single time point for the measurement of dye efflux.

Measurement of Rh-123 efflux was preferred to dye accumulation studies, which are more convenient but theoretically less sensitive (Canitrot et al., 1996;

Huet et al., 1998). Even though, our functional assay may not be sensitive enough to detect very low levels of P-gp activity, which may be a problem in rats. Rh-123 has been shown to be one of the most specific and sensitive dyes for evaluation of P-gp function (Broxterman, 1999). However, it was unable to allow detection of P-gp activity in some tumour cell lines expressing low P-gp levels (Canitrot et al., 1996). Another factor limiting the sensitivity of the method may be a heterogeneous efflux of Rh-123 by the overall population of lymphocytes. This was observed on sample histograms from men, goats, cattle, sheep and pigs obtained after incubation of stained lymphocytes in dye-free medium without inhibitors. Previous reports have indicated a differential expression of P-gp in subsets of human lymphocytes (Drach et al., 1992; Klimecki et al., 1994; Ludescher et al., 1998). These results suggest that this is also probably the case in the studied animal species.

Verapamil and cyclosporin A are widely used as P-gp inhibitors in transport studies. However, the resulting inhibition of R-123 efflux may not reflect the overall P-gp activity. In the present study, no statistical difference in inhibition was observed between verapamil, cyclosporin A and ivermectin at the applied concentrations. Furthermore, in human lymphocytes, the monoclonal P-gp antibody MRK-16 blocked Rh-123 efflux to a similar extent than the three P-gp modulators, suggesting that this inhibition was representative for the P-gp function. To ensure that the antibody had correctly bound to P-gp, the comparison in the decrease of Rh-123 cellular fluorescence was done 30 min after resuspending the stained lymphocytes in dye-free medium. Calculation of AUCs from t = 30 min to t = 2.5 h gave a percentage of inhibition of approximately 78% for ivermectin, cyclosporin A and verapamil in human lymphocytes. This percentage of inhibition is higher than that obtained by calculation of AUCs from t = 0 to t = 2.5 h (53%). One explanation for this may be that the applied inhibitors did not immediately block the P-gp pumps at t = 0. It is also possible that the actual contribution of P-gp in dye efflux was initially lower due to a higher passive diffusion of Rh-123 and/or the saturation of P-gp at the initially high intracellular Rh-123 concentrations. The analysis of Rh-123 efflux from t = 30 min to t = 2.5 h gave higher percentages of inhibition in all animal species (82% inhibition in goats, 52% in cattle, 51% in sheep, 48% in pigs and 33% in horses) but did not change the conclusions of the comparative study.

In all cases, neither the human specific antibody MRK-16 nor the P-gp modulators were able to block Rh-123 efflux completely. The efflux of Rh-123 is the result of both active transport mechanisms and passive diffusion, but the involvement of transporters other than P-gp cannot be excluded. Rh-123 has been

suggested to be a relatively specific substrate for P-gp but not MRP (multidrug resistance-associated proteins) in multidrug resistant tumour cells (Feller et al., 1995) and human lymphocytes (Parasrampuria et al., 2001). This remains to be documented in lymphocytes for the present animal species.

P-gp expression in lymphocytes and small intestinal tissue (jejunum) was assessed using the human monoclonal antibody C219. This antibody recognises a highly conserved amino acid sequence (Georges et al., 1990) found in all P-gp isoforms characterised to date, and has been successively used for detection of P-gp expression in tissues or cell lines derived from rat, cattle and pig (Schinkel et al., 1994; Fontaine et al., 1996; Nobmann et al., 2001). However, possible differences in C219 affinity for P-gp across animal species hamper any quantitative interspecies comparison, which explains the interest of functional studies for quantitative investigations. Furthermore, as the specificity of C219 for P-gp is certainly lower in animal species than in man, aspecific binding may be a problem. For this reason, Western-blot analysis appeared as the most appropriate technique for detection of P-gp expression (expected molecular weight of approximately 170 kDa). Finally, although an amplified detection system was used for detection of the primary antibody, it is noteworthy that a lack of detection by the C219 antibody does not exclude P-gp expression, as this may be due to a very low binding affinity of the antibody for the protein. In the present study, the results of Western-blot analysis correlate well with those of the functional studies. P-gp was clearly expressed in peripheral blood lymphocytes from goats and could be detected in lymphocytes from cattle, pigs and sheep at the level of the positive control (Caco-2 cells, 170 kDa). In contrast, P-gp could not be detected in lymphocytes from rats and horses, which is consistent with the low levels of P-gp activity observed. Western-blot analysis of jejunal tissue revealed a large band of about 170 kDa for goats, sheep, pigs and horses, and of 90-110 kDa for rats and cattle. In cattle and rats, the 90-110 kDa band of protein corresponds most likely to P-gp, considering the intensity of the signal and the absence of aspecific binding. Furthermore, it is known that P-gp is expressed in rat jejunum (Brady et al., 2002). A degradation of P-gps into 90-kDa proteins has been already reported in the literature (Doi et al., 2001), and it is likely that the P-gps of rats and cattle are more sensitive to degradation than P-gps of other species.

Even if only low levels of P-gp activity were detected in rat and horse lymphocytes, the present study suggests the expression of functional P-gps in all animal species studied. High interindividual variations in P-gp activity were observed in rats, goats, sheep, cattle and pigs, similar to those that have been reported in human lymphocytes (Schluesener et al., 1992). Young castrated pigs

showed a significantly lower Rh-123 efflux and inhibition than older boars, suggesting that P-gp activity could vary with the age or hormonal status of animals. This is consistent with previous reports in the literature, indicating an upregulated P-gp function in lymphocytes with age in mice (Witkowski and Miller, 1993) and humans (Gupta, 1995), and suggesting a higher P-gp activity in lymphocytes from men than women (Steiner et al., 1998). Further investigations at a large scale are required to confirm this trend.

In addition to functional studies, P-gp expression was found in intestinal tissue (jejunum) of all animal species. Taken together, the present results suggest that P-gps equivalent to those identified in humans and rodents exist in cattle, goats, sheep, pigs and horses. P-gp has been shown to play a major role in the disposition (distribution, elimination) of many important veterinary drugs such as ivermectin (Schinkel et al., 1994; Laffont et al., 2002), a worldwide used antiparasitic drug in livestock, and fluoroquinolones (Yamaguchi et al., 2002). Interspecies differences in the pharmacokinetics of these drugs could, therefore, be partly explained by interspecies differences in P-gp activity, in addition to possible differences in metabolism. Whether the proposed classification of the species with respect to P-gp applies to tissues other than lymphocytes remains to be documented. However, it is striking that the highest P-gp activity was found in lymphocytes from goats, known to eliminate ivermectin much more rapidly than other farm animal species (Scott et al., 1990).

High levels of P-gp activity were observed in lymphocytes from goats, cattle, sheep and generally pigs. Consequently, lymphocytes offer the possibility to study transport of veterinary drugs and drug-drug interactions in the target animal species. Although the relevance of the lymphocyte-based model needs to be further documented, it is certainly more predictive that *in vitro* conventional models, which cannot take into account interspecies differences. Furthermore, it has been reported that the expression of P-gp could vary following drug exposure (Matheny et al., 2001) or under inflammatory conditions (Hartmann et al., 2001). Thus, the lymphocyte-based model could also be used to evaluate the influence of various physiological or pathological factors on the P-gp function, and monitor the effects of drugs (particularly long-acting drugs such as ivermectin) on P-gp. Since lymphocytes can be easily isolated from a single blood sampling, this model is suitable for a population approach.

In conclusion, our study shows the existence of a physiological P-gp function in peripheral blood lymphocytes from goats, sheep, cattle, pigs and horses, that has yet not been reported. However, in contrast to the other species, only low levels of P-gp activity were detected in horse lymphocytes. The lymphocyte-based model

offers new perspectives to study and monitor the P-gp function and expression in cattle, goats, sheep and pigs. P-gp expression was also shown in intestinal tissues of all animal species studied.

References

Beck J, Gekeler V, Ringger M, Handgretinger R and Niethammer D (1996) Rhodamine 123-efflux from hematopoietic subpopulations and leukaemic blast populations marked by PerCP-conjugated monoclonal antibodies. *Cancer Letters* **99:**197-207.

Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71:**537-92.

Brady JM, Cherrington NJ, Hartley DP, Buist SC, Li N and Klaassen CD (2002) Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metab Dispos* **30:**838-844.

Broxterman HJ (1999) Measurement of P-glycoprotein function in Methods in Molecular Medicine, Vol. 28: Cytotoxic drug resistance mechanisms (Brown R and Boger-Brown U ed) pp 53-61, Humana Press Inc., Totowa, NJ.

Canitrot Y, Lahmy S, Buquen JJ, Canitrot D and Lautier D (1996) Functional study of multidrug resistance with fluorescent dyes. Limits of the assay for low levels of resistance and application in clinical samples. *Cancer Lett* **106:**59-68.

Childs S and Ling V (1996) Duplication and evolution of the P-glycoprotein genes in pig. *Biochim Biophys Acta* **1307:**205-212.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at the blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86:**695-698.

Doi AM, Holmes E and Kleinow KM (2001) P-glycoprotein in the catfish intestine: inducibility by xenobiotics and functional properties. *Aquatic Toxicol* **55:**157-170.

Drach D, Zhao S, Drach J, Mahadevia R, Gattringer C, Huber H and Andreeff M (1992) Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* **80:**2729-2734.

Eneroth A, Astrom E, Hoogstraate J, Schrenk D, Conrad S, Kauffmann HM and Gjellan K (2001) Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. *Eur J Pharm Sci* **12:**205-214.

Feller N, Kuiper CM, Lankelma J, Ruhdal JK, Scheper RJ, Pinedo HM and Broxterman HJ (1995) Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. *Br J Cancer* **72:**543-549.

Fontaine M, Elmquist WF and Miller DW (1996) Use of rhodamine 123 to examine the functional activity of P-glycoprotein in primary cultured brain microvessel endothelial cell monolayers. *Life Sci* **59:**1521-1531.

Ford JM and Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:**155-199.

Georges E, Bradley G, Gariepy J and Ling V (1990) Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc Natl Acad Sci USA* **87:**152-156.

Gupta S (1995) P-glycoprotein expression and regulation. Age-related changes and potential effects on drug therapy. *Drugs Aging* **7:**19-29.

Hartmann G, Kim H and Piquette-Miller M (2001) Regulation of the hepatic multidrug resistance gene expression by endotoxin and inflammatory cytokines in mice. *Int Immunopharmacol* **1:**189-199.

Huet S, Marie JP, Gualde N and Robert J (1998) Reference method for detection of P-gp mediated multidrug resistance in human hematological malignancies: a method validated by the laboratories of the French Drug Resistance Network. *Cytometry* **34:**248-256.

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455:**152-162.

Klimecki WT, Futscher BW, Grogan TM and Dalton WS (1994) P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood* **83:**2451-2458.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680-685.

Laffont CM, Toutain PL, Alvinerie M and Bousquet-Melou A (2002) Intestinal secretion is a major route for parent ivermectin elimination in the rat. *Drug Metab Dispos* **30:**626-630.

Lifschitz A, Virkel G, Sallovitz J, Imperiale F, Pis A and Lanusse C (2002) Loperamide-induced enhancement of moxidectin availability in cattle. *J Vet Pharmacol Ther* **25:**111-120.

Longley M, Phua SH, van Stijn TC and Crawford AM (1999) Isolation and mapping of the first ruminant multidrug resistance genes. *Anim Genet* **30:**207-210.

Ludescher C, Pall G, Irschick EU and Gastl G (1998) Differential activity of P-glycoprotein in normal blood lymphocyte subsets. *Br J Haematol* **101:**722-727.

Matheny CJ, Lamb MW, Brouwer KL and Pollack GM (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy* **21:**778-796.

Miller DS, Nobmann SN, Gutmann H, Toeroek M, Drewe J and Fricker G (2000) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol Pharmacol* **58:**1357-1367.

Nobmann S, Bauer B and Fricker G (2001) Ivermectin excretion by isolated functionally intact brain endothelial capillaries. *Br J Pharmacol* **132:**722-728.

Parasrampuria DA, Lantz MV and Benet LZ (2001) A human lymphocyte based ex vivo assay to study the effect of drugs on P-glycoprotein (P-gp) function. *Pharm Res* **18:**39-44.

Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussault E, Lacroix B, Simon-Asman P, Haffen K, Fogh J and Zweibaum A (1983) Enterocyte-like differentiation and polarisation of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* **47:**323-330.

Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E (1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **53:**17-25.

Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, Berns AJM and Borst P (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:**491-502.

Schinkel AH, Wagenaar E, van Deemter L, Mol CA and Borst P (1995) Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96:**1698-1705.

Schluesener HJ, Koeppel C and Jung S (1992) Multidrug transport in human autoimmune T line cells and peripheral blood lymphocytes. *Immunopharmacolog* **23:**37-48.

Scott EW, Kinabo LD and McKellar QA (1990) Pharmacokinetics of ivermectin after oral or percutaneous administration to adult milking goats. *J Vet Pharmacol Ther* **3:**432-435.

Steiner H, Polliack A, Kimchi-Sarfaty C, Libster D, Fibach E and Rund D (1998) Differences in rhodamine-123 efflux in B-type chronic lymphocytic leukemia suggest possible gender and stage variations in drug-resistance gene activity. *Ann Hematol* **76:**189-194.

Tanigawara Y (2000) Role of P-glycoprotein in drug disposition. *Therapeutic drug monitoring* **22:**137-140.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84:**7735-7738.

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350-4354.

Wang E-J, Casciano CN, Clement RP and Johnson WW (2000) In vitro flow cytometry method to quantitatively assess inhibitors of P-glycoprotein. *Drug Metab Dispos* **28:**522-528.

Witkowski JM and Miller RA (1993) Increased function of P-glycoprotein in T lymphocyte subsets of aging mice. *J Immunol* **150:**1296-1306.

Yamaguchi H, Yano I, Saito H and Inui K (2002) Pharmacokinetic role of P-glycoprotein in oral bioavailability and intestinal secretion of grepafloxacin in vivo. *J Pharmacol Exp Ther* **300:**1063-1069.

CHAPTER 5

Licking Behaviour and Environmental Contamination Arising from Pour-on Ivermectin for Cattle

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Abstract

Pour-on formulations of endectocides are extensively used to treat and control systemic parasitic diseases in cattle, worldwide. The purpose of the present study was to investigate the influence of the natural licking behaviour of cattle on the plasma and faecal disposition of topically-administered ivermectin. Twelve Holstein cattle were given one single i.v. (200 µg/kg) and topical (500 µg/kg) administration of ivermectin at a 5-month interval. For the pour-on administration, the animals were allocated into two groups (n = 6): one control group (lickers) and one group where licking was prevented (non-lickers). Ivermectin plasma (total) clearance (270 ± 57.4 mL/kg/day) was very homogeneous among the 12 cattle. In contrast, major differences between lickers and non-lickers were observed following pour-on administration. Prevention of licking resulted in an extended terminal plasma half-life (363 ± 16.2 *vs* 154 ± 7.4 h in lickers) and in a lower and less variable systemic availability of ivermectin (19 ± 4.9 *vs* 33 ± 18.5% in lickers). More importantly, nearly 70% of the pour-on dose was recovered as parent drug in the faeces of lickers *vs* only 6.6% in non-lickers. Altogether, these results are consistent with an oral rather than percutaneous absorption of topical ivermectin in control animals, the non-systemically available fraction of ingested ivermectin providing a major contribution (80%) to the drug faecal output. The consequences of licking on the disposition of pour-on ivermectin are discussed in terms of environment, given the known ecotoxicity of this drug, and of cross-contamination. Animals licking themselves and each other could result in unexpected residues in edible tissues of untreated animals and in possible subtherapeutic drug concentrations, a factor in drug resistance. According to the Precautionary Principle, these considerations elicit concern over the use of topical drug formulations in cattle.

Introduction

In both livestock and companion animals, many different antiparasitic drugs, including pyrethroid compounds, organophosphates and later, endectocides (such as ivermectin, doramectin, eprinomectin and moxidectin) are administered topically to treat different parasitic conditions. Pour-on formulations of endectocides limit the risk of injury to user and animal and are particularly convenient for farmers who can apply the product easily themselves (Hennessy, 1997). For these reasons, pour-on have largely displaced therapeutically equivalent injectable formulations in farming practice and are routinely used to treat millions of cattle per year, worldwide.

Ivermectin and structurally related drugs are extensively excreted as metabolites and parent drug in the faeces of treated animals, regardless of administration route (Campbell, 1985; Chiu et al., 1990*)*. Unchanged (active) compound in the faeces may be toxic to non-target organisms such as dung-breeding insects and the fauna involved in the degradation of livestock dung on the pasture (Wall and Strong, 1987*)*. The issue of environmental impact of endectocides used in large scale has been debated for almost 20 years (Fincher, 1992, 1996; Sommer et al., 1993), and there is still little sign of consensus since different studies have shown conflicting results with varying degrees of ecotoxicity (Strong and Wall, 1994*)*. Contradictory data can be partly explained by the observation that different routes of administration (s.c., topical, oral) lead to different ivermectin excretion profiles (Herd et al., 1996*)*. Interestingly, higher faecal concentrations of ivermectin have been reported following pour-on application than following s.c. injection (Herd et al., 1996*)*, which is unexpected considering the lower plasma concentrations. Indeed, if it is assumed that the plasma concentration of ivermectin is the only driving force for ivermectin excretion into faeces, lower faecal concentrations of parent drug should be expected after the pour-on administration. To explain this apparent inconsistency, we decided to explore the influence of the natural grooming behaviour of domestic cattle on the disposition of ivermectin pour-on formulation. This grooming behaviour consists predominantly of self-licking or licking another animal (so called allo-licking). It serves an important physiological function in skin and hair hygiene, can be stimulated by the presence of ectoparasites and is also a factor in the establishment and cohesion of herd social structure (Simonsen, 1979; Sato et al., 1991, 1993; Krohn, 1994*)*. The present experiment was designed to test the hypothesis that a relevant fraction of the ivermectin topically-administered to cattle was actually ingested by licking.

