Bioanalysis and Clinical Pharmacology of Tamoxifen in Breast Cancer

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Bioanalysis and Clinical Pharmacology of Tamoxifen in Breast Cancer

Bioanalyse en klinische farmacologie van tamoxifen bij borstkanker
(met een samenvatting in het Nederlands)

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Nynke Geeske Lokke Jager

geboren op 7 oktober 1985
te Dordrecht

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Not everything that counts can be counted,
and not everything that can be counted counts.

- Albert Einstein

Contents

Preface

Preface

Each year, around 1.4 million cases of breast cancer are diagnosed worldwide [1]. Around 70% of the breast cancers are estrogen receptor (ER) positive; the growth of these tumors depends on the binding of estrogen to the ER on tumor cells. The cornerstone of systemic treatment for patients with ER positive breast cancer is endocrine therapy, with tamoxifen being the most widely used endocrine agent. Tamoxifen binds to the ER, thereby blocking the proliferative actions of estrogen on mammary tissue. It has been used for over 40 years, for both pre- and postmenopausal patients and in all stages of management. Until today, tamoxifen remains an important medicine for many ER positive breast cancer patients across the world. Tamoxifen is usually prescribed for long-term use of five years, and recent research even suggests to extent the use of tamoxifen to ten years [2,3]. However, despite the significantly improved recurrence and mortality rates when using endocrine therapy, around 25-30% of the breast cancer patients relapse within ten years and will eventually die from the disease [4]. In order to optimize treatment, many researchers have sought to find factors associated with treatment outcome, however this quest has not been finished yet. Studies on the pharmacology of tamoxifen revealed extensive biotransformation and showed that some of its metabolites, especially 4-hydroxytamoxifen and *N*-desmethyl-4-hydroxytamoxifen (endoxifen), have a much higher affinity for the ER than tamoxifen itself. Recently, endoxifen is identified as the metabolite of primary interest, considering it is present at a much higher steady-state serum concentration in patients using tamoxifen than 4-hydroxytamoxifen. Although there is a wide inter-patient variability in tamoxifen pharmacokinetics, in general all patients use a standard dose of 20 mg tamoxifen per day. These findings have provided a new option for treatment optimization; individual dosing based on active metabolites levels. The results of a recent large clinical trial encouraged the use of active metabolite levels; a significant correlation between endoxifen serum levels and recurrence free survival was demonstrated and a minimum therapeutic threshold endoxifen serum level was suggested [5].

The aim of this research is to enable and implement dose individualization in clinical practice, by the use of active metabolite concentrations measured in patient samples. In order to ease the logistics of the sampling and as a patient-friendly alternative to venous sampling, a dried blood spot sampling (DBS) method, where patient samples are obtained by means of a finger prick, has been developed.

Outline of this thesis

In Chapter 1, two bioanalytical assays for the quantification of tamoxifen and its metabolites in patient samples are described. In Chapter 1.1 a selective LC-MS/MS assay for the quantification of tamoxifen and five of its phase I metabolites in serum samples is presented, followed by a report highlighting the importance of highly selective analytical methods for the accurate quantification of analytes in Chapter 1.2. Chapter 1.3 and 1.4 focus on DBS sampling. In Chapter 1.3, the bioanalytical challenges for DBS assays and the validation procedures for these assays are reviewed. Chapter 1.4 describes the development and validation of a bioanalytical assay for the determination of tamoxifen and its metabolite endoxifen in DBS, with the focus on the previously discussed challenges.

Chapter 2 concerns the clinical application of the bioanalytical assays described in the previous chapter, starting with an evaluation of the metabolite levels that are found in a large cohort of patients from the outpatient clinic of the Netherlands Cancer Institute in Chapter 2.1. In Chapter 2.2, a bridging study for DBS and serum concentrations of tamoxifen and endoxifen is described. The aim of this study is to establish a simple conversion formula to calculate serum concentrations from analyzed DBS concentrations, to allow the use of DBS sampling in clinical practice. In Chapter 2.3, the feasibility of DBS self-sampling at home is assessed.

The third part of this thesis focuses on clinical pharmacological aspects of tamoxifen and its main metabolites, where in Chapter 3.1 the relationship between the main side effect of tamoxifen, hot flashes, and serum concentrations of tamoxifen and metabolites is presented. Finally, in Chapter 3.2, the position of active metabolite concentration-guided dosing in tamoxifen treatment is discussed.

Altogether, this thesis presents several aspects of tailor-made tamoxifen treatment based on individual metabolite levels; from the development of bioanalytical assays to the implementation of these assays in clinical practice and clinical pharmacologic aspects of tamoxifen pharmacokinetics.