Materials and Methods

Experimental design

Six pairs of monozygotic twin Holstein cattle (567 ± 24 kg body weight, 3 years old), obtained by micro-manipulation (Ozil et al., 1982*)* and maintained under identical conditions, were given a single i.v. administration of injectable ivermectin (Ivomec® injectable, Merial; 200 µg/kg). After a 5-month washout period, each animal was given one single administration of topical ivermectin (Ivomec® pour-on bovin, Merial) at the recommended dose of 500 µg/kg. For the pour-on administration, each pair of twins was separated into two groups of six animals. One group (the lickers) was kept in individual tie-stalls, each animal being tethered with a loose chain so that it could lick itself and its immediate neighbours. In the other group (the non-lickers), each animal was isolated from the others by a screen and was fitted with a wooden neck collar to prevent self-licking. The collar was removed 44 days after administration. Blood was collected regularly for 44 days and 31 days following pour-on and i.v. administration, respectively. An additional blood sample was obtained in the 12 cattle on day 56 following pour-on application (12 days after removal of collars). Once collected, the blood samples were chilled on wet ice and promptly centrifuged. The plasma was removed from the tubes, and stored at – 20°C until analysis. Total faeces were collected over 24 h on days 4, 7 and 14 after the i.v. administration. Following the pour-on administration, faeces were collected for 6 h (from 09:00 to 15:00 h) on days 1, 2, 3, 4, 7, 14, 18, 22 and 28 after application. Wet faeces were weighed, homogenized, and a 50-g aliquot was collected and stored at – 20°C until analysis.

Analytical method

Ivermectin (22,23-dihydroavermectin B1a) concentrations in plasma and faeces were measured using a high-performance liquid chromatography (HPLC) technique (Alvinerie et al., 1987*)*. The lower limit of quantification for ivermectin was 0.05 ng/mL for the plasma and 0.5 ng/g for the wet faecal samples. Accuracy and precision (intra-assay variation) expressed as relative standard deviation were less than 8 and 6%, respectively.

Pharmacokinetic analysis

Data were analysed using a non-compartmental approach. The areas under the plasma concentration-time curve AUC ($0\text{-}t_{last}$) (from 0 to the last sample *e.g.* 31 days (i.v.) or 44 days (pour-on)) were computed using the trapezoidal rule.

Ivermectin total (plasma) clearance was calculated by dividing the administered dose by the AUC (0-t_{last}) obtained for the i.v. route (Eq. 1):

$$Cl_{tot} = \frac{Dose^{i.v.}}{AUC^{i.v.}_{0-tlast}} \quad \text{Eq.1}$$

The systemic availability for topical ivermectin was calculated using the ratio of the AUC (0-t_{last}) obtained after topical ($AUC^{pour-on}_{0-tlast}$) and i.v. administration ($AUC^{i.v.}_{0-tlast}$), corrected by the ratio of the administered doses (Eq. 2):

$$F(\%) = \frac{AUC^{pour-on}_{0-tlast}}{AUC^{i.v.}_{0-tlast}} \times \frac{Dose^{i.v.}}{Dose^{pour-on}} \times 100 \quad \text{Eq. 2}$$

The faecal excretion rate of ivermectin at each time point (t) was obtained by dividing the total amount of parent drug eliminated in faeces within the collection interval ($Q_{faeces,\,\tau}$) by the time of collection τ (6 or 24 h):

$$faecal\ excretion\ rate\ (t) = \frac{Q_{faeces,\,\tau}}{\tau} \quad \text{Eq. 3}$$

where $Q_{faeces,\,\tau}$ was the product of the weight of wet faeces and the faecal concentration of ivermectin (µg/g wet faeces) over the collection period. The total amount of parent drug eliminated in faeces within 28 days post-topical administration was estimated by integration of the faecal excretion rate profile in function of time between 0 and 28 days using the trapezoidal rule.

Faecal clearances were calculated at each time point (t) following the equation given below:

$$Cl_{faecal}\ (t) = \frac{faecal\ excretion\ rate\ (t)}{C_{plasma,\,\tau}} \quad \text{Eq. 4}$$

where $C_{plasma,\,\tau}$ was the corresponding plasma concentration over τ.

Statistics

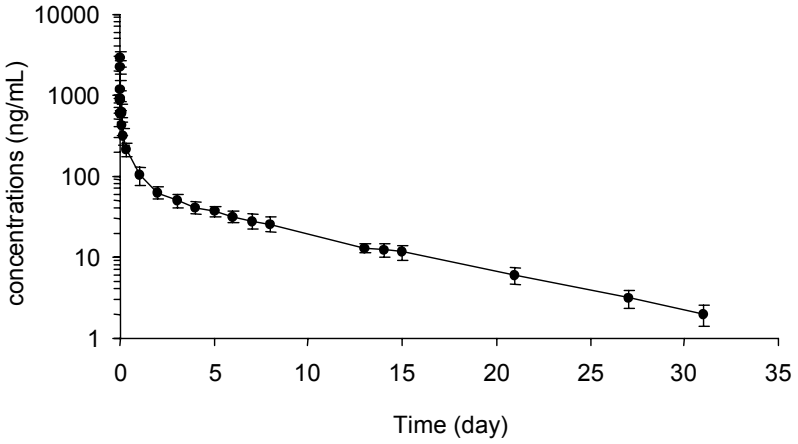
The arithmetic means and standard deviations (SD) of the different parameters were calculated. For terminal plasma half-life, the harmonic means and SD were computed using the Jackknife technique (Lam et al., 1985). Comparison between lickers and non-lickers was carried out using paired *t*-test for terminal half-lives, AUC (0-t_{last}), total (plasma) clearance and C_{max}, and a using non-parametric paired test (Wilcoxon) for T_{max} and F% (SYSTAT ® 8.0, SPSS Inc., Chicago, IL). A $p < 0.05$ was considered as significant.

Results

Plasma disposition

Ivermectin plasma concentration-time profiles obtained in cattle following i.v. and pour-on administrations are presented in Figs. 1 and 2, respectively. Table 1 gives the mean values of ivermectin pharmacokinetic parameters for both i.v. and pour-on administrations in the licker and non-licker groups. Ivermectin total (plasma) clearance following i.v. administration was found to be homogeneous among the 12 cattle, and equal to 270 ± 57.4 mL/kg/day (n = 12). The terminal plasma half-life was similar between pour-on and i.v. administrations in lickers, but was much longer after pour-on (363 ± 16.2 h) than following i.v. administration

Figure 1. *Ivermectin mean plasma concentration-time profile over 31 days in the 6 pairs of monozygotic twin cattle simultaneously administered with 200 µg/kg ivermectin i.v.*



Each point represents the mean ± SD obtained in the 12 animals.

Table 1. *Pharmacokinetic parameters (mean ± SD) of ivermectin following intravenous and topical administration of ivermectin (200 and 500 μg/kg, respectively) to six licking and six non-licking monozygotic twin cattle.*

Parameter	Lickers (n = 6)		Non-lickers (n = 6)	
	i.v.	pour-on	i.v.	pour-on
$t_{1/2}$ (h)	137 ± 2.7	154 ± 7.4	144 ± 3.0	363 ± 16.2 **, #
AUC (ng.h/mL)	18429 ± 3652	14283 ± 6424	18749 ± 3036	9146 ± 3078 *
Cl (mL/kg/day)	274 ± 68.8	—	264 ± 47.4	—
F (%)	—	33 ± 18.5	—	19 ± 4.9 *
C_{max} (ng/mL)	—	39 ± 20.9	—	16 ± 6.4 ***
T_{max} (day)	—	147 ± 43.6	—	191 ± 15.2

* ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) indicate a significant difference between the licker and non-licker groups for this parameter.

The plasma terminal half-life ($t_{1/2}$) differed significantly between i.v. and pour-on administrations ($p < 0.01$).

A paired *t*-test was used for plasma terminal half-lives, AUC, Cl, and C_{max}, whereas a non-parametric paired test (Wilcoxon) was used for F(%) and T_{max}.

(144 ± 3.0 h) in the non-licker group, indicating a flip-flop phenomenon in non-licking cattle. The systemic availability for topical ivermectin was higher and more variable in lickers than in non-lickers (33 ± 18.5 % *vs* 19 ± 4.9 %).

At the end of the 44-day trial, the collars were removed from the six non-licking cattle, and measurement of ivermectin plasma concentrations 12 days later indicated an obvious rebound in three of the six animals. In these three animals, the plasma concentrations on day 56 were increased by a factor of 39, 56 and 135% compared to the plasma concentrations measured on day 44, which could not be attributed to the variability of the analytical method.

Faecal disposition

Comparison of ivermectin excretion profiles in faeces (Fig. 3) showed a major difference between lickers and non-lickers. On day 4 post-administration, for example, the faecal elimination rate of ivermectin in the licker group was 33-times

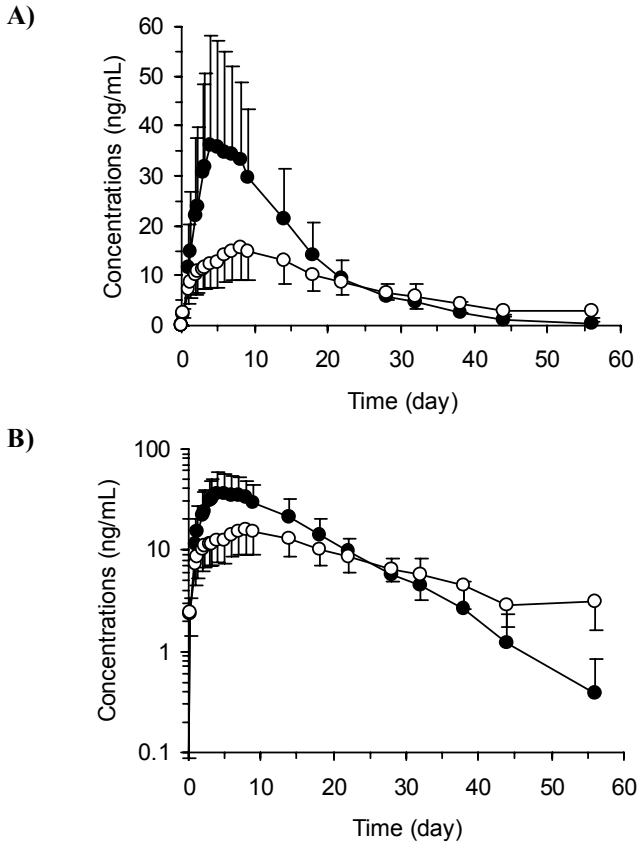


Figure 2. Comparative ivermectin plasma concentration-time profiles in six licking (filled symbol) and six paired non-licking (open symbol) monozygotic twin cattle over a 56-day period, following a single 500 µg/kg topical administration with ivermectin pour-on formulation. Each point represents the mean ± SD obtained in the six animals of each group. **A)** Arithmetic scale shows a higher and more variable ivermectin bioavailability in lickers than in non-lickers. **B)** Semi-logarithmic scale shows a different slope for the plasma terminal phase in lickers and non-lickers.

higher than in the non-licker group (825 ± 227.5 *vs* 25 ± 10.0 µg/h) and 10-times higher than after the i.v. administration (83 ± 10.4 µg/h). The estimated amount of ivermectin eliminated in the faeces over 28 days was 346 ± 60.5 µg/kg body weight (69% of the administered dose; Fig. 3B) in the lickers *vs* 33 ± 11.7 µg/kg body weight in non-lickers (6.6% of the dose; Fig. 3B).

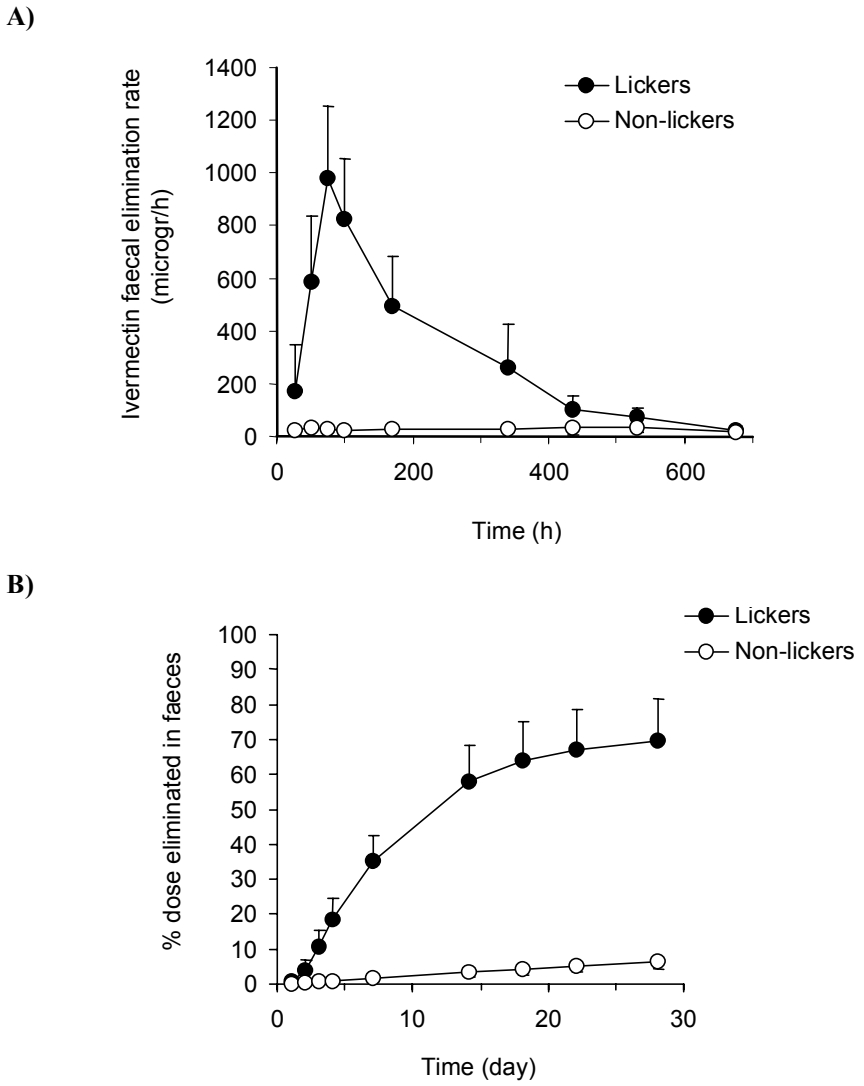


Figure 3. Comparative excretion profiles of unchanged ivermectin in the faeces of six licking (filled symbol) and six paired non-licking (open symbol) monozygotic twin cattle over 28 days, following a single 500 µg/kg administration of pour-on ivermectin. **A)** Faecal elimination rates (mean ± SD) of ivermectin as parent drug. **B)** Cumulative amounts of ivermectin eliminated in faeces expressed as percentages of the administered dose (mean ± SD).

In non-lickers (Fig. 4A), individual faecal clearances of ivermectin were similar after pour-on (89 ± 24.5 mL/kg/day) and i.v. administrations (102 ± 23.5 mL/kg/day). In contrast, in the licker group (Fig. 4B), the individual apparent faecal clearances of ivermectin following pour-on administration (ranging from 203 ± 170.6 to 1671 ± 724.1 mL/kg/day) were much higher than the faecal clearance obtained for the i.v. route (106 ± 33.5 mL/kg/day) throughout the 28 days of investigation.

The values of ivermectin faecal clearance obtained in the 12 cattle on day 4, 7 and 14 after i.v. administration were very homogeneous (104 ± 28.6 mL/kg/day, n = 12), representing 38% ($CI_{95\%}$: [36%;40.5%]) of the plasma (total) clearance (270 ± 57.4 mL/kg/day, n = 12).

Discussion

Our results indicated that i.v. faecal clearance accounted for 38% of total (plasma) clearance following i.v. administration, which implies that 38% of the i.v. dose was actually excreted as parent drug in the faeces. This is consistent with the

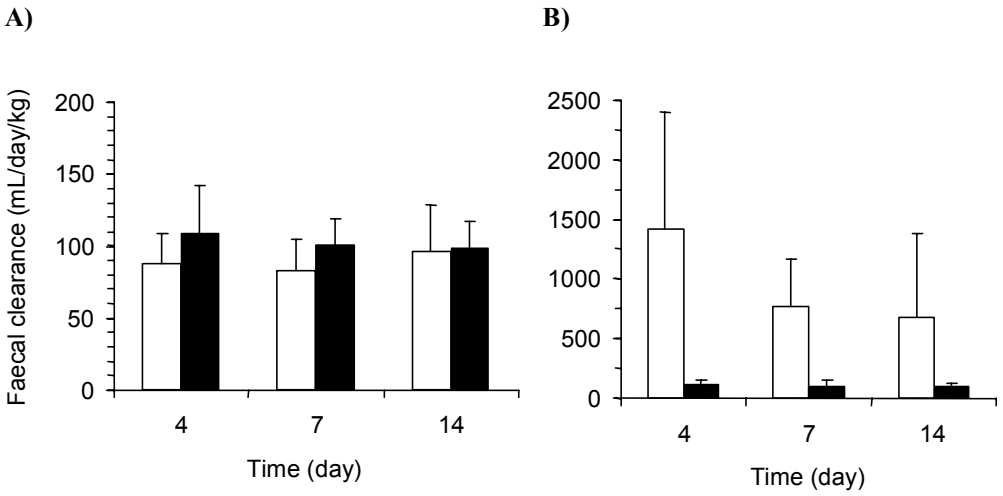


Figure 4. Ivermectin faecal clearance on days 4, 7 and 14 post-administration, following an i.v. injection of ivermectin at 200 µg/kg (filled column) and a pour-on application of ivermectin at 500 µg/kg (open column). **A)** mean ± SD in the non-licker group. **B)** mean ± SD in the licker group. The figures show that ivermectin faecal clearance was similar after i.v. and pour-on administrations in non-lickers, but much higher after pour-on than following i.v. administration in lickers due to the large ingestion of ivermectin by licking.

study performed in cattle and with radiolabelled drug by Halley et al. (1989), who showed that 39-45% of the faecal output consisted of parent drug and 59% of metabolites after subcutaneous administration.

After the pour-on administration, a marked difference was observed between licker and non-licker groups for both plasma and faecal disposition of ivermectin. In the non-licker group, faecal clearance of ivermectin following pour-on application was similar to faecal clearance found after i.v. administration, which is consistent with the assumption that the plasma concentration was the only driving force controlling ivermectin faecal elimination in non-lickers. The percentage of the administered dose eliminated in faeces from systemic blood can be theoretically assessed as the product of the faecal-to-total clearance ratio (0.38) and the fraction systemically available. In non-lickers, this theoretical percentage was 7% (0.38×0.19), which is very close to the value estimated by integration of the excretion rate profile against time (6.6%). In lickers, we would expect 13% of the dose (0.38×0.33) to be removed as parent drug from blood in faeces. In contrast, we observed that nearly 70% of the pour-on dose (500 µg/kg) was eliminated unchanged in the dung. In addition, pour-on faecal clearance in lickers was much higher than the faecal clearance obtained for the i.v. route throughout the 28 days of investigation, and could exceed up to nine times the i.v. plasma (total) clearance in the same animals, which is theoretically impossible. Altogether, these findings demonstrate that over this time period, a large fraction of the ivermectin eliminated in the faeces of licking animals (nearly 57% of the dose *e.g.* 80% of the faecal output) could not be of plasma origin. It is concluded that an important amount of topically administered drug was actually ingested by licking and transited directly through the digestive tract into faeces.

The systemic availability for topical ivermectin was highly variable in lickers (coefficient of variation of 56%), which is in line with previous studies (Gayrard et al., 1999). The prevention of licking resulted in a lower and less variable systemic availability (coefficient of variation of 26% in non-lickers) and in an extended elimination half-life (363 h in non-lickers *vs* 154 h in lickers). The terminal plasma half-life in the non-licker group was also much longer than after the i.v. administration (144 h). This indicates that ivermectin absorption through the skin was a very slow process limiting the drug plasma elimination. In contrast, the terminal plasma half-life of ivermectin in the licker group was very similar to that observed after the i.v. administration. These results conclusively demonstrate that the rate and extent of ivermectin absorption differed between licking and non-licking animals, which is consistent with a difference in absorption mechanism. Considering that a large amount of drug transited through the digestive tract, it is

strongly suggested that a large fraction of topical ivermectin gained access to systemic circulation by the oral route, rather than percutaneous absorption, as a consequence of the licking behaviour. We found a relatively high systemic availability in the licker group (33%). By comparison with the s.c. route, the systemic availability for the oral route was estimated to be 12.5% for the sustained release bolus (Alvinerie et al., 1998) and 26% for the intra-ruminal bolus (Chiu et al., 1990). This suggests that perlingual absorption of ivermectin cannot be ruled out in licking cattle.

The obvious rebound in plasma concentration observed in three of the six animals (non-licking group) 12 days after removal of the collar suggests that some ivermectin was still present on the skin of the animals and available for licking. This implies that ivermectin did not undergo complete degradation over 44 days, which is surprising given the alleged photolability of the drug.

The present findings are consistent with the observations reported by others but not previously understood (Sommer et al., 1992; Herd et al., 1993, 1996). Our results provide clear evidence that the natural grooming behaviour of cattle has a major influence on the plasma disposition of topical ivermectin. Self- and allo-grooming are governed by various social, nutritional, physiological, pathological, environmental and managerial factors (Sato et al., 1991, 1993), which makes the systemic availability of topical ivermectin more variable and unpredictable. More importantly, allo-grooming might result in cross-contamination of animals, giving rise to unexpected drug residues in edible tissues of untreated cattle, and undesirable subtherapeutic concentrations in both treated and untreated cattle, which can contribute to the development of drug resistance. Finally, our study demonstrates that the prevention of licking can lead to 10-times lower amounts of parent drug in faeces, under our experimental conditions. This suggests that the licking behaviour of cattle should be taken into consideration in the environmental risk assessment of endectocides. It must also be stressed that the poor and erratic bioavailability of pour-on formulations has led to increased dose rates for ivermectin, doramectin and moxidectin by a factor of 2.5 compared with the s.c. formulation. However, with approximately 70% of the dose recovered in the faeces of licking cattle, increasing the dose contributes to a higher and unnecessary environmental burden of parent drug. In contrast, the therapeutically equivalent s.c. formulation would provide a lower faecal output of ivermectin. Indeed, the maximum faecal excretion of parent drug following s.c. administration (200 µg/kg) can be estimated using our i.v. faecal clearance to be about 78 µg/kg *vs* 346 ± 60.5 µg/kg in lickers given topical ivermectin. Altogether, these considerations elicit concern over the topical route for endectocide administration in cattle, and it is

strongly suggested that the use of safe and efficacious injectable preparations be encouraged.

References

Alvinerie M, Sutra JF, Galtier P and Toutain PL (1987) Determination of ivermectin in milk by high performance liquid chromatography. *Vet Res* **18:**269-74.

Alvinerie M, Sutra JF, Galtier P, Lifschitz A, Virkel G, Sallovitz J and Lanusse C (1998) Persistence of ivermectin in plasma and faeces following administration of a sustained-release bolus to cattle. *Res Vet Sci* **66:**57-61.

Campbell WC (1985) Ivermectin: an update. *Parasitol Today* **1:**10-16.

Chiu L, Green ML, Baylis FP, Eline D, Rosegay A, Meriwether H and Jacob TA (1990). Absorption, tissue distribution, and excretion of tritium-labeled ivermectin in cattle, sheep, and rat. *J Agric Food Chem* **38:**2072-2078.

Fincher GT (1992) Injectable ivermectin for cattle: effects on some dung-inhabiting insects. *Environ Entomol* **21:**871-876.

Fincher GT (1996) Ivermectin pour-on for cattle: effects on some dung-inhabiting insects. *Southwest Entomol* **21:**445-450.

Gayrard V, Alvinerie M and Toutain PL (1999) Comparison of pharmacokinetic profiles of doramectin and ivermectin pour-on formulations in cattle. *Vet Parasitol* **81:**47-55.

Halley BA, Nessel RJ and Lu AYH (1989) In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York. pp, 162-172.

Hennessy DR (1997) Modifying the formulation or delivery mechanism to increase the activity of anthelmintic compounds. *Vet Parasitol* **72:**367-390.

Herd R, Strong L and Wardhaugh K (1993) Research recommendations. *Vet Parasitol* **48:**337-340.

Herd RP, Sams RA and Ashcraft SM (1996) Persistence of ivermectin in plasma and faeces following treatment of cows with ivermectin sustained-release, pour-on or injectable formulations. *Int J Parasitol* **26:**1087-1093.

Krohn CC (1994) Behaviour of dairy cows kept in extensive (loose housing/pasture) or intensive (tie stall) environments. III. Grooming, exploration and abnormal behaviour. *Appl Anim Behav Sci* **42:**73-86.

Lam FC, Hung CT and Perrier DC (1985). Estimation of variance for harmonic mean half-life. *J Pharm Sci* **74:**229-231.

Ozil JP, Heyman Y and Renard JP (1982). Production of monozygotic twins by micromanipulation and cervical transfer in the cow. *Vet Rec* **110:**126-127.

Sato S, Sako S and Maeda A (1991). Social licking patterns in cattle (Bos taurus): influence of environmental and social factors. *Appl Anim Behav Sci* **32:**3-12.

Sato S, Tarumizu K and Hatae K (1993). The influence of social factors on allogrooming in cows. *Appl Anim Behav Sci* **38:**235-244.

Simonsen HB (1979). Grooming behaviour of domestic cattle. *Nord Vet Med* **31:**1-5.

Sommer C, Steffansen B, Overgaard Nielsen B, Gronvold J, Vagn Jensen KM, Brochner Jespersen J, Springborg J and Nansen P (1992). Ivermectin excreted in cattle dung after s.c. injection or pour-on treatment: concentrations and impact on dung fauna. *B*

Entomol Res **82:**257-264.

Sommer C, Gronvold J, Holter P and Nansen P (1993). Effects of ivermectin on two afrotropical dung beetles, Onthophagus gazella and Diastellopalpus quinquedens (Coleoptera: Scarabaeidae). *Vet Parasitol* **48:**171-179.

Strong L and Wall R (1994). Effect of ivermectin and moxidectin on the insects of cattle faeces. *B Entomol Res* **84:**403-409.

Wall R and Strong L (1987) Environmental consequences of treating cattle with the antiparasitic drug ivermectin. *Nature* **327:**418-421.

CHAPTER 6

Pharmacokinetic Modeling of the Disposition of Topical Ivermectin in Cattle as determined by Animal Behaviour

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Abstract

Animal behaviour is generally not recognised as a major determinant of drug disposition in animals. In the present study, pharmacokinetic modeling was applied to quantify the contribution of physiological cattle behaviour (licking) on the plasma and faecal disposition of topical ivermectin, a worldwide-used antiparasitic drug largely administered to cattle as a percutaneous formulation (pour-on). Six pairs of monozygotic twin cattle were given successively a single i.v. and two pour-on administrations of ivermectin at a 3-5-month interval. For the second pour-on administration, the twins were separated into two groups: a control group (lickers) and a group where self- and allo-licking were prevented. Ivermectin data obtained after i.v. and the second pour-on administrations in licking and non-licking twins were fitted simultaneously to a set of differential equations describing a seven-compartment model. Licking conferred a high intra- and inter-individual variability of systemic exposure after topical administration. We show that 58-87% of the pour-on dose was ingested, while only 10% of the dose was absorbed percutaneously. It results that 50-77% of the drug systemic absorption was achieved by the oral route. Approximately 72% of the ingested ivermectin transited directly into faeces, increasing by 5-fold the environmental burden of parent drug. This is of ecological interest, given the potential of ivermectin to adversely affect dung-degrading insects. We conclude that topical administration does not guarantee a controlled drug delivery in cattle. More importantly, simulations revealed that non-treated cattle could get easily contaminated by allo-licking, raising the public health problem of unexpected drug residues in edible tissues.

Introduction

Licking is an important part of the natural grooming behaviour of animals in many species. In cattle, it serves an important physiological function in skin and hair hygiene, and plays a major role in the establishment and maintenance of the herd social structure (Simonsen, 1979; Sato et al., 1991, 1993; Krohn, 1994). However, despite the increasing use of percutaneous drug formulations in both companion and food-producing animals (see Magnusson et al., 2001), the influence of licking on the disposition of topical drugs has never been documented. Over the last twenty years, the percutaneous route has received considerable interest in veterinary medicine for local and/or systemic delivery of drugs (Hennessy, 1997; Magnusson et al., 2001; Riviere and Papich, 2001). This application technique is particularly convenient for the owner who can easily apply the treatment himself with minimal risk of injury and minimal animal distress (Hennessy, 1997; Rothen-Weinhold et al., 2000). Furthermore, topical administration avoids the problems of hepatic first pass metabolism and of drug degradation in the gastrointestinal tract following oral administration, and therefore may be a good alternative to the oral route to achieve systemic therapeutic effects (Hennessy, 1997; Magnusson et al., 2001).

Ivermectin is a worldwide-used antiparasitic drug, which is routinely administered to millions of cattle per year for systemic effects. As for other endectocides (doramectin, moxidectin, eprinomectin), the topical "pour-on" formulation of ivermectin has largely displaced the conventional injectable formulation in farming practice. In a recent study (Laffont et al., 2001), we showed that the disposition of pour-on ivermectin in cattle was markedly modified with restriction of animal licking. Prevention of licking resulted in a significantly lower absolute bioavailability of ivermectin, in a two-times longer plasma elimination half-life, and overall in a ten-times lower elimination of the parent drug in faeces (Laffont et al., 2001). Altogether, these results suggested that under normal licking conditions, a large amount of the topical drug reached the systemic circulation by oral rather than percutaneous absorption. The raw data of this study on ivermectin in non-licking and control cattle are used in the present paper for the purpose of pharmacokinetic modeling. The aim was to quantify the actual contribution of licking to the systemic availability of pour-on ivermectin and to the excretion of the parent drug in faeces. The extent of ivermectin faecal excretion is indeed of special concern, given the potential negative environmental impact caused by the effect of the parent drug against some beneficial dung-breeding and dung-degrading insects (for review, see McKellar, 1997). We also present original data

on another pour-on administration of ivermectin performed in the same animals, in order to assess the intra-individual variability of exposure of the treated cattle.

This paper addresses the general problem of controlled drug delivery by topical application in animals, and discusses the consequences of animal social licking in terms of public health and food safety.

Materials and Methods

Study design and animals

The material and methods have been extensively described in the original paper (Laffont et al., 2001), except for information related to the first pour-on administration (PO1). Briefly, six pairs of monozygotic twin Holstein cattle (467 ± 19 kg b.wt., 3 years old) obtained by micromanipulation (Ozil et al., 1982) were used in the experiments. Each cattle received a single i.v. administration of injectable ivermectin (IVOMEC® injectable, Merial, Lyon, France; 200 µg/kg b.wt.) and two topical administrations of ivermectin (IVOMEC® pour-on, Merial, Lyon, France) at the standard dose of 500 µg/kg b.wt. All administrations were performed at a 3- to 5-month interval. For the second pour-on administration (PO2), the twins were separated into two groups of six animals. In the control group (the lickers), animals were kept in individual tie-stalls, each cattle being tethered with a loose chain so it could lick itself and its immediate neighbours. In the other group (the non-lickers), each animal was isolated from the others by a screen and was fitted with a wooden neck collar to prevent self-licking. Blood was collected regularly at 17 times up to 28 days after application of PO1, at 22 times up to 44 days after application of PO2, and at 20 times up to 31 days following i.v. administration. Total faeces were collected over 24 hours on days 4 and 14 after application of PO1. Following application of PO2, total faeces were collected for a period of 6 h (from 09:00 to 15:00 h) on days 1, 2, 3, 4, 7, 14, 18, 22 and 28 post-administration.

Analytical method

Concentrations of ivermectin (22,23-dihydroavermectin B1a) in plasma and in wet faeces were measured using a high-performance liquid chromatography (HPLC) technique (Alvinerie et al., 1987). The lower limit of quantification for ivermectin was 0.05 ng/mL for the plasma and 0.5 ng per g of wet faeces for the faecal samples. The limit of detection for ivermectin in plasma was 0.01 ng/mL. Accuracy and precision (intra-assay variation) expressed as relative standard

deviation were less than 8 and 6%, respectively.

Pharmacokinetic analysis

Non-compartmental approach. Individual areas under the plasma concentration-time curve, AUC(0-infinite), were computed using the trapezoidal rule. The extrapolated area (from the last sample to infinity) was calculated by dividing the last plasma concentration measured by the slope of the terminal phase (estimated in a semi-logarithmic scale).
Ivermectin (total) plasma clearance (CL_{tot}) was calculated as the ratio of the administered dose (200 µg/kg) divided by the AUC (0-infinite) obtained for the i.v. route :

$$CL_{tot} = Dose_{i.v.} / AUC_{i.v.} \quad \text{Eq. 1}$$

The systemic availability for topical ivermectin (F_{tot}) was calculated using the ratio of the AUCs (0-infinite) obtained after topical ($AUC_{pour\text{-}on}$) and i.v. administrations ($AUC_{i.v.}$) corrected by the ratio of the administered doses :

$$F_{tot}\ (\%) = (AUC_{pour\text{-}on} / AUC_{i.v}) \times (Dose_{i.v.} / Dose_{pour\text{-}on}) \times 100 \quad \text{Eq. 2}$$

Cumulative amounts of parent ivermectin eliminated in faeces were calculated by integration of the faecal excretion rate profile as a function of time, using the trapezoidal rule. The faecal excretion rates were obtained as the total amount of parent drug eliminated in faeces within the collection interval (*i.e.*, the product of the faecal concentration of ivermectin and the weight of wet faeces), divided by the time of collection.

Data modeling. Twin cattle were considered as the same animal taken under normal and restricted licking conditions. Each pair of twins was fitted separately. The pharmacokinetic model presented in Fig. 1 was selected among other different models to fit simultaneously the plasma and faecal ivermectin data obtained in the licking and non-licking twins after pour-on administration (PO2) and the i.v. plasma ivermectin data of the two twin cattle. The model was assumed dose-independent, but was not time-independent since the rate constant of ingestion of topical ivermectin (Ka) was described as a biexponential function of time. It was defined by the following equations :

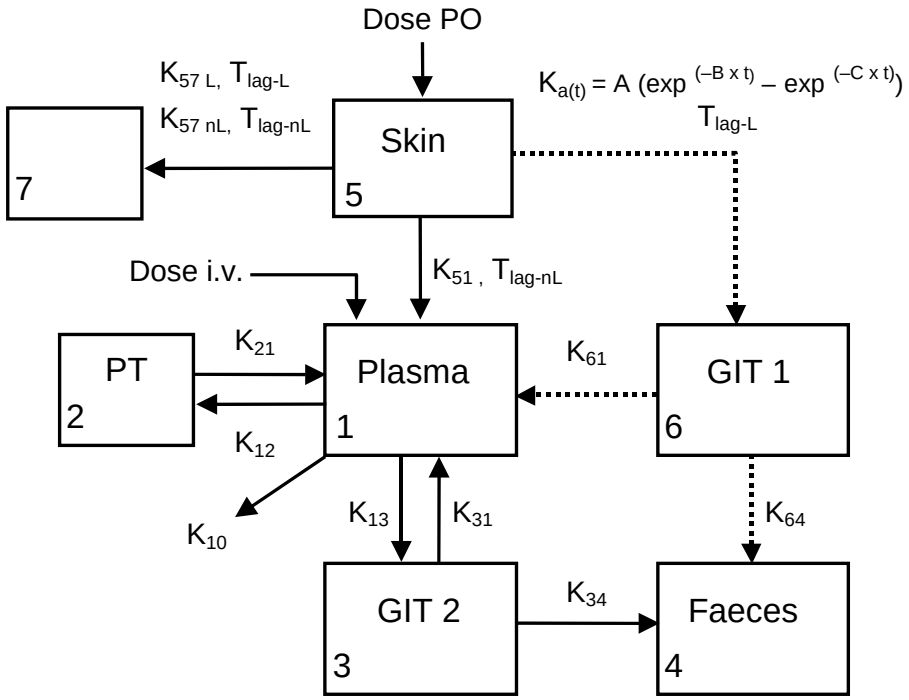


Figure 1. Disposition model for ivermectin after i.v. and topical (pour-on) administration in one pair of licking and non-licking monozygotic twin cattle**.** K_{ij} is the first-order rate constant of transfer from compartment i to compartment j. K_{10} is the first-order rate constant of elimination from compartment 1. The rate constant Ka traduces the ingestion of topical ivermectin by licking and is modelised as a biexponential function of time. The i.v. dose (200 µg/kg) is introduced in compartment 1 (plasma), while the pour-on dose (500 µg/kg) is introduced in compartment 5 (at the surface of the skin). The subscripts " L " and " nL " refer to the "licking" (control) or "non-licking" twin, respectively. "GIT 1", gastrointestinal compartment 1. "GIT 2", gastrointestinal compartment 2. " PT ", peripheral tissues.

$$Cp = X1 / V1 \quad \text{Eq. 3}$$

$$dX1/dt = K21 \times X2 + K31 \times X3 + K51 \times X5 + K61 \times X6 - (K10 + K12 + K13) \times X1 \quad \text{Eq. 4}$$

$$dX2/dt = K12 \times X1 - K21 \times X2 \quad \text{Eq. 5}$$

$$dX3/dt = K13 \times X1 - (K31 + K34) \times X3 \quad \text{Eq. 6}$$

$$dX4/dt = K34 \times X3 + K64 \times X6 \quad \text{Eq. 7}$$

$$dX5/dt = -(K51 + Ka(t) + K57) \times X5 \quad \text{Eq. 8}$$

$$dX6/dt = Ka(t) \times X5 - (K61 + K64) \times X6 \quad \text{Eq. 9}$$

$$dX7/dt = K57 \times X5 \quad \text{Eq. 10}$$

where Cp is the concentration of ivermectin in plasma (compartment 1, volume V1), Xi is the amount of ivermectin in compartment i, Kij is the first-order rate constant of transfer from compartment i to compartment j, and K10 is the first-order rate constant of elimination from compartment 1.
For the disposition of topical ivermectin in non-lickers, Ka (the constant of transfer from compartment 5 to compartment 6) was set to zero. For the disposition of topical ivermectin in control animals (the lickers), Ka was empirically described as a function of time (Eq. 11), increasing up to a maximum and then decreasing (see discussion).

$$Ka(t) = A \times [\exp^{(-B \times t)} - \exp^{(-C \times t)}] \quad \text{Eq. 11}$$

where the parameters A, B and C are expressed in h^{-1}.