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1

Bioanalysis of tamoxifen and its metabolites

Development and validation of a quantitative assay for the determination of tamoxifen and its five main phase I metabolites in human serum using liquid chromatography coupled with tandem mass spectrometry

1.1

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[‡] These authors contributed equally

Abstract

Background. A sensitive bioanalytical assay for the quantitative determination of tamoxifen and five of its phase I metabolites (*N*-desmethyltamoxifen, *N*-desmethyl-4-hydroxytamoxifen, *N*-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen) in serum is described. The method has been fully validated at ranges covering steady-state serum concentrations in patients receiving therapeutic dosages of tamoxifen.

Methods. The bioanalytical assay is based on reversed phase liquid chromatography coupled with tandem mass spectrometry in the positive ion mode using multiple reaction monitoring for drug (-metabolite) quantification. The sample pretreatment consists of protein precipitation with acetonitrile using only 50 µL of serum. In the past, numerous assays have been developed by other groups for the quantification of tamoxifen and its phase I metabolites. However, the number of metabolites included in these studies is very limited and only very few of these assays have been fully validated. A liquid chromatography tandem mass spectrometry assay for the quantification of tamoxifen and four phase I metabolites in human serum that was previously developed by our group is now explicitly improved and described herein.

Results. Time of analysis has been reduced by 50% and sensitivity was increased by reduction of the lower limit of quantification from 1.0 ng/mL to 0.4 ng/mL for 4-hydroxytamoxifen and to 0.2 ng/mL for 4′-hydroxytamoxifen. Additionally, two phase I metabolites that have never been quantified in human serum hitherto, namely 4′-hydroxytamoxifen and *N*-desmethyl-4′-hydroxytamoxifen, were included in this assay. Validation results demonstrate an accurate and precise quantification of tamoxifen, *N*-desmethyltamoxifen, *N*-desmethyl-4-hydroxytamoxifen, *N*-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen in human serum. The applicability of the assay was demonstrated and it is now successfully used to support clinical studies in which patient-specific dose optimization is performed based on serum concentrations of tamoxifen metabolites.

Introduction

The selective estrogen receptor modulator tamoxifen is widely applied in estrogen receptor positive breast cancer treatment [1,2]. During phase I metabolism, metabolites of tamoxifen are formed that are more active than tamoxifen itself. Tamoxifen is therefore considered a prodrug.

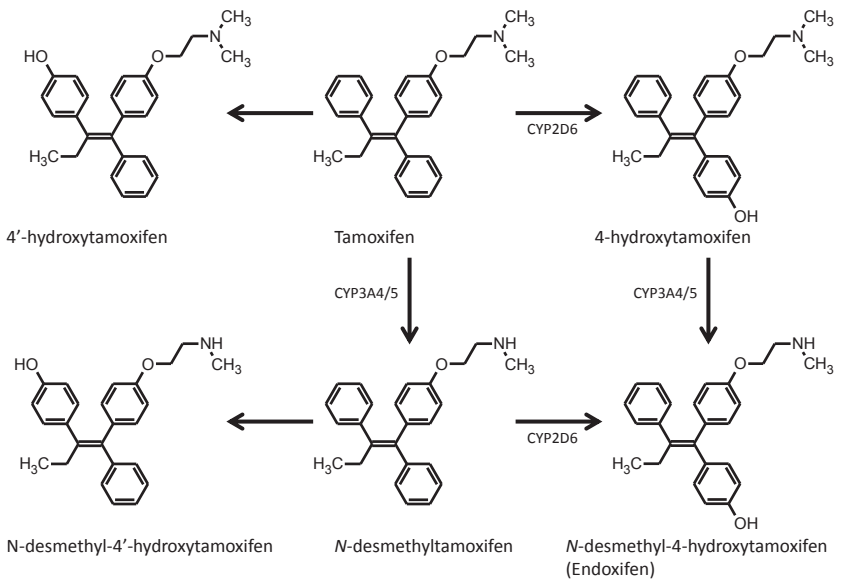
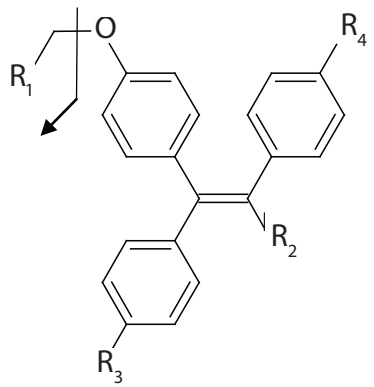


Figure 1 Part of the metabolism of tamoxifen. The enzymes mentioned in the figure are responsible for the primary metabolism