The i.v. dose (200 µg/kg) was introduced into the plasma compartment, while the pour-on dose (500 µg/kg) was introduced into compartment 5 representing the skin surface. K12, K21, K13, K31 are the distribution rate constants between the plasma and the peripheral compartments 2 and 3. The rate constant K10 relates to the elimination of ivermectin from plasma by metabolism, while the rate constant K34 traduces the removal of parent ivermectin from the gastrointestinal tract (GIT 2;

compartment 3) into faeces (compartment 4). K51 is the rate constant for the systemic absorption of topical ivermectin *via* the skin. It was assumed that K51 did not differ between lickers and non-lickers (see discussion). The rate constant $K57_{nL}$ accounts for the fraction of topical ivermectin which was not systemically available by the percutaneous route in the non-licking cattle (*e.g.* left at the skin surface, photo-degraded, or metabolised by the enzymes of the skin). In control animals (the lickers), the topical drug could be absorbed through the skin (K51) or ingested by licking (Ka). One fraction of the ingested ivermectin was then absorbed in plasma (K61), while the remaining fraction transited unchanged into faeces (K64). The model assumes that the local disposition of ivermectin is not the same at all sites of the digestive tract (see discussion). The rates of drug absorption and elimination were indeed different in the gastrointestinal compartments 3 and 6 (GIT 1 and GIT 2, respectively). In lickers, the rate constant $K57_L$ refers to the fraction of topical ivermectin which was neither ingested nor systemically-available by the percutaneous route. First pass metabolism of ivermectin by the oral route was considered negligible, given the low value of the drug total clearance (overall coefficient of extraction < 1% in cattle). $T_{lag\text{-}L}$ is the delay to oral absorption, and $T_{lag\text{-}nL}$ is the delay to percutaneous absorption.

The differential equations were solved numerically using the SCIENTIST® program (MicroMath® Scientific Software, Inc., Version 2.01). The goodness of fit was assessed by examination of the lines of best fit and of residual patterns, taking into account a modified Akaike information criterion. The data points were weighed by the inverse of the squared observed values ($1/Y^2$) for ivermectin plasma concentrations, and by the inverse of the observed values (1/Y) for the amounts of ivermectin excreted in faeces. The fitting was performed stepwise: 1) the i.v. plasma concentrations of ivermectin obtained in the two twins were fitted simultaneously to equations 3-6, 2) the i.v. plasma data of the twins and the pour-on (plasma and faecal) data of the non-licker were fitted simultaneously using equations 3-10 (the estimates yielded by the first fitting were used as initial values for the parameters), and 3) the pour-on (plasma and faecal) data of the licking and non-licking twins and the i.v. plasma data of the two cattle were fitted simultaneously using the equations 3-11. The estimates yielded by the second fitting were taken as initial values for the parameters.

Pharmacokinetic parameters were calculated from the final estimates of the model parameters. Ivermectin total (plasma) clearance, $CL_{tot\ IVM}$, was calculated for each pair of twins according to the following equation (for demonstration, see

Nakashima and Benet, 1988):

$$CL_{tot\ IVM} = V1 \times [\ K10\ +\ K34\ \times K13 / (K31+K34)\ ] \qquad \text{Eq. 12}$$

The relative importance of the oral and percutaneous routes in the removal of ivermectin from the skin was assessed for each licking cattle by simulation of X1, X6 and X7 using equations 4 and 8-10 with K10, K12, K13, K61, K64 equal to zero. Simulations were performed over a period of 6000 h, until no ivermectin was left in compartment 5. The fraction of topical ivermectin absorbed though the skin, ingested ($f_{ingested}$), and remaining (left on the skin or degraded) were calculated as the final amount of ivermectin obtained by simulation in compartment 1, 6 and 7, respectively, divided by the applied dose of pour-on (500 µg/kg).
The absolute oral bioavailability of ivermectin (F_{oral}) was estimated by modeling :

$$F_{oral}\ (\%) = [\ K61 / (K61 + K64\ )] \times 100 \qquad \text{Eq. 13}$$

The percentage of topical ivermectin systemically-available by the oral route was obtained as $f_{ingested} \times F_{oral}$ (%).

Simulation of the ingestion of topical ivermectin with increased rates of percutaneous absorption

The influence of the rate of percutaneous absorption on the extent of drug ingestion was examined. Cumulative amounts of ivermectin ingested and absorbed through the skin after topical application were simulated for various magnitudes of percutaneous absorption rates. Simulations were performed using the estimated parameters obtained in each pair of twins (see Table 2). Equations 4 and 8-10 were used for the simulations with K10, K12, K13, K61, K64 equal to zero.

Simulation of the exposure of non-treated animals following cross-contamination by allo-licking

The possibility of the cross-contamination of non-treated cattle by licking of those topically-treated was examined. Simulations were carried out to evaluate the minimal amount of ivermectin that had to be licked to achieve non-negligible plasma exposure of untreated cattle. Residues of ivermectin in milk were given as an indicator of the contamination of food. The simulations were performed for each pair of twins using the final estimated parameters (listed in Table 2). For simulation of a single oral uptake, the dose was introduced into compartment 6. In the case of

the ingestion of topical ivermectin by multiple oral uptake (modelised by Ka), the dose was introduced into compartment 5 with K51 and $K57_L$ equal to zero, and equations 3-9 and 11 were used for the simulations. Residues of ivermectin in milk were predicted from the corresponding plasma concentrations of ivermectin, given a milk/plasma concentration ratio of 0.766 (Toutain et al., 1988).

Statistical analysis

Statistical analysis was performed using the SYSTAT® 8.0 (SPSS Inc., Chicago, IL) software. ANOVA was used to compare the within-pair and between-pair variability of plasma exposure (AUC) and of ivermectin total clearance after i.v. administration in the 6 pairs of twins. ANOVA was also applied to compare the intra- and inter-individual variability of plasma exposure (AUC) in the licker group after pour-on administration. A $p < 0.05$ was considered as significant. The results were expressed as means ± SD.

Results

Intravenous administration of ivermectin

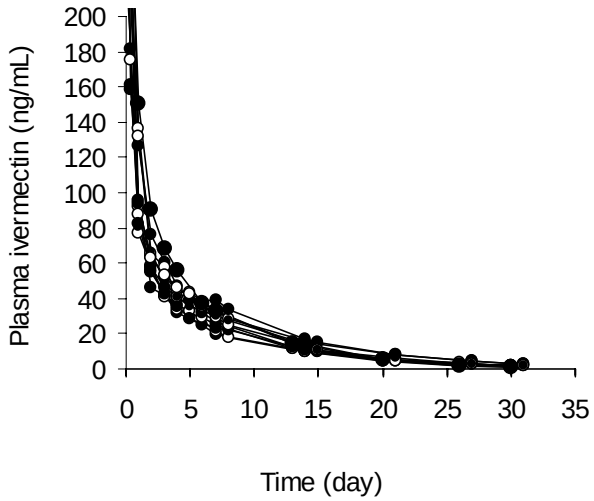
The plasma concentration profiles of ivermectin obtained in the twelve cattle were very homogenous (Fig. 2A). It must be noted that for all pairs of twins, the plasma concentration-time curves of the two twins were exactly superposed (see Fig. 3 for a representative pair of twins). Furthermore, the variability in plasma AUC was significantly lower between the twins (coefficient of variation (CV) of 5%) than that between the different pairs of twins (CV of 25%) ($p < 0.01$). Similar results were obtained for ivermectin total clearance, with a lower within-pair than between-pair variability ($p < 0.01$). Altogether, these results consolidate the choice of using monozygotic twins for the purpose of modeling.

The plasma data were well fitted by the model, as it is shown for a representative pair of twin cattle in Fig 3. The individual total clearances of ivermectin calculated by non-compartmental analysis were consistent with those estimated by modeling (Table 1; see the original article for other pharmacokinetic parameters: Laffont et al., 2001).

Pour-on administration of ivermectin

The plasma concentration-time profiles of ivermectin obtained after application of PO2 are presented in Figure 2B. By comparison with the i.v. route, the systemic exposure of animals was highly variable, depending on their ability to lick or not.

A)



B)

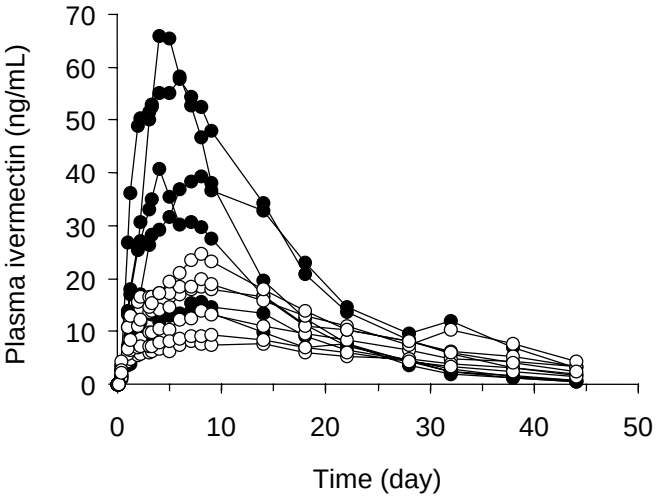
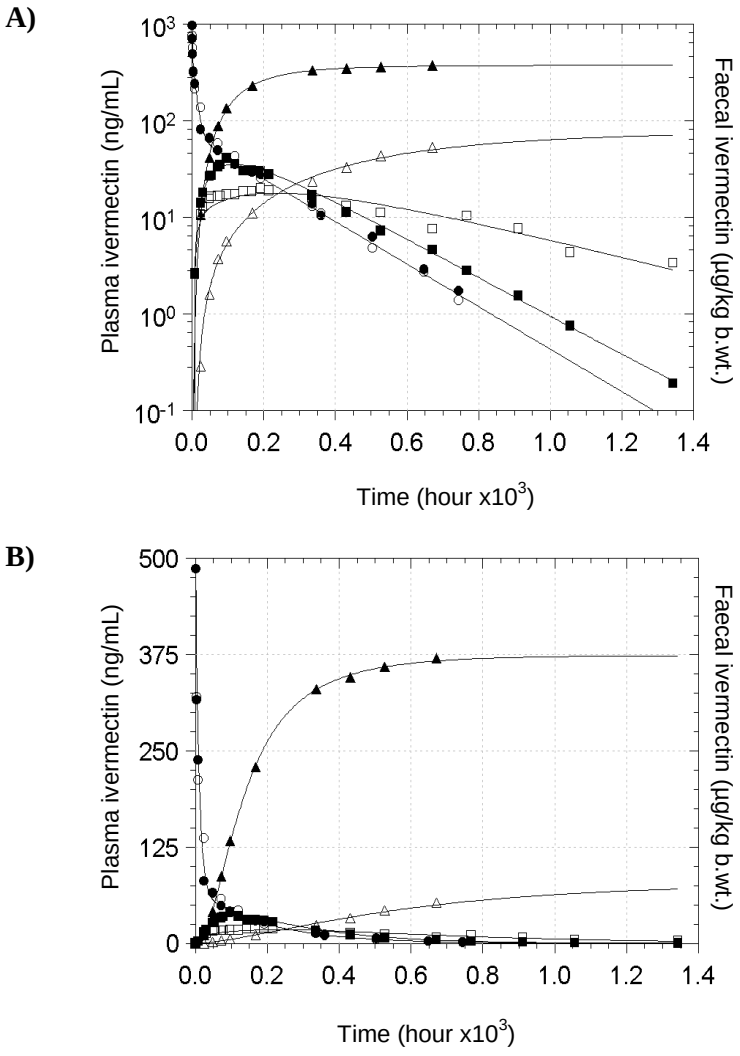
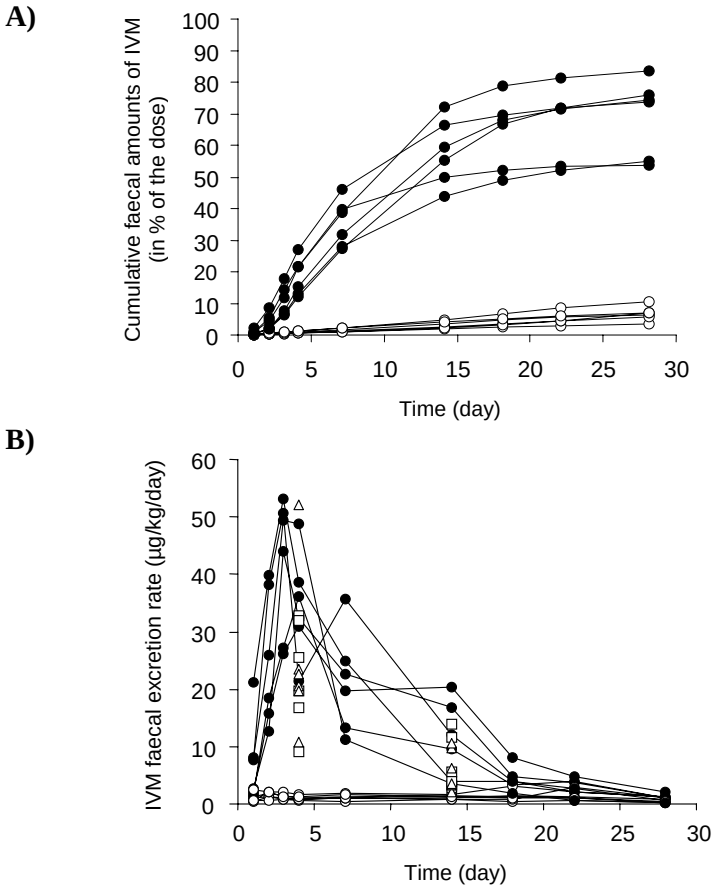


Figure 2. Ivermectin plasma concentration-time curves in 6 pairs of monozygotic twin cattle after i.v. administration (**Fig. 2A**) and topical (pour-on) administration (**Fig. 2B**). The twins were separated in two groups of 6 animals: the licker group (filled symbols) and the non-licker group (open symbols). For the pour-on administration, self- and allo-licking were prevented in the cattle of the non-licker group.

Figure 3A shows different slopes for the plasma terminal phase between i.v. and pour-on administrations in non-lickers. This “flip-flop” phenomenon (extensively described in the original article: Laffont et al., 2001) does not occur under normal licking conditions and shows that, in contrast to oral absorption, the percutaneous absorption of ivermectin is a very slow process limiting the drug plasma elimination. Cumulative amounts of parent ivermectin excreted in faeces under normal or restricted liking conditions are presented in Fig. 4A. They show major differences in the faecal excretion of the parent drug between licking and non-licking animals (see also Table 1).





▲ **Figure 4.** Faecal excretion of parent ivermectin (IVM) following topical application of 6 pairs of monozygotic twin cattle (PO2). Twins were separated into two groups of 6 animals: the non-licker group (open circles), in which self-and allo-licking were prevented, and the licker group (filled circles, control group). **A)** Cumulative amounts of ivermectin excreted in faeces. **B)** Ivermectin faecal excretion rates. Open triangles and open squares refer to a previous pour-on administration (PO1) in the same animals (licker and non-licker groups, respectively), with this time no restriction of licking in any of the two groups.

◀ **Figure 3.** Experimental (symbols) and simulated (line) ivermectin data after i.v. and topical (pour-on) administration in a representative pair of monozygotic twin cattle. Circle symbols, plasma concentrations of ivermectin after i.v. administration. Triangle symbols, cumulative amounts of parent ivermectin excreted in faeces after topical administration. Square symbols, plasma concentrations of ivermectin after topical administration. Licking was prevented in one of the two twins (open symbols; non-licking cattle), the other twin serving as a control (filled symbols; licking cattle). **A)** semi-logarithmic scale. **B)** arithmetic scale.

For the cattle of the non-licker group, it was possible to compare the disposition of pour-on ivermectin in the same animal under normal (PO1) and restricted (PO2) licking conditions. As shown in Fig. 4B, the faecal excretion rates observed in these cattle after application of PO1 match those found in their twins (licker group) following administration of PO1 and PO2. The overall bioavailability of pour-on ivermectin was not significantly increased with licking (24 ± 6.4 % in PO1 *vs* 22 ± 6.6 % in PO2), but more importantly no flip-flop phenomenon was observed.

Taken under normal licking conditions, all cattle showed a high variability of plasma exposure. After application of PO1, the difference in ivermectin bioavailability within the same pair of twins could be as high as 70%. In the licker group, the plasma AUC of ivermectin in a same animal was multiplied by a factor of 0.6 to 2.3 from one pour-on application to the other. The intra-individual variability (CV of 41%) was not significantly lower than the inter-individual variability (CV of 55%).

The plasma and faecal data obtained for ivermectin after pour-on administration were well fitted by the model for all pairs of twins (Table 1; see Fig. 3 for a representative pair of twins). In one pair of twins however, the predicted faecal data underestimated by 35% the observed amounts of parent drug recovered in the faeces of the licker, which will be further discussed. The estimated parameters of the overall disposition model presented in Fig. 1 are listed in Table 2. It is noteworthy that the parameters showing the highest coefficients of variation were those associated with the ingestion of the topical drug by licking (A, B, C, K61, K64, K57, $T_{lag\text{-}L}$). Following topical administration, pharmacokinetic modeling gave a mean absolute bioavailability of ivermectin of 32 ± 13.8% in lickers and of 23 ± 7.5% in non-lickers (see Table 3), which is very close to the values obtained by non-compartmental analysis (33 ± 18.6% and 22 ± 6.6%, respectively).

The model indicates that 58-87% of the topically-applied ivermectin was actually ingested by licking (Table 3), and that 50-77% of the drug systemic absorption was achieved by the oral route (corresponding to 22% of the applied dose; Table 3) *vs* 23-50% by the percutaneous route (10% of the applied dose; Table 3). Oral absolute bioavailability of ivermectin was estimated by modeling (Eq. 8), and tended to be higher than for the percutaneous route (28 ± 13.2% *vs* 22 ± 6.6%, respectively). Together with the large ingestion of drug, this explains the higher overall bioavailability of pour-on ivermectin in lickers (F_{tot} of 32 ± 13.8%), compared to non-licking cattle (F_{tot} of 23 ± 7.5%) (Table 3).

Table 1. *Ivermectin total clearance (CL_{total}) in cattle, and evaluation of the contribution of licking to the faecal excretion of parent ivermectin after topical application to cattle.*

Pair number	CL_{total} (mL/day/kg)		% dose eliminated unchanged in the faeces of lickers			
	non-comp.	comp.	non-comp. (0-28 days)	comp. (0-28 days)	comp. (0-infinite)	By the oral route
Non-lickers						
1	230.8	227.9	6.5	6.7	9.0	—
2	286.0	322.9	3.3	4.3	6.4	—
3	332.7	325.1	5.7	5.3	7.1	—
4	283.6	296.3	6.9	4.6	6.5	—
5	236.7	258.2	10.6	10.0	15.5	—
6	225.6	233.0	6.8	7.7	10.2	—
mean ± SD	266 ± 42.2	277 ± 43.6	6.6 ± 2.3	6.4 ± 2.2	9.1 ± 3.5	—
Lickers						
1	211.1	227.9	74.2	74.8	80.5	59.4
2	322.1	322.9	76.0	75.4	77.5	70.4
3	328.3	325.1	55.0	54.2	56.5	48.2
4	355.5	296.3	83.2	54.0	55.7	44.0
5	214.4	258.2	74.1	73.4	74.8	61.3
6	210.2	233.0	53.6	56.8	57.6	43.9
mean ± SD	274 ± 68.5	277 ± 43.6	69 ± 12.1	65 ± 10.7	67 ± 11.6	55 ± 10.8

Compartmental (comp.) and non-compartmental (non-comp.) analysis were performed in the 6 pairs of monozygotic twin cattle.

Table 2. *Estimated parameters of the seven-compartment model (Fig. 1) selected to describe the i.v and topical disposition of ivermectin in cattle.*

Parameters	pair number						mean	%CV
	1	2	3	4	5	6		
V1 (L/kg)	0.078	0.084	0.080	0.093	0.074	0.058	0.078	15
K10 (h^{-1})	0.054	0.096	0.101	0.105	0.077	0.094	0.088	22
K12 (h^{-1})	0.288	0.293	0.439	0.271	0.283	0.358	0.322	20
K21 (h^{-1})	0.016	0.016	0.019	0.020	0.017	0.021	0.018	13
K64 (h^{-1})	0.345	0.057	0.038	1.929	0.145	0.021	0.423	177
K61 (h^{-1})	0.140	0.008	0.008	1.868	0.044	0.011	0.347	216
$K57_{L}$ (h^{-1})	0.00011	0.00058	0.00181	0.00000	0.00071	0.00223	0.00091	101
$K57_{nL}$ (h^{-1})	0.00269	0.00192	0.00245	0.00176	0.00143	0.00216	0.00207	22
K13 (h^{-1})	1.760	3.872	3.693	2.096	1.673	3.242	2.723	37
K31 (h^{-1})	0.642	0.883	0.918	0.484	0.491	0.889	0.718	28
K34 (h^{-1})	0.026	0.015	0.018	0.006	0.021	0.021	0.018	37
$TLAG_{L}$ (h)	37.1	14.3	27.4	20.0	0.0	5.4	17.4	79
$TLAG_{nL}$ (h)	6.8	5.4	5.0	2.0	7.1	5.7	5.3	35
A (h^{-1})	0.0186	0.0480	0.0042	0.0093	0.0108	0.0114	0.0170	93
B (h^{-1})	0.00325	0.00215	0.00096	0.00179	0.00158	0.00241	0.00202	39
C (h^{-1})	0.00731	0.00308	0.04622	0.02854	0.01658	0.02418	0.02098	75
K51 (h^{-1})	0.00052	0.00037	0.00052	0.00079	0.00070	0.00065	0.00059	25

The plasma (i.v., pour-on) and faecal (pour-on) ivermectin data of six pairs of monozygotic twin cattle were used for the compartmental analysis. Twins were separated as "lickers" and "non-lickers": licking was prevented in one twin, the other twin serving as a control. Each pair of twins was fitted separately. "L", Lickers; "nL", Non-lickers.

Table 3. *Absolute bioavailability (F_{total}) of pour-on ivermectin in cattle, and evaluation of the extent of ingestion, oral absorption, and percutaneous absorption of ivermectin after topical application.*

Pair number	F_{total} (%)		% of the dose			
	non-comp. analysis	comp. analysis	ingested	absorbed orally	absorbed through the skin	left
Non-lickers						
1	17.4	16.2	—	—	16.2	83.8
2	15.6	16.2	—	—	16.2	83.8
3	16.9	17.5	—	—	17.5	82.5
4	30.1	31.0	—	—	31.0	69.0
5	29.9	32.8	—	—	32.8	67.2
6	24.2	23.2	—	—	23.2	76.8
mean ± SD	22 ± 6.6	23 ± 7.5	—	—	23 ± 7.5	77 ± 7.5
Lickers						
1	35.6	37.8	83.4	24.0	13.8	2.6
2	18.2	18.0	80.5	10.1	7.9	11.7
3	21.4	20.4	58.4	10.2	10.2	31.4
4	68.4	56.0	86.6	42.6	13.4	0.0
5	22.9	28.4	80.1	18.7	9.7	10.3
6	29.6	31.1	67.8	23.9	7.3	25.0
mean ± SD	33 ± 18.6	32 ± 13.8	76 ± 10.8	22 ± 12.0	10 ± 2.7	13 ± 12.4

Compartmental (comp.) and non-compartmental (non-comp.) analysis were performed in the 6 pairs of monozygotic twin cattle.

Approximately 72% of the ingested ivermectin was not absorbed and transited directly into faeces, providing a major contribution (82%) to the excretion of parent drug in the dung (72 = (0.55 / 0.76) × 100; 82 = (0.55 / 0.67) × 100; see Tables 1 and 3).

Individual time profiles of the ingestion rate constant, Ka, were generated by modeling and are presented in Fig. 5A. Peak values of Ka were reached between 3 and 16 days after pour-on application. In general, high Ka peak values were associated with a rapid decrease of Ka, whereas lower Ka peak values were associated with a slower decrease of Ka. Half of the ingestion was achieved between 3 and 7 days post-administration, and 90% of the ingestion was achieved between 9 and 17 days post-administration. The differences observed among cattle are consistent with the large variability expected from the licking behaviour of animals.

Simulations of ivermectin ingestion profiles with increased rates of percutaneous absorption

Ingestion profiles were simulated for different magnitudes of percutaneous absorption rates, using the final estimated parameters obtained for each pair of twins. The results of the simulations are presented in Fig 5B for a representative pair of twin cattle. They indicate that the rate constant of percutaneous absorption, K51, must be increased by a factor 6 - 10 to obtain less ivermectin ingested by licking than absorbed through the skin, and by at least a factor 50 to reduce the ingestion of ivermectin by one order of magnitude (down to 8 ± 2.3% of the dose). A 8-fold higher rate constant of percutaneous absorption resulted in a higher bioavailability of topical drug *via* the skin (48 ± 6.4% instead of 10 ± 2.7%), and in lower amounts of ivermectin remaining as a cutaneous depot or degraded (8 ± 7.4% of the dose instead of 13 ± 12.4%).

Simulation of the contamination of non-treated animals with ivermectin by allo-licking

In the case of multiple oral uptake (modelised by Ka), plasma concentrations of 1 ng/mL were achieved following total ingestion of 2 - 3.7% of the pour-on dose in 4 of the 6 pairs of twins, and of 10-11% in the two other pairs. This resulted in detectable (> 0.01 ng/mL) and persistent (> 44 days) concentrations of ivermectin in milk, with maximal values of 0.8 ng/mL. Only 0.02 - 0.11% of the dose had to be ingested in total to recover detectable concentrations of ivermectin in plasma (0.01 ng/mL). In the case of single oral uptake, plasma concentrations of 1 ng/mL

(0.8 ng/mL in milk) were achieved following total ingestion of 0.3 - 2.5% of the pour-on dose in the 6 pairs of twins. Total ingestion of 0.003 - 0.025% of the pour-on dose was sufficient to recover detectable concentrations of ivermectin in plasma.

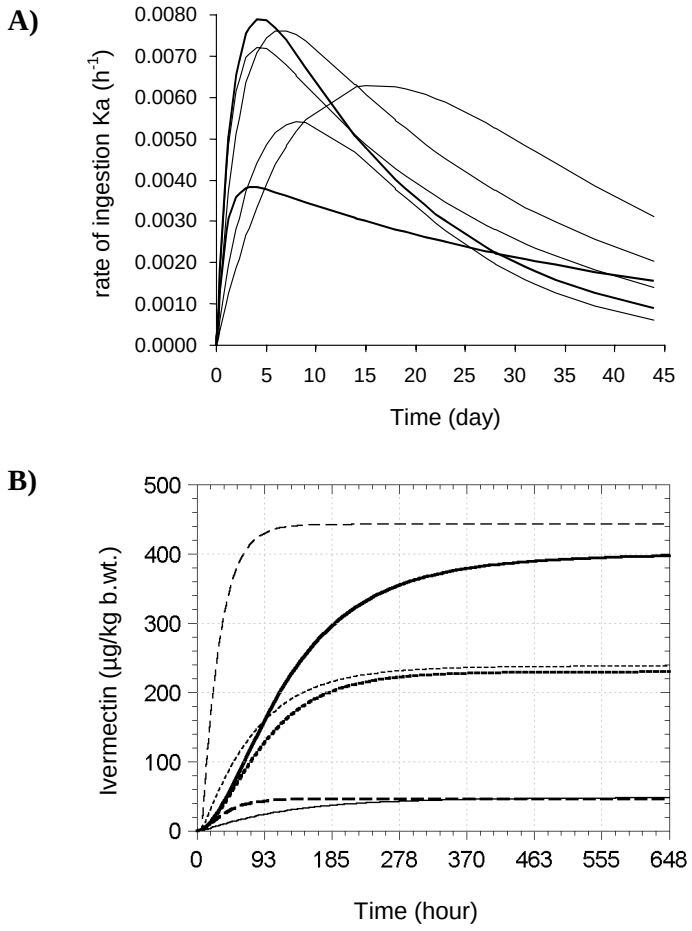


Figure 5. Ingestion of ivermectin following topical application to cattle along the dorsal midline. **A)** The rate constant of ingestion, Ka, obtained by modeling is plotted as a function of time, for each of the 6 licking cattle. **B)** Simulations of the percutaneous absorption (**normal-width line**) and ingestion (**thick line**) of topical ivermectin with increased rates of percutaneous absorption in one representative licking cattle. **Solid line,** control values. **Short-dashed line,** percutaneous rate multiplied by 8. **Medium-dashed line,** percutaneous rate multiplied by 50.

Discussion

In contrast to what could be expected for a "percutaneous" formulation, the present study shows that the main route for the systemic absorption of topical ivermectin is not percutaneous but oral, as a result of cattle licking behaviour. This is the first time such an interaction between the individual/social behaviour of animals and the pharmacokinetics of parenterally-administered drugs is reported.

The selected pharmacokinetic model adequately describes the experimental plasma and faecal data observed after i.v. and pour-on administrations of ivermectin in licking and non-licking cattle. The predicted values for the absolute bioavailability of pour-on ivermectin (F_{tot}) and for the drug total clearance (CL_{tot}) are very close to those obtained by non-compartmental analysis. It was assumed that the rate constant of percutaneous absorption, K51, did not differ between lickers and non-lickers. This is supported by a non-significant difference in the estimates of $K51_L$ and $K51_{nL}$ when considered as separate parameters in the model, which suggests that the absence of licking for 44 days did not impair the process of ivermectin absorption through the skin.

It is worthwhile to mention that the pour-on data obtained in lickers were totally misfitted by a model containing a single gastrointestinal compartment pooling compartments GIT 1 and GIT 2. Selection of a model with two separate gastrointestinal compartments suggests that the local disposition of ivermectin is not the same at all sites of the gastrointestinal tract in terms of (re-)absorption and elimination. This is in line with the experimental finding of a different ivermectin bioavailability in the rumen and in the abomasum in sheep (Prichard et al., 1985). The low bioavailability of ivermectin after intraruminal administration (75% lower than after intra-abomasal administration) was first attributed to an extensive degradation or metabolisation of drug in the rumen (Prichard et al., 1985). This explanation is however difficult to conciliate with our results, since the unabsorbed fraction of ingested ivermectin was fully recovered in the faeces of lickers. Moreover, several studies argue for the stability of ivermectin in rumen fluids in cattle and in sheep (Bogan and McKellar, 1988; Andrew and Halley, 1996), and show that there is rather an extensive adsorption of ivermectin to the digesta particulates of the rumen (Ali and Hennessy, 1996). Our model stipulates that the fraction of drug which is not absorbed in compartment GIT 1 will not be absorbed in compartment GIT 2. A possible interpretation is that ivermectin remains adsorbed to the particulate phase of the digesta during intestinal transit, and thus is not available anymore for absorption in intestinal fluids. Such a situation has already been described for other compounds such as phenylbutazone (Bogan et al.,

1984; Lees et al., 1988), and is consistent with the high organic-carbon binding constant of ivermectin (K_{oc} = 12600-15700; Halley et al., 1989) and its high hydrophobicity (Pouliot et al., 1997). Ivermectin oral bioavailability was estimated from the model to 28 ± 13.2%, which is in line with the bioavailability found by Chiu et al. (1990) in cattle for an intraruminal bolus of ivermectin relative to the subcutaneous route (26%). However, the contribution of a buccal absorption of drug cannot be excluded.

Pharmacokinetic modeling showed that approximately 76% of the pour-on dose was ingested by licking. As indicated by preliminary deconvolution studies, the rate constant of ingestion, Ka, could be modelised as a biexponential function of time, with an increase up to a maximum followed by a gradual decrease. Ivermectin is a highly lipid soluble drug (Fisher and Mrozik, 1989), which markedly accumulates in the ear wax of pigs (Scott and McKellar, 1992). It is thus possible that ivermectin spreads over the skin of cattle by diffusion within the skin lipid layer, as shown for the antiparasitic agents cypermethrin (Jenkinson et al., 1986), flumethrin (Stendel et al., 1992) and parathion (Brimer et al., 1994) in sheep, cattle or pigs. The first increase of Ka would then be explained by a higher licking efficiency, due to a progressive increase in skin surface exposure. Conversely, the decrease of Ka would correspond to a reduction in licking efficiency, consistent with the decrease of ivermectin concentrations on the skin. This decrease would be more or less rapid depending on individual licking activity. Kinetic modeling shows an ingestion of topical ivermectin up to 19 days post-administration, suggesting that ivermectin was available at the skin surface for a long period of time. This is in agreement with previously published results, indicating that ivermectin was still present on the skin of non-licking cattle 44 days post-administration (Laffont et al., 2001). It is though possible that ivermectin partitions into the various layers of the skin as for other lipid-soluble compounds (chlorpyrifos, Griffin et al., 2000), and is released over a prolonged period at the skin surface. Distribution of the drug in the skin after systemic absorption cannot be excluded, given the high concentrations of ivermectin found in the epidermis and dermis of cattle after subcutaneous administration (Lifschitz et al., 2000).

The model indicates that 50-77% of ivermectin absorption in plasma was achieved by the oral route, compared to 23-50% by the percutaneous route. This infers that the systemic exposure of animals most likely depends on their ability to lick themselves (self-licking) or each other (allo-licking). This would explain the erratic bioavailability of pour-on ivermectin observed in the licker group (coefficient of variation of 55% in lickers *vs* 29% in non-lickers after application of PO2) and in previous studies (Gayrard et al., 1999), as well as the large intra-

individual and within-pair variability of plasma exposure (up to 230% and 70%, respectively) which could not be of genetic origin. More generally, the extent of licking depends on various social, nutritional, physiological, pathological, environmental and managerial factors (Sato et al., 1991, 1993), which makes the delivery of drug even more unpredictable. An increased licking activity has been reported in parasitised cattle (Sato et al., 1991), which could be in favour of a higher plasma exposure of the animals and a better efficacy of the pour-on treatment. On the other hand, potential systemic underexposure could result in subtherapeutic plasma concentrations, which may promote the development of drug resistance (Geerts and Gryseels, 2000). At last, the results question the relevance of performing *in vivo* bioequivalence assays for evaluation of topical drug formulations in licking animals.

For simplicity reasons, allo-licking was not taken into account in the model. However, although the cattle of the licking group were housed in individual boxes, contacts by allo-licking were observed during the experiments between the immediate neighbours. In one pair of twins, the pour-on dose (500 µg/kg) applied on the back of the licker did not provide a sufficient amount of ivermectin to explain the high faecal data (no ivermectin was left on the skin). This suggests a supplementary source of topical drug consistent with allo-licking.