Among the bio-activated metabolites, 4-hydroxytamoxifen has been shown to be a potent anti-estrogen (30 to 100-fold more potent than tamoxifen itself) [3,4]. *N*-desmethyl-4-hydroxytamoxifen (endoxifen) is equipotent to 4-hydroxytamoxifen in estrogen receptor (ER)-alpha and ER-beta binding, as well as in inhibition of 17β-estradiol induced proliferation in human breast cancer cells [5–7]. Endoxifen, however, is on average present at a much higher steady-state concentration in serum of patients than 4-hydroxytamoxifen [5,7,8]. It is known from the literature that tamoxifen and its metabolites are subject to large inter-patient variation in steady-state serum concentrations [7,9,10]. Numerous studies reported a relationship between *in vivo* metabolite concentrations and patient survival, single nucleotide polymorphisms (SNPs) and cytochrome P450 activity [11–16]. It is of pivotal importance to be able to

quantify tamoxifen metabolites *in vivo* as the serum concentrations of active metabolites vary widely among individuals, due to a large variation in metabolism. This variation might be caused by genotype and co-medication that influences the CYP450 system (e.g. SSRIs) [7,9,10]. Numerous assays have been developed for the quantification of tamoxifen and its phase I metabolites [17]. However, the number of metabolites included in these studies is very limited and only very few of these assays have been fully validated according to FDA guidelines [18]. We have previously designed and fully validated a liquid chromatography tandem mass spectrometry assay for the quantification of tamoxifen and four phase I metabolites in human serum [8]. This assay now requires improvement to optimally support clinical studies for which large numbers of samples need to be analyzed. Therefore, the speed of analysis was reduced by 50%, the sensitivity was increased by a reduction of the lower limit of quantification from 1.0 to 0.2 ng/mL, additional tamoxifen metabolites were included and the validated range was reduced to improve accuracy. The range was chosen based solely on steady-state serum concentrations of women receiving the commonly used daily dose of 20 or 40 mg tamoxifen. The dynamic range in the previously developed assay was much wider, as that method was developed to support both clinical studies in patients as well as toxicokinetic studies in mice receiving high doses of tamoxifen. Besides reducing the validated ranges, the lower limit of quantification (LLOQ) was lowered significantly; 1.0 to 0.4 ng/mL for 4-hydroxytamoxifen and to 0.2 ng/mL for 4'-hydroxytamoxifen to ensure accurate *in vivo* quantification. This required a mass spectrometer capable of detecting analytes with a concentration as low as 0.2 ng/mL. Additionally, the phase I metabolites selected for quantification were changed. This is the result of profound new insight into the phase I metabolism of tamoxifen obtained from supporting clinical studies using the previously developed assay. In all serum samples obtained from these clinical studies, in which the patients received 20 mg tamoxifen once daily, two additional tamoxifen metabolites were consistently observed. These were 4'-hydroxytamoxifen and *N*-desmethyl-4'-hydroxytamoxifen. Their structure and steady-state serum concentration is very similar to 4-hydroxytamoxifen and *N*-desmethyl-4-hydroxytamoxifen (endoxifen), respectively; two metabolites that are acknowledged to be the active metabolites of tamoxifen. To the best of our knowledge, these two metabolites have never been quantified in human specimens hitherto. We included them in this assay for quantification in human serum. Other tamoxifen phase I metabolites were not included in this assay as their steady-state serum concentration was low; around the detection limit of the MS detector (or no reference standard was available). A full validation of the developed assay was performed according to the FDA guidelines [18]. Additionally, the applicability of the assay in clinical samples was demonstrated.

Table 1 Trivial names, chemical structures, molecular masses and selected fragmentation position of tamoxifen, five of its metabolites and its stable isotope labelled internal standards



Trivial name	R_1	R_2	R_3	R_4	Mol. formula	Mol. mass
Tamoxifen	$N(CH_3)_2$	CH_2-CH_3	H	H	$C_{26}H_{29}NO$	371.5
Tamoxifen-*d5*	$N(CH_3)_2$	C^2H_2-C^2H_3	H	H	$C_{26}H_{24}{}^2H_5NO$	376.5
N-desmethyltamoxifen	NH-CH_3	CH_2-CH_3	H	H	$C_{25}H_{27}NO$	357.5
N-desmethyltamoxifen-*d5*	NH-CH_3	C^2H_2-C^2H_3	H	H	$C_{25}H_{22}{}^2H_5NO$	362.5
N-desmethyl-4-hydroxytamoxifen	NH-CH_3	CH_2-CH_3	OH	H	$C_{25}H_{27}NO_2$	373.5
N-desmethyl-4-hydroxytamoxifen-*d5*	NH-CH_3	C^2H_2-C^2H_3	OH	H	$C_{25}H_{22}{}^2H_5NO_2$	378.5
N-desmethyl-4'-hydroxytamoxifen	NH-CH_3	CH_2-CH_3	H	OH	$C_{25}H_{27}NO_2$	373.5
4-Hydroxytamoxifen	$N(CH_3)_2$	CH_2-CH_3	OH	H	$C_{26}H_{29}NO_2$	387.5
4-Hydroxytamoxifen-*d5*	$N(CH_3)_2$	C^2H_2-C^2H_3	OH	H	$C_{26}H_{24}{}^2H_5NO_2$	392.5
4'-Hydroxytamoxifen	$N(CH_3)_2$	CH_2-CH_3	H	OH	$C_{26}H_{29}NO_2$	387.5