Under field conditions, allo-licking may result in the contamination of the non-treated cattle in contact with those topically treated. Our simulations (multiple oral uptake) showed a significant (detectable) plasma exposure of non-treated animals which would lick small amounts of ivermectin from the skin of one or several treated cattle (0.02-0.11% of the pour-on dose in total). It is noteworthy that a continuous licking corresponding to 2-11% of the pour-on dose in total would result in detectable concentrations in the milk of non-treated cattle for more than 44 days, with maximal plasma concentrations of 1 ng/mL. These amounts depend obviously on the individual licking activity (traduced by the Ka function), but are all very low compared to the 76% of the dose actually ingested by the treated cattle under the same conditions. Altogether, these results suggest an easy contamination of non-treated cattle by social contact with treated animals and elicit concern over possible insecticide residues in their milk and edible tissues.

The skin provides an excellent barrier against the environment and foreign substances, and many drugs used in human and veterinary medicine exhibit a low rate and extent of dermal absorption (Barry, 2001; Magnusson et al., 2001; Riviere and Papich, 2001). In the present study however, we show that a low rate of absorption through the skin may not ensure a controlled and optimised delivery of drug to the systemic or local circulation. In control cattle, licking reduced by a

factor two the total amount of ivermectin delivered to the plasma *via* the skin (from 20% of the dose in non-lickers to 10% in lickers) and led to an unpredictable absorption of topical drug by the oral route. As indicated by the simulations, the rate constant of percutaneous absorption K51 would need to be increased by a factor of at least 50 to warrant a good percutaneous absorption of drug, independently of animal licking behaviour or other external events (evaporation, accidental removal of the drug …).

In conclusion, we demonstrate that the social/individual licking behaviour of animals can markedly interfere with the disposition of topical drugs and can contribute to a higher excretion of active compound into the environment. We also suggest that allo-licking can result in drug exposure of non-treated animals. This route of contamination has to be considered as a general risk for topically-applied drug formulations in food-producing animals.

References

Ali DN and Hennessy DR (1996) The effect of level of feed intake on the pharmacokinetic disposition and efficacy of ivermectin in sheep. *J Vet Pharmacol Ther* **19:**89-94.

Alvinerie M, Sutra JF, Galtier P and Toutain PL (1987) Determination of ivermectin in milk by high performance liquid chromatography. *Ann Rech Vet* **18:**269-274.

Andrew NW and Halley BA (1996) Stability of ivermectin in rumen fluids. *J Vet Pharmacol Ther* **19:**295-299.

Barry BW (2001) Novel mechanisms and devices to enable successful transdermal drug delivery. *Eur J Pharm Sci* **14:**101-114.

Bogan JA, Galbraith A, Baxter P, Ali NM and Marriner SE (1984) Effect of feeding on the fate of orally administered phenylbutazone, trimethoprim and sulphadiazine in the horse. *Vet Rec* **115:**599-600.

Bogan JA and McKellar QA (1988) The pharmacodynamics of ivermectin in sheep and cattle. *J Vet Pharmacol Ther* **11:**260-268.

Brimer L, Gyrd-Hansen N and Rasmussen F (1994) Disposition of parathion after dermal application in pigs. *J Vet Pharmacol Ther* **17:**304-308.

Chiu L, Green ML, Baylis FP, Eline D, Rosegay A, Meriwether H and Jacob TA (1990) Absorption, tissue distribution and excretion of tritium-labeled ivermectin in cattle, sheep and rat. *J Agric Food Chem* **38:**2072-2078.

Fisher MH and Mrozik H (1989) Chemistry. In: Ivermectin and Abamectin (Campbell WC ed). New York: Springer, 1-23.

Gayrard V, Alvinerie M and Toutain PL (1999) Comparison of pharmacokinetic profiles of doramectin and ivermectin pour-on formulations in cattle. *Vet Parasitol* **81:**47-55.

Geerts S and Gryseels B (2000) Drug resistance in human helminths: Current situation and lessons from Livestock. *Clin Microbiol Rev* **13:**207-222.

Griffin P, Payne M, Mason H, Freedlander E, Curran AD and Cocker J (2000) The in vitro percutaneous penetration of chlorpyrifos. *Hum Exp Toxicol* **19:**104-107.

Halley BA, Nessel RJ and Lu AYH (1989) Environmental aspects of ivermectin usage in livestock: general considerations. In: Ivermectin and Abamectin (Campbell WC ed). New York: Springer, 162-172.

Hennessy DR (1997) Modifying the formulation or delivery mechanism to increase the activity of anthelmintic compounds.*Vet Parasitol* **72:**367-382.

Jenkinson DM, Hutchison G, Jackson D and McQueen L (1986) Route of passage of cypermethrin across the surface of sheep skin. *Res Vet Sci* **41:**237-241.

Krohn CC (1994) Behaviour of dairy cows kept in extensive (loose housing/pasture) or intensive (tie-stall) environments. III. Grooming, exploration and abnormal behaviour. *Appl Anim Behav Sci* **42:**73-86.

Laffont CM, Alvinerie M, Bousquet-Melou A and Toutain PL (2001) Licking behaviour and environmental contamination arising from pour-on ivermectin for cattle. *Int*

J Parasitol **31:**1687-1692.

Lees P, Taylor JB, Higgins AJ and Sedgwick AD (1988) In vitro and in vivo binding of phenylbutazone and related drugs to equine feeds and digesta. *Res Vet Sci* **44:**50-56.

Lifschitz A, Virkel G, Sallovitz J, Sutra JF, Galtier P, Alvinerie M and Lanusse C (2000) Comparative distribution of ivermectin and doramectin to parasite location tissues in cattle. *Vet Parasitol* **87:**327-338.

Magnusson BM, Kenneth A and Roberts MS (2001). Veterinary drug delivery: potential for skin penetration enhancement. *Adv Drug Deliv Rev* **50:**205-227.

McKellar QA (1997) Ecotoxicology and residues of anthelmintic compounds. *Vet Parasitol* **72:**413-435.

Nakashima E and Benet LZ (1988) General treatment of mean residence time clearance, and volume parameters in linear mammillary models with elimination from any compartment. *J Pharmacokinet Biopharm* **16:**475-492.

Ozil JP, Heyman Y and Renard JP (1982) Production of monozygotic twins by micromanipulation and cervical transfer in the cow. *Vet Rec* **110:**126-127.

Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E (1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **10:**17-25.

Prichard RK, Steel JW, Lacey E and Hennessy DR (1985) Pharmacokinetics of ivermectin in sheep following intravenous, intra-abomasal or intraruminal administration. *J Vet Pharmacol Ther* **8:**88-94.

Riviere JE and Papich MG (2001) Potential and problems of developing transdermal patches for veterinary applications. *Adv Drug Deliv Rev* **50:**175-203.

Rothen-Weinhold A, Gurny R and Dahn M (2000) Formulation and technology aspects of controlled drug delivery in animals. *Pharm Sci Technol Today* **3:**222-231.

Sato S, Sako S and Maeda A (1991) Social licking patterns in cattle (Bos taurus): influence of environmental and social factors. *Appl Anim Behav Sci* **32:**3-12.

Sato S, Tarumizu K and Hatae K (1993) The influence of social factors on allogrooming in cows. *Appl Anim Behav Sci* **38:**235-244.

Scott EW and McKellar QA (1992) The distribution and some pharmacokinetic parameters of ivermectin in pigs. *Vet Res Commun* **16:**139-146.

Simonsen HB (1979) Grooming behaviour of domestic cattle. *Nord Vet Med* **31**:1-5.

Stendel W, Hamel HD, Sieveking HU and Bruhne D (1992) Analytical determination of the distribution of flumethrin on the body surface of cattle following topical pour-on application. *Vet Parasitol* **42:**137-143.

Toutain PL, Campan M, Galtier P and Alvinerie M (1988) Kinetic and insecticidal properties of ivermectin residues in the milk of dairy cows. *J Vet Pharmacol Ther* **11:**288-291.

CHAPTER 7

General discussion

General discussion

Avermectins and milbemycins are potent antiparasitic drugs, which are widely used in veterinary medicine to treat and control various parasitic conditions. They present a unique spectrum of activity against both ecto- and endoparasites, which resulted into the embracing name of "endectocides". Ivermectin was the first endectocide to be introduced onto the international health market in 1981 and is still the most widely used across animal species, worldwide. It is also currently used in human medicine and has become the drug of choice for the treatment of onchocerciasis.

Ivermectin is extensively eliminated in faeces, irrespective of the species and administration route, with less than 2% excreted in the urine (Campbell, 1985). A large amount of ivermectin is eliminated unchanged from blood (40% of a i.v. dose in cattle; **chapter 5**), but the routes by which parent ivermectin reaches the digestive tract are still not fully understood. As for other endectocides (Hennessy et al., 2000), it is currently believed that biliary secretion is the main elimination pathway of the parent drug (Chiu et al., 1990). However, the possibility of an intestinal secretion of ivermectin by the gut wall has been raised. In a previous study in sheep and cattle, Bogan and McKellar (1988) found indeed three-times higher concentrations of ivermectin in duodenal and ileal fluids than in fluids sampled proximal to the bile duct opening. Ivermectin was present at even higher concentrations in intestinal mucous, with no significant difference between proximal and distal small intestine.

The existence of an intestinal secretion of parent ivermectin was demonstrated in the first part of the thesis, using two validated *in vitro* and *in situ* models. The mechanisms involved in the secretion process are discussed, with special emphasis on the drug transporting P-glycoprotein. A better understanding of these routes and mechanisms of elimination is of clinical interest, since they influence the persistence of ivermectin in plasma and determine the exposure of gastrointestinal parasites after systemic absorption or parenteral administration.

Ivermectin elimination from blood, however, cannot always explain the rate and extent of ivermectin excretion into faeces after parenteral administration. Topical application results indeed in higher faecal concentrations of ivermectin than for the subcutaneous route, which is inconsistent with the lower plasma concentrations (Herd et al., 1996). No explanation for this apparent discrepancy has been provided. Our hypothesis was that topically-treated cattle could ingest ivermectin by licking. This hypothesis was examined in the second part of the thesis and proved to be relevant.

Intestinal elimination of ivermectin and implication of P-glycoprotein in the secretion process

The possibility of an intestinal secretion of ivermectin was examined *in vitro* using the human colon adenocarcinoma cell line Caco-2 (**chapter 2**). Caco-2 cells form confluent monolayers of well-differentiated cells, which display many morphological and functional characteristics of the human enterocyte (reviewed by Boulenc, 1997). Among other intestinal transporters, they express P-glycoprotein (P-gp) and the multidrug resistance-associated protein 2 (MRP2) (Hosoya et al., 1996; Boulenc, 1997; Hunter and Hirst, 1997; Hirohashi et al., 2000; Walgren et al., 2000). P-gp and MRP2 are two drug efflux transporters localised in the apical (luminal) membrane, which have been shown to mediate the intestinal secretion of several compounds (Hunter and Hirst, 1997; Van Asperen et al., 1998; König et al., 1999; Gotoh et al., 2000; Suzuki and Sugiyama, 2000; Walgren et al., 2000; Borst and Elferink; 2002). The Caco-2 cell line is now a well-established *in vitro* model of the intestinal epithelium and is widely used to characterise the intestinal behaviour of drugs (absorption, secretion). We showed that ivermectin was preferentially transported from the basolateral side (representing systemic blood) to the apical side (representing the intestinal lumen) of Caco-2 cell monolayers (**chapter 2**). The secretory flux was 2- to 10-times higher than that in the opposite direction, depending on the concentration of ivermectin in the donor compartment. This study provides therefore evidence for a transepithelial secretion of parent ivermectin from blood into the intestinal lumen.

The *in vivo* relevance of this secretory transport was confirmed by our *in situ* studies in the rat using the intestinal closed-loop model (**chapter 3**). This model allows direct quantification of both biliary and intestinal secretions of parenterally-administered drugs, by cannulation of the common bile duct and *in situ* isolation of intestinal loops with their intact blood supplies. This study demonstrated the existence of an intestinal secretion of parent ivermectin by the small intestine, which was 5-fold higher than that into bile at all tested dose rates (100-200-400 µg/kg i.a.). The secreted ivermectin was extensively associated with intestinal mucous, in agreement with the observations of Bogan and McKellar (1988) in sheep and cattle (previously described). Ivermectin small intestinal clearance accounted for 27% of the drug total clearance, whereas the biliary clearance of ivermectin represented only 5.5% of the drug total clearance. Since no glucuronide conjugates of ivermectin have ever been identified *in vivo*, the ivermectin secreted into bile was the only contribution of biliary secretion to the total amount of parent drug in the intestinal lumen. It results that the major route for the elimination of

parent ivermectin in the rat is not biliary but intestinal secretion, in contrast to previous beliefs. Besides, it is noteworthy that the intestinal clearance of ivermectin was calculated solely from the small intestine, but that other regions of the digestive tract may participate as well to the elimination process. The colon, for instance, displays high levels of P-gp expression (Thiebaut et al., 1987). Therefore, it could contribute significantly to the intestinal elimination of ivermectin, as has been shown for other xenobiotics (Mayer et al., 1996; Ramon et al., 1996; Makhey et al., 1998; Van Asperen et al., 2000).

The mechanisms involved in the overall intestinal elimination of ivermectin in the rat are not clear. Partial inhibition (50%) of the jejunal secretion of parent ivermectin by the P-gp inhibitor verapamil suggests that P-gp plays a role in the secretion process (**chapter 3**). However, verapamil did not significantly affect the secretion of ivermectin by the duodenum and ileum in the same animals (**chapter 3**). This seems quite unexpected considering the continuous and increased expression of P-gp from proximal to distal small intestine (Brady et al., 2002). On the other hand, it has been suggested that there are not single but multiple intestinal P-gps, with different substrate affinities depending on their location along the gastrointestinal tract (Saitoh and Aungst, 1995; Makhey et al., 1998). Although the use of verapamil *in vivo* is not exclusive enough in demonstrating a P-gp-mediated process, these results point towards the involvement of other transport mechanisms. Examination of the mechanisms of secretion *in vitro* (**chapter 2**) confirmed the implication of P-gp in the intestinal secretion of ivermectin, which was significantly inhibited by the P-gp modulators verapamil, testosterone, and cyclosporin A. The secretory flux was also partially inhibited by the MRP-selective inhibitor MK571, suggesting that MRP2 could be involved as well in the secretion process. It is noteworthy that the potent P-gp modulator cyclosporin A, which totally abolished the net secretion of ivermectin across Caco-2 cell monolayers, has been shown to also inhibit MRP2 at high concentrations (König et al., 1999). The involvement of MRP2 remains to be confirmed *in vitro* using MRP2-transfected vesicles as well as *in vivo*. *In vivo*, MRP2 is highly expressed in the proximal small intestine, with a gradual decrease in expression from jejunum to distal ileum (Mottino et al., 2000), and could therefore significantly contribute to the overall intestinal elimination of the drug. Finally, a high passive diffusion of ivermectin and/or the involvement of other efflux transporters cannot be excluded.

In both studies, intestinal P-gps were identified as an important mechanism of intestinal secretion (**chapters 2** and **3**). This is in line with previous reports showing that ivermectin is a substrate of P-gp and that P-gp is implicated in the disposition of this drug in mice and rats (Schinkel et al., 1994, 1995; Didier and

Loor, 1995; Lankas et al., 1997; Pouliot et al., 1997; Alvinerie et al., 1999; Kwei et al., 1999; Smith et al., 2000). *In vivo*, P-gp is not only expressed in the intestines but in various other tissues, including the biliary canalicular membrane of hepatocytes, the apical membrane of renal proximal tubule cells, the lumenal membrane of capillary endothelial cells at the blood-brain and blood-testis barriers, and the apical membrane of placental trophoblasts (Thiebault et al., 1987; Cordon-Cardo et al., 1989). This transporter was shown to protect the brain and the foetus against the toxic effects of ivermectin (or abamectin) by limiting the entry of the drug in these organs (Schinkel et al., 1994; Didier and Loor, 1995; Lankas et al., 1997, 1998; Kwei et al., 1999). However, the involvement of P-gp in ivermectin elimination had never been clearly investigated. P-gp-deficient mice with no cannulated gallbladder showed a 30% lower secretion of [3H]-ivermectin in bile plus intestinal contents than wild-type mice after i.v. administration (Kwei et al., 1999), suggesting that P-gp was somehow involved in the elimination process.

A 3-fold increase in ivermectin plasma concentrations was observed in P-gp-deficient mice compared to wild-type mice after oral administration (Schinkel et al., 1994; Lankas et al., 1997; Kwei et al., 1999). Thus, it has been suggested that P-gp could limit the absorption of orally-administered ivermectin by pumping the drug back into the intestinal lumen (Schinkel et al., 1994; Lankas et al., 1997; Kwei et al., 1999). This hypothesis is consistent with our studies in Caco-2 cells, which showed a directional and active transport of ivermectin in the secretory direction (**chapter 2**). On the other hand, it is noteworthy that ivermectin is relatively well absorbed in mice following oral administration (over 60% of the dose; Kwei et al., 1999), suggesting that this increase in plasma concentrations could be due to a reduced elimination of ivermectin as well. As for other P-gp substrates, a good intestinal absorption of ivermectin could be explained by the saturation of P-gps at the local drug concentrations, high rates of passive diffusion, or the existence of gastrointestinal sites with a lower expression of P-gp where drugs are easily absorbed (Makhey et al., 1998; Van Asperen et al., 1998; Aungst, 1999; Doppenschmit et al., 1999). In contrast to mice, a low oral bioavailability of ivermectin has been reported in cattle, sheep, pig and horse (for review, see McKellar and Benchaoui, 1996). However, the existence of a relevant first pass-effect (metabolic and transport processes) in these species is doubtful. Indeed, the total clearance of ivermectin is low in most species (rat, cattle, and sheep; **chapters 3** and **5**; Prichard et al., 1985), corresponding to a body extraction ratio below 1%. Even considering the possibility of a synergistic action of intestinal cytochrome P450 3A and P-gp in reducing even more systemic availability (Wacher et al., 1995; Benet and Cummins, 2001), it is unlikely that such a low extraction ratio

(metabolic and transport processes) has an impact on the overall oral bioavailability of the drug. Other factors are probably more relevant in explaining the poor bioavailability of ivermectin by the oral route in these species. In sheep, the bioavailability of ivermectin given intraruminally was shown to be 75% lower that that after intra-abomasal administration (Prichard et al., 1985). Given the stability of ivermectin in ruminal fluids (Bogan and McKellar, 1988, Andrew and Halley, 1996), this has been attributed to an extensive binding of ivermectin to particulate phase of rumen digesta (>95% in cattle and sheep; Steel, 1993; Ali and Hennessy, 1996). We even suggest that once ivermectin is bound to digesta particulates in the rumen, it is not available anymore for further absorption in the gastrointestinal tract (**chapter 6**).

There has been so far no direct demonstration of a P-gp-mediated process for ivermectin secretion into bile. *In vitro* studies using primary cultures of hepatocytes suggested that hepatic P-gps could mediate the efflux of the structural analogue moxidectin (Dupuy et al., 2001). Our own studies (**chapter 3**) in anaesthetised rats showed no significant reduction of the biliary secretion of ivermectin following systemic co-administration of the P-gp modulator verapamil. However, it is possible that a reduced secretion of drug had been masked by a metabolic interaction between ivermectin and verapamil (inhibition of P450 3A isoenzymes), resulting in a compensatory increase in the secretion of parent ivermectin. A recent study using a MDR3-transfected cell line showed that the MDR3 gene product could also transport ivermectin, albeit to a lesser extent than P-gp (Smith et al., 2000). MDR3 is a close homologue of the MDR1 gene encoding P-gp (for review, see Borst and Elferink, 2002), which is expressed in the canalicular membrane of hepatocytes. The MDR3 P-gp has been shown to play a major role in hepatobiliary function by transporting phosphatidylcholine into bile (Smit et al., 1993). Its possible role in drug transport has just been raised (Smith et al., 2000).

Active secretion of ivermectin by renal P-gps was demonstrated *in vitro* using kidney proximal tubules of killifish (Fricker et al., 1999). These results are, however, quite inconsistent with the poor excretion of ivermectin in the urine observed *in vivo* (< 2% in all species; Campbell, 1985), suggesting that there is either a very low renal secretion process or a major reabsorption of the secreted ivermectin into blood. This study shows that the importance of secretory transport *in vivo* can be sometimes over-emphasised from *in vitro* data.

Altogether, **chapters 2** and **3** support the existence *in vivo* of an intestinal elimination of parent ivermectin through the gut wall. They provide further evidence of the role of the intestines in the elimination of drugs (Hunter and Hirst, 1997; Van Asperen et al., 1998; Matheny et al., 2001) and offer new directions into

the pharmacokinetics of endectocides to understand their clinical efficacy against gastrointestinal parasites. Indeed, many parasites do not feed on plasma, and an important secretion of active drug along the digestive tract could be of major clinical significance. The characterisation of a P-gp-mediated process offers also the possibility to modulate the elimination of biologically-active ivermectin in the gut and in faeces.

Existence of functional P-gps in the target animal species

Our previous results are of relevance in rat (**chapter 3**) and probably in humans (**chapter 2**) but the question arises whether or not they could be extrapolated to other animal species such as cattle, sheep, goat, pig and horse, which are the target species for ivermectin use in veterinary medicine.

In both previous studies (**chapters 2** and **3**), P-gp was identified as an important mechanism of ivermectin intestinal secretion. In this respect, an indication for the existence of functional P-gps in the target animal species would be of major interest. Five P-gp genes have been identified in pig, four of which show similarity with the Class I P-gp isoforms(s) in man (MDR1) and rodents (mdr1a, mdr1b) (Childs and Ling, 1996). Recently, the first ruminant P-gp gene has been cloned and sequenced from sheep (Longley et al., 1999). In cattle, the presence of three P-gp genes is expected based on genomic DNA analysis (Southern blotting) (Childs and Ling, 1996). It is noteworthy that Murray Grey cattle are particularly sensitive to ivermectin, suggesting that like Collie dogs and CF-1 mice, this breed of cattle may be naturally deficient with P-gp (Seaman et al., 1987).

However, little information is available on the P-gp function in these animal species, in contrast to humans and laboratory animals, in which the P-gp function has been extensively documented (Matheny et al., 2001; Borst and Elferink, 2002). Expression of functional P-gps has been shown in freshly isolated brain capillaries or primary brain capillary endothelial cells from pig and cattle (Rose et al., 1998; Miller et al., 2000; Nobmann et al., 2001). In contrast, no or very low P-gp activity was detected in the porcine kidney proximal tubule cell line (LLC-PK1) (Schinkel et al., 1995). It is difficult though to appreciate the extent of the P-gp function *in vivo* from immortalised cell lines or primary cells, as protein expression is often modified during cell culture, varying from total loss of expression to overexpression.

An alternative to the conventional *in vitro* models could be the use of freshly isolated peripheral blood lymphocytes. These have been shown to express functional P-gps in humans (Drach et al., 1992; Klimecki et al., 1994; Ludescher et

al., 1998) and are widely used for evaluation and monitoring of multidrug resistance in leukaemia patients (Beck et al., 1996). Peripheral blood lymphocytes were therefore isolated from cattle, sheep, goats, pigs, horses, rats, and men (taken as a reference) for evaluation of the P-gp function (**chapter 4**). Lymphocytes were stained with the fluorescent dye Rhodamine 123 (Rh-123), a prototypic P-gp substrate, after which they were resuspended in dye-free medium in the absence or presence of P-gp modulators (verapamil, cyclosporin A, ivermectin) to allow dye efflux. The ability of the cells to extrude Rh-123 was evaluated indirectly, by measuring intracellular dye retention using flow cytometric analysis.

Rh-123 has been shown to be a reliable marker of P-gp activity in human lymphocytes, on the basis of both dye efflux and its inhibition by P-gp modulators (Beck et al., 1996; Huet et al., 1998; Broxterman, 1999). According to these criteria, three groups of species were identified (**chapter 4**). Goat lymphocytes showed a significantly higher P-gp activity than lymphocytes of other species, under the same experimental conditions. A relatively high but lower P-gp activity was observed in lymphocytes from cattle, sheep, pigs and men. In contrast, only low levels of P-gp activity could be detected in lymphocytes from rats and horses. These interspecies differences suggest that the capacity and/or affinity of P-gp may differ from one species to another. This is consistent with the study of Schinkel et al. (1995), who showed that certain drugs (morphine) could be transported to a different extent by the human MDR1 P-gp and the murine Mdr1 P-gp in transfected cell lines. In addition to interspecies differences, large interindividual variations in P-gp activity were observed (**chapter 4**). Our data in pigs even suggested that the P-gp function could be influenced by various physiological factors such as the age and the sex, as has been reported in humans using the same lymphocyte-based model (Gupta, 1995; Steiner et al., 1998). Finally, P-gp expression was confirmed in small intestine tissue (jejunum) of goats, cattle, sheep, pigs and horses (**chapter 4**).

Altogether, our findings suggest that P-gps equivalent to those identified in humans and rodents exist in cattle, sheep, goats, pigs and even horses, providing thereby the mechanistic support for an intestinal secretion of ivermectin. The large interspecies differences in P-gp activity suggest, however, that the actual contribution of this secretion mechanism may differ from one species to another. The high P-gp activity observed in goat lymphocytes is particularly striking, since goats are known to eliminate ivermectin much more rapidly than any other farm animal species (Scott and McKellar, 1990; McKellar and Benchaoui, 1996). Differences in metabolic capacities have to be considered as well.

It is noteworthy that ivermectin inhibited significantly the efflux of Rh-123 in

lymphocytes of all species to a similar extent as verapamil and cyclosporin A. This infers that P-gps of goats, cattle, sheep, pigs and horses could interact with ivermectin, and it can be expected that they transport ivermectin as well. This would be consistent with a previous *in vivo* study in ivermectin-treated sheep, showing a transient increase in ivermectin plasma concentrations following i.v. administration of verapamil (data not shown). Furthermore, in moxidectin-treated cattle, the clearance of the structural analogue moxidectin was significantly decreased (33%) by co-administration of loperamide (Lifschitz et al., 2002), another P-gp substrate (Schinkel et al., 1996).

High levels of P-gp activity were observed in lymphocytes from goats, cattle, sheep and generally pigs (**chapter 4**). Consequently, lymphocytes offer the possibility to study transport of veterinary drugs in the target animal species as well as drug-drug interactions. Although the relevance of the lymphocyte-based model needs to be further documented, it is certainly more predictive than other conventional *in vitro* models consisting of immortalised or primary cell lines. Finally, it has been shown in humans that P-gp expression could vary among individuals, depending on natural polymorphisms, exposure to P-gp substrates or inducers, and physiological and pathological (inflammatory) factors (Hartmann et al., 2001; Matheny et al., 2001; Borst and Elferink, 2002). Thus, the lymphocyte-based model could also be used to document the effect of these factors on the P-gp function. Since lymphocytes can be easily isolated from a single blood sampling, this model is suitable for a population approach.

Influence of animal behaviour on the plasma and faecal disposition of topical ivermectin

The elimination of parent ivermectin from the bloodstream may not always explain the rate and extent of ivermectin excretion into faeces following parenteral administration. It has been shown that topical (pour-on) administration of ivermectin to cattle results in lower ivermectin plasma concentrations but in higher ivermectin faecal concentrations than subcutaneous administration (Herd et al., 1996). We confirmed this discrepancy by measuring the faecal clearance of ivermectin after topical and i.v. administrations to cattle. Ivermectin faecal clearance was indeed 2 to 16-times higher after topical application than following i.v. administration (**chapter 5**), which clearly indicated that the plasma concentration was not the only driving force for the elimination of parent ivermectin in faeces. Our hypothesis was that cattle could ingest a part of the topical drug by licking, and that a fraction of the ingested ivermectin could transit

directly into faeces. This hypothesis proved to be relevant, since we found that 58-87% of the dose applied over the back of cattle was actually ingested by licking (**chapters 5** and **6**). In contrast, only 10% of the pour-on dose (500 µg/kg) was absorbed through the skin (**chapters 5** and **6**). Approximately 72% of the ingested ivermectin transited directly into faeces (mean oral bioavailability of 28%), which accounted for approximately 80% of the total faecal output (**chapter 6**).

Licking is an important part of the natural grooming behaviour of cattle. It serves an important physiological function in skin and hair hygiene, and plays a major role in the formation and maintenance of social bounds (Simonsen, 1979; Sato et al., 1991, 1993; Krohn, 1994). However, a possible influence of cattle licking behaviour on the disposition of topical drugs had never been documented. Topical drugs are generally applied over the backline of animals, in a region where they cannot theoretically scratch, snatch or lick. This recommendation was made without considering the particular ability of cattle to turn and stretch to lick, as has been observed during our experiments. Furthermore, topical drugs can spread over the skin from the site of application to a larger surface and thus be exposed to licking. This has been shown for many antiparasitic agents in sheep, cattle, or pig (cypermethrin, flumethrin, parathion; Jenkinson et al., 1986; Stendel et al., 1992; Brimer et al., 1994) and is also probably the case of ivermectin (**chapter 6**).

When licking was prevented, the absorption of ivermectin into the systemic circulation was very slow, resulting in a much longer plasma elimination half life than following i.v. administration (so-called flip-flop effect) (**chapters 5** and **6**). This shows that topical ivermectin is in fact very slowly absorbed through the skin of cattle. In normal licking cattle, half to two-third of the topical ivermectin entering the systemic circulation resulted from oral absorption (**chapter 6**). There was therefore a major contribution of the oral route, which explains that the absorption of ivermectin in plasma was rapid (as for an oral dosing; Steel, 1993) and that the plasma elimination half life was similar to that obtained after i.v. administration (no flip-flop effect) (**chapters 5** and **6**). Taken together, our results suggest that the plasma exposure of topically-treated animals depends mainly on their ability to lick themselves (self-licking) or each other (allo-licking). When animals were successively given two pour-on administrations of ivermectin (500 µg/kg) at a washout interval, the plasma exposure of a same cattle could vary from 60% to 240% from one pour-on application to the other (**chapter 6**). Intra-individual variability of drug exposure was not significantly lower than inter-individual variability, which is a very uncommon feature in pharmacology (**chapter 6**). Furthermore, it is noteworthy that the extent of licking is influenced by various physiological, nutritional, managerial and pathological factors including

parasitism of animals (Sato et al., 1991; 1993). Thus, it can be expected that under field conditions, the exposure of animals is even more variable and unpredictable. The possibility of underexposure of animals has to be considered in the case of high allo-licking and/or low self-licking activity. Underexposure of animals is of special concern, since subtherapeutic concentrations may promote the development of drug resistance (Smith et al., 1999; Geerts and Gryseels 2000).

Finally, allo-licking was observed during our experiments (**chapters 5** and **6**), which raises the possibility of a transfer of ivermectin from treated to non-treated cattle. Our simulations indicated that a continuous licking accounting for only 2 % of the pour-on dose in total could be sufficient to obtain non-negligible systemic exposure of non-treated cattle (maximum plasma concentration of 1 ng/mL), along with a significant and persistent elimination of ivermectin in milk (**chapter 6**). These results elicit concerns over the possible occurrence of violative residues of ivermectin in milk and edible tissues of untreated cattle that would have been in contact with those topically-treated.

In conclusion, it appears that the relevance of cattle licking behaviour has been largely underestimated. Possible consequences in terms of public health suggest that animal licking behaviour should be more carefully addressed in the future in food-producing animals. One solution to reduce the relevance of licking on the disposition of topical ivermectin in cattle would be to increase the rate of drug absorption through the skin. Simulations indicated that the rate of percutaneous absorption should be increased by a factor 8 to have as much ivermectin absorbed trough the skin as ingested, and by at least a factor 50 to reduce the extent of drug ingestion below 10% of the dose (**chapter 6**). It must be stressed, however, that a reliable and safe alternative to the pour-on formulation exists, which is the subcutaneous formulation.

Summarising conclusions and perspectives

It was the aim of the presented investigations to contribute to the understanding of the diverse factors involved in the pharmacokinetics of ivermectin, a very potent antiparasitic drug widely used in both human and veterinary medicine.

Convincing evidence for an intestinal secretion of ivermectin from blood into the intestinal lumen was provided by experiments *in vitro* (Caco-2 cells) and *in situ* (rat intestinal loops). In the rat, intestinal secretion was even more important than biliary secretion in the overall elimination of the parent drug. These findings may initiate a re-appraisal of the disposition of ivermectin, particularly its distribution in the digestive tract, and thus allow a better understanding of its clinical efficacy

against gastrointestinal parasites.

The above mentioned investigations also pointed towards the prominent role of P-gp in the intestinal secretion process, although other transport mechanisms (MRP2) may be involved.

The relevance of these findings for veterinary target species was demonstrated by examining the P-gp function in cattle, sheep, goats, pigs and horses using a lymphocyte-based *ex vivo* model. P-gp activity was found in peripheral blood lymphocytes of all animal species studied, albeit at different levels. In addition, P-gp expression was shown in the intestines for all species. These results strongly suggest the presence of P-gp proteins in cattle, sheep, goats, horses, and pigs, equivalent to those identified in humans and rodents. They provide therefore a mechanistic support for the intestinal secretion of ivermectin and other P-gp substrates. Since wide interspecies variations in P-gp activity were observed, it can be expected that the contribution of this secretion mechanism may differ from one species to the other.

Finally, we identified that external, behavioural factors such as animal licking does obviously contribute to the disposition of ivermectin in cattle following topical application. Under our experimental conditions, 58-87% of the dose (500 µg/kg) applied topically over the back of cattle was actually ingested by licking, while only 10% of the dose was absorbed through the skin. Thus, the systemic exposure of topically-treated animals depends most likely on their ability to lick themselves or each other. Furthermore, social contacts with other cattle (allo-licking) may result in the cross-contamination of non-treated cattle present in the same herd.

In conclusion, our results provide evidence for

- the intestinal secretion of ivermectin from blood into the intestinal lumen (*in vitro* and *in vivo* data).
- the involvement of P-gp in the intestinal secretion process, as shown in the Caco-2 cell model and further supported by our findings in the rat.
- the existence of functional P-gps in the target animal species (cattle, sheep, goat, pig and horse) using a lymphocyte-based model, together with the expression of P-gp in their intestinal tissues.
- the contribution of cattle licking behaviour to the plasma and faecal disposition of topical ivermectin, resulting in a higher faecal excretion of the parent drug.

These results may stimulate further investigations towards the contribution of

intestinal secretion to the overall elimination of endectocides in target animal species. The examination of the role of P-gp and P-gp-like transporters (MRP2) in the relative disposition of different endectocides may be useful to understand differences in their pharmacokinetics and support the development of new pharmaceutical formulations tailored towards individual compounds and target animal species.

References

Ali DN and Hennessy DR (1996) The effect of level of feed intake on the pharmacokinetic disposition and efficacy of ivermectin in sheep. *J Vet Pharmacol Ther* **19:**89-94.

Alvinerie M, Dupuy J, Eeckhoutte C and Sutra JF (1999) Enhanced absorption of pour-on ivermectin formulation in rats by co-administration of the multidrug-resistant-reversing agent verapamil. *Parasitol Res* **85:**920-922.

Andrew NW and Halley BA (1996) Stability of ivermectin in rumen fluids. *J Vet Pharmacol Ther* **19:**295-299.

Aungst BJ (1999) P-glycoprotein, secretory transport, and other barriers to the oral delivery of anti-HIV drugs. *Adv Drug Deliv Rev* **39:**105-116.

Beck J, Gekeler V, Ringger M, Handgretinger R and Niethammer D (1996) Rhodamine 123-efflux from hematopoietic subpopulations and leukaemic blast populations marked by PerCP-conjugated monoclonal antibodies. *Cancer Letters* **99:**197-207.

Benet LZ and Cummins CL (2001) The drug efflux-metabolism alliance: biochemical aspects. *Adv Drug Deliv Rev* **50:** S3-S11.

Bogan JA and McKellar QA (1988) The pharmacodynamics of ivermectin in sheep and cattle. *J Vet Pharmacol Ther* **11:**260-268.

Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71:**537-92.

Boulenc X (1997) Intestinal cell models: their use in evaluating the metabolism and absorption of xenobiotics. *S.T.P Pharma Sciences* **7:**259-269.

Brady JM, Cherrington NJ, Hartley DP, Buist SC, Li N and Klaassen CD (2002) Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metab Dispos* **30:**838-844.

Brimer L, Gyrd-Hansen N and Rasmussen F (1994) Disposition of parathion after dermal application in pigs. *J Vet Pharmacol Ther* **17:**304-308.

Broxterman HJ (1999) Measurement of P-glycoprotein function in Methods in Molecular Medicine, Vol. 28: Cytotoxic drug resistance mechanisms (Brown R and Boger-Brown U ed) pp 53-61, Humana Press Inc., Totowa, NJ.