Experimental

Reagents and chemicals

Tamoxifen, tamoxifen-*d5*, 4-hydroxytamoxifen, 4-hydroxytamoxifen-*d5*, 4'-hydroxy-tamoxifen, *N*-desmethyltamoxifen-HCl, *N*-desmethyltamoxifen-*d5*, *N*-desmethyl-4-hydroxytamoxifen (1:1, *E*/*Z* mixture), *N*-desmethyl-4-hydroxytamoxifen-*d5* (1:1, *E*/*Z* mixture) and *N*-desmethyl-4'-hydroxytamoxifen were purchased from Toronto Research Chemicals (North York, ON, Canada). The chemical structures of the analytes and internal standards are shown in Table 1. Acetonitrile and methanol were obtained from Biosolve Ltd. (Amsterdam, the Netherlands). Ammonium formate was purchased from Acros Organics (Geel, Belgium). Formic acid and LiChrosolv water for HPLC were purchased from Merck (Darmstadt, Germany). Small volumes of control drug-free human serum, obtained from the Slotervaart Hospital (Amsterdam, the Netherlands), were pooled and used for validation purposes.

Instrumentation

HPLC

An Agilent HPLC system was used consisting of an 1100 series binary pump, column oven, on-line degasser and autosampler (Agilent Technologies, Palo Alto, CA, USA). Mobile phase A was prepared by adjusting a 4.0 mM ammonium formate solution to pH 3.5 with a 98% formic acid solution. Mobile phase B consisted of 100% acetonitrile. Mobile phases A and B were pumped through a Kinetex C18 100 Å column (150 x 2.1 mm I.D., 2.6 µm; Phenomenex) at a flow rate of 0.4 mL/min using a gradient as shown in Table 2. The analytical column was protected by a KrudKatcher inline filter (Phenomenex, Torrance, CA, USA). The separation was performed at 60˚C. Volumes of 15 µL were injected using the autosampler thermostatted at 7˚C. The column was equilibrated for 3 minutes before the next injection, leading to a total run time of 10 minutes. The autosampler needle was rinsed with acetonitrile before and after each injection. During the first and last 1.0 minute the eluate was directed to waste using a divert valve to prevent the introduction of endogenous compounds into the mass spectrometer.

Table 2 HPLC gradient parameters used for the separation of tamoxifen and its metabolites using a Phenomenex Kinetex C18 column (150 x 2.1 mm I.D., 2.6µm) thermostatted at 60˚C

Time (min)	Flow rate (mL/min)	Mobile phase A* (%)	Mobile phase B** (%)
0.00	0.40	70.0	30.0
6.00	0.40	47.5	52.5
6.01	0.40	20.0	80.0
7.00	0.40	20.0	80.0
7.01	0.40	70.0	30.0
10.00	0.40	70.0	30.0

*Mobile phase A: 4.0 mM ammonium formate buffer pH 3.5
**Mobile phase B: acetonitrile

MS

An API 4000 triple quadrupole mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization (APCI) source (AB Sciex, Foster City, CA, USA) operating in the positive ion mode was used as a detector. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired and processed using Analyst® software (AB Sciex). The quadrupoles were operating at unit resolution (0.7 Da). APCI-MS/MS operating parameters and mass transitions are listed in Table 3.

Table 3 APCI-MS/MS operating parameters

Parameter		Setting
Run duration		10 min
Ion spray voltage		5500 V
Collision gas/ curtain gas/ turbo gas		8/ 10/ 45 arbitrary units
APCI temperature		400°C
Tamoxifen	Mass transition	372 -> 72 *m/z*
	Collision energy	45 V
	Declustering potential	91 V
	Collision exit potential	14 V
N-desmethyltamoxifen	Mass transition	358 -> 58 *m/z*
	Collision energy	45 V
	Declustering potential	71 V
	Collision exit potential	10 V
N-desmethyl