Campbell WC (1985) Ivermectin: an update. *Parasitol Today* **1:**10-16.

Childs S and Ling V (1996) Duplication and evolution of the P-glycoprotein genes in pig. *Biochim Biophys Acta* **1307:**205-212.

Chiu L, Green ML, Baylis FP, Eline D, Rosegay A, Meriwether H and Jacob TA (1990) Absorption, tissue distribution and excretion of tritium-labeled ivermectin in cattle, sheep and rat. *J Agric Food Chem* **38:**2072-2078.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at the blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86:**695-698.

Didier AD and Loor F (1995) Decreased biotolerability for ivermectin and cyclosporin A in mice exposed to potent P-glycoprotein inhibitors. *Int J Cancer* **63:**263-

267.

Doppenschmitt S, Spahn-Langguth H, Gunnar Regardh C and Langguuth P (1999) Role of P-glycoprotein-mediated secretion in absorptive drug permeability: An approach using passive membrane permeability and affinity to P-glycoprotein. *J Pharm Sci* **88:**1067-1072.

Drach D, Zhao S, Drach J, Mahadevia R, Gattringer C, Huber H and Andreeff M (1992) Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* **80:**2729-2734

Dupuy J, Larrieu G, Sutra JF, Eeckhoutte C and Alvinerie M (2001) Influence of verapamil on the efflux and metabolism of 14C moxidectin in cultured rat hepatocytes. *J Vet Pharmacol Ther* **24:**171-177.

Fricker G, Gutmann H, Droulle A, Drewe J and Miller DS (1999) Epithelial transport of anthelmintic ivermectin in a novel model of isolated proximal kidney tubules. *Pharm Res* **16:**1570-1575.

Geerts S and Gryseels B (2000) Drug resistance in human helminths: Current situation and lessons from Livestock. *Clin Microbiol Rev* **13:**207-222.

Gotoh Y, Suzuki H, Kinoshita S, Hirohashi T, Kato Y and Sugiyama Y (2000) Involvement of an organic anion transporter (canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2) in gastrointestinal secretion of glutathione conjugates in rats. *J Pharmacol Exp Ther* **292:**433-439.

Gupta S (1995) P-glycoprotein expression and regulation. Age-related changes and potential effects on drug therapy. *Drugs Aging* **7:**19-29.

Hartmann G, Kim H and Piquette-Miller M (2001) Regulation of the hepatic multidrug resistance gene expression by endotoxin and inflammatory cytokines in mice. *Int Immunopharmacol* **1:**189-199.

Hennessy DR, Page SW and Gottschall D (2000) The behaviour of doramectin in the gastrointestinal tract, its secretion in bile and pharmacokinetic disposition in the peripheral circulation after oral and intravenous administration to sheep. *J Vet Pharmacol Ther* **23:**203-213.

Herd RP, Sams RA and Ashcraft SM (1996) Persistence of ivermectin in plasma and faeces following treatment of cows with ivermectin sustained-release, pour-on or injectable formulations. *Int J Parasitol* **26:**1087-1093.

Hirohashi T, Suzuki H, Chu XY, Tamai I, Tsuji A and Sugiyama Y (2000) Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). *J Pharmacol Exp Ther* **292:**265-270.

Hosoya KI, Kim KJ and Lee VH (1996) Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharm Res* **13:**885-890.

Huet S, Marie JP, Gualde N and Robert J (1998) Reference method for detection of P-gp mediated multidrug resistance in human hematological malignancies: a method validated by the laboratories of the French Drug Resistance Network. *Cytometry* **34:**248-256.

Hunter J and Hirst BH (1997) Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv Drug Deliv Rev* **25:**129-157.

Jenkinson DM, Hutchison G, Jackson D and McQueen L (1986) Route of passage of cypermethrin across the surface of sheep skin. *Res Vet Sci* **41:**237-241.

Klimecki WT, Futscher BW, Grogan TM and Dalton WS (1994) P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood* **83:**2451-2458.

König J, Nies AT, Cui Y, Leier I and Keppler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* **1461:**377-394.

Krohn CC (1994) Behaviour of dairy cows kept in extensive (loose housing/pasture) or intensive (tie stall) environments. III. Grooming, exploration and abnormal behaviour. *Appl Anim Behav Sci* **42:**73-86.

Kwei GY, Alvaro RF, Chen Q, Jenkins HJ, Hop CE, Keohane CA, Ly VT, Strauss JR, Wang RW, Wang Z, Pippert TR and Umbenhauer DR (1999) Disposition of ivermectin and cyclosporin A in CF-1 mice deficient in mdr1a P-glycoprotein. *Drug Metab Dispos* **27:**581-587.

Lankas GR, Cartwright ME and Umbenhauer D (1997) P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicol Appl Pharmacol* **143:**357-365.

Lankas GR, Wise LD, Cartwright ME, Pippert T and Umbenhauer DR (1998) Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol* **12:**457-463.

Lifschitz A, Virkel G, Sallovitz J, Imperiale F, Pis A and Lanusse C (2002) Loperamide-induced enhancement of moxidectin availability in cattle. *J Vet Pharmacol Ther* **25:**111-120.

Longley M, Phua SH, van Stijn TC and Crawford AM (1999) Isolation and mapping of the first ruminant multidrug resistance genes. *Anim Genet* **30:**207-210.

Ludescher C, Pall G, Irschick EU and Gastl G (1998) Differential activity of P-glycoprotein in normal blood lymphocyte subsets. *Br J Haematol* **101:**722-727.

Makhey VD, Guo A, Norris DA, Hu P, Yan J and Sinko PJ (1998) Characterisation of the regional intestinal kinetics of drug efflux in rat and in human intestine and Caco-2 cells. *Pharm Res* **15:**1160-1167.

Matheny CJ, Lamb MW, Brouwer KL and Pollack GM (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy* **21:**778-796.

Mayer U, Wagenaar E, Beijnen JH, Smit JW, Meijer DKF, Van Asperen J, Borst P and Schinkel A.H (1996) Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr1a P-glycoprotein. *Br J Pharmacol* **119:**1038-1044.

McKellar QA and Benchaoui HA (1996) Avermectins and milbemycins. *J Vet Pharmacol Ther* **19:**331-351.

Miller DS, Nobmann SN, Gutmann H, Toeroek M, Drewe J and Fricker G (2000) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol Pharmacol* **58:**1357-1367.

Mottino AD, Hoffman T, Jennes L and Vore M (2000) Expression and localization of multidrug resistant protein mrp2 in rat small intestine. *J Pharmacol Exp Ther* **293:**717-723.

Nobmann S, Bauer B and Fricker G (2001) Ivermectin excretion by isolated functionally intact brain endothelial capillaries. *Br J Pharmacol* **132:**722-728.

Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E *(*1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **53:**17-25.

Prichard RK, Steel JW, Lacey E and Hennessy DR (1985) Pharmacokinetics of ivermectin in sheep following intravenous, intra-abomasal or intraruminal administration. *J Vet Pharmacol Ther* **8:**88-94.

Ramon J, Dautrey S, Farinoti R, Carbon C and Rubinstein E (1996) Excretion of ciprofloxacin into the large bowel of the rabbit. *Antimicrob Agents Chemother* **40:**11-13.

Rose JM, Peckham SL, Scism JL and Audus KL (1998) Evaluation of the role of P-glycoprotein in ivermectin uptake by primary cultures of bovine brain microvessel endothelial cells. *Neurochem Res* **23:**203-209.

Saitoh H and Aungst BJ (1995) Possible involvement of multiple P-glycoprotein-mediated efflux systems in the transport of verapamil and other organic cations across rat intestine. *Pharm Res* **12:**1304-1310.

Sato S, Sako S and Maeda A (1991) Social licking patterns in cattle (Bos taurus): influence of environmental and social factors. *Appl Anim Behav Sci* **32:**3-12.

Sato S, Tarumizu K and Hatae K (1993) The influence of social factors on allogrooming in cows. *Appl Anim Behav Sci* **38:**235-244.

Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, Berns AJM and Borst P (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:**491-502.

Schinkel AH, Wagenaar E, van Deemter L, Mol CA and Borst P (1995) Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96:**1698-1705.

Schinkel AH, Wagenaar E, Mol CA and van Deemter L (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest* **97:**2517-2524.

Scott EW, Kinabo LD and McKellar QA (1990) Pharmacokinetics of ivermectin after oral or percutaneous administration to adult milking goats. *J Vet Pharmacol Ther* **13:**432-435.

Seaman JT, Eagleson JS, Carrigan MJ and Webb RF (1987) Avermectin B1 toxicity in a herd of Murray Grey cattle. *Aust Vet J* **64:**284-285.

Simonsen HB (1979) Grooming behaviour of domestic cattle. *Nord Vet Med* **31:**1-5.

Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA, et al. (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75:**451-462.

Smith G, Grenfell BT, Isham V and Cornell S (1999) Anthelmintic resistance revisited: underdosing, chemoprophylactic strategies, and mating probabilities. *Int J Parasitol* **29:**77-91.

Smith AJ, van Helvoort A, van Meer G, Szabo K, Welker E, Szakacs G, Varadi A, Sarkadi B and Borst P (2000) MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. *J Biol Chem* **275:**23530-23539.

Steel JW (1993) Pharmacokinetics and metabolism of avermectins in livestock. *Vet Parasitol* **48:**45-57.

Steiner H, Polliack A, Kimchi-Sarfaty C, Libster D, Fibach E and Rund D (1998) Differences in rhodamine-123 efflux in B-type chronic lymphocytic leukemia suggest possible gender and stage variations in drug-resistance gene activity. *Ann Hematol* **76:**189-194.

Stendel Hamel HD, Sieveking HU and Bruhne D (1992) Analytical determination of the distribution of flumethrin on the body surface of cattle following topical pour-on application. *Vet Parasitol* **42:**137-143.

Suzuki H and Sugiyama (2000) Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur J Pharm Sci* **12:**3-12.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* **84:**7735-7738.

Van Asperen J, Van Tellingen O and Beijnen JH (1998) The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacol Res* **37:**429-435.

Van Asperen J, Van Tellingen O and Beijnen JH (2000) The role of mdr1a P-glycoprotein in the biliary and intestinal secretion of doxorubicin and vinblastine in mice. *Drug Metab Dispos* **28:**264-267.

Wacher VJ, Wu CY and Benet LZ (1995) Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol Carcinog* **13:**129-134.

Walgren RA, Lin JT, Kinne RK and Walle T (2000) Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. *J Pharmacol Exp Ther* **294:**837-843.

Nederlandse Samenvatting

Korte nederlandse samenvatting van de onderzoeken.

Het doel van het hier beschreven onderzoek was een bijdrage te leveren aan het begrip van de diverse factoren betrokken in de farmacokinetiek van ivermectine, een potent anti-parasitair medicijn dat veel gebruikt wordt in zowel de humane geneeskunde als de diergeneeskunde. Het onderzoek omvat twee verschillende onderdelen: *in vivo* en *in vitro* onderzoek.

Middels *in vitro* (humane Caco-2 cellen) en *in situ* (darmsegmenten van ratten) experimenten kon worden aangetoond dat er intestinale uitscheiding van ivermectine vanuit het bloed naar het darm-lumen plaats vindt. In de rat was zelfs de uitscheiding van systemisch toegediend ivermectine via de darm belangrijker dan via de gal. Deze bevindingen geven aanleiding tot een heroverweging van de kinetiek van ivermectine, met name de verdeling in de digestie tractus, en daarmee tot een beter begrip van de klinische effectiviteit van ivermectine tegen maag-darm parasieten.

Bovendien duiden de resultaten van de onderzoeken op een prominente rol van het transport eiwit Pg-p in de intestinale secretie, hoewel ook andere transporters, zoals MRP2, betrokken kunnen zijn.

Het belang van de bevindingen met de humane Caco-2 cellen voor de doeldieren is aangetoond door de functie van P-gp in rundvee, schapen, geiten, varkens en paarden te onderzoeken met behulp van een *ex vivo* model van lymfocyten uit het perifere bloed. Activiteit van P-gp is gevonden in de lymfocyten van alle genoemde species, echter met verschil in grootte. Daarnaast is de aanwezigheid (expressie) van P-gp aangetoond in de darmen van deze diersoorten. Onze resultaten suggereren de aanwezigheid van P-gp eiwitten in rundvee, schapen, geiten, varkens en paarden gelijk aan die in mensen en knaagdieren. Ze voorzien daarmee in een mechanisme dat de intestinale secretie van ivermectine en andere P-gp substraten kan verklaren. Aangezien er duidelijke variaties in P-gp aktiviteit tussen de diersoorten zijn waargenomen, kan het verwacht worden dat de bijdrage van dit mechanisme verschilt van de ene diersoort tot de andere.

In het tweede deel van het proefschrift hebben we laten zien dat externe gedragsfactoren zoals likken eveneens bijdragen aan de lotgevallen van ivermectine in rundvee na topicale toediening op de huid. Onder onze experimentele omstandigheden werd 58-87% van de dosis (500 μg/kg LG) toegediend op de rug van koeien eigenlijk oraal opgenomen door likken, terwijl maar 10% van de dosis door de huid werd opgenomen. Dit houdt in dat de systemische beschikbaarheid van topicaal behandelde dieren zeer waarschijnlijk afhankelijk is van de mogelijkheid om zichzelf en/of elkaar te likken. De sociale

contacten met andere koeien (allo-licking) resulteren in een besmetting van niet-behandelde koeien in hetzelfde koppel.

Ter conclusie, onze resultaten voeren bewijs aan voor:

- Intestinale secretie van ivermectine vanuit het systemische bloed naar het darm lumen (*in vitro* en *in vivo* data).
- De betrokkenheid van P-gp in het intestinale secretie proces, zoals aangetoond in het Caco-2 cel model en ondersteund door de bevindingen in het ratten model (darmsegmenten in situ).
- Het bestaan van functioneel P-gp in de doel-diersoorten (rundvee, schapen, geiten, varkens en paarden), door het gebruik van het lymfocyten model samen met de expressie van P-gp in het darm weefsel.
- De bijdrage van het likgedrag van koeien aan de bloedplasma en mest spiegels na topicale toediening van ivermectine op de huid.

Deze resultaten geven aanleiding tot verder onderzoek naar de bijdrage van intestinale secretie in de totale eliminatie van endecticiden (Avermectinen) in de doeldieren. Onderzoek naar de rol van P-gp en andere transport eiwitten, zoals MRP2, met betrekking tot de lotgevallen van verschillende endectosiden in het lichaam, kan het begrip van de verschillen in hun farmacokinetiek verduidelijken en de ontwikkeling van nieuwe farmaceutische formuleringen, die op maat gemaakt zijn om een optimaal effect in een bepaalde diersoort te bewerkstelligen, ondersteunen.

Curriculum vitae

Céline Laffont was born on May 4th, 1975, in Toulouse, France. After finishing high school, she commenced her studies at the National Veterinary School of Toulouse in 1994 and graduated in 1998 from the same Veterinary School as veterinarian. She started as a Ph.D. student in November 1998 at the laboratory of Physiology and Therapeutics at the National Veterinary School of Toulouse under the supervision of Prof. Dr. P.-L. Toutain and Dr. A. Bousquet-Mélou. In this period, a number of relevant qualifications were achieved, including a post-graduate degree in pharmacokinetics and a certificate in cellular pharmacology, pharmacokinetics and pharmacogenetics. Professional training included also courses conducted by the ECVPT (European College for Veterinary Pharmacology and Toxicology) such as workshops on *"Animal pain and inflammation and their control"* and *"Pharmacokinetics"*. The experimental Ph.D. work was continued in 2001 at the Department of Veterinary Pharmacology, Pharmacy and Toxicology (VFFT) at the Faculty of Veterinary Medicine, Utrecht University (The Netherlands) under the supervision of Prof. Dr. J. Fink-Gremmels. The entire Ph.D. project focused on various internal (transport mechanisms) and external (animal behaviour) factors affecting the plasma and faecal disposition of ivermectin in target species. The results of the work are described in the present thesis, which will be defended in public on December 4, 2002.

List of Publications

Laffont CM, Alvinerie M, Bousquet-Melou A and Toutain PL (2001) Licking behaviour and environmental contamination arising from pour-on ivermectin for cattle. *Int J Parasitol* **31:**1687-1692.

Laffont CM, Toutain PL, Alvinerie M and Bousquet-Melou A (2002) Intestinal secretion is a major route for parent ivermectin elimination in the rat. *Drug Metab Dispos* **30:**626-630.

Laffont CM, Maas R, Bousquet-Mélou A and Fink-Gremmels J. Active secretion of the anthelmintic ivermectin across human intestinal epithelial Caco-2 cell monolayers. *Submitted.*

Laffont CM, de Vrieze G, Maas R, Bousquet-Mélou A and Fink-Gremmels J. Comparative evaluation of P-gp activity in cattle, sheep, goats, pigs, horses and rats using a lymphocyte-based ex vivo model. *Submitted.*

Laffont CM, Bousquet-Mélou A, Bralet D, Alvinerie M, Fink-Gremmels J and Toutain PL. Pharmacokinetic modeling of the disposition of topical ivermectin in cattle as determined by animal behaviour. *Submitted.*

Bousquet-Mélou A, **Laffont CM**, Laroute V and Toutain PL (2002) Modelling the loss of metabolic capacities of cultured hepatocytes - Application to measurement of Michaelis-Menten kinetic parameters in *in vitro* systems. *Xenobiotica* **32:**895-906.

Van der Linden M, **Laffont CM** and Sloet van Oldruitenborgh-Oosterbaan MM. Survival analysis to determine prognosis in equine colic: both surgical and medical cases. Accepted in *J Vet Int Med.*

Van der Harst MR, Bull S, **Laffont CM**, Klein WR and Fink-Gremmels J. Transient nephrotoxicity following the administration of therapeutic doses of gentamycin to healthy ponies. *Submitted.*

Abstracts

Laffont CM, Bousquet-Melou A, Alvinerie M and Toutain PL. Bioavailability of

ivermectin pour-on formulation in cattle. 8th International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT), Jerusalem, august 2000.

Laffont CM, Bousquet-Melou A, Alvinerie M and Toutain PL. Comportement social des bovins et pharmacocinétique des endectocides. Journées INRA (Institut National de la Recherche Agronomique), Biarritz, France, October 2001.

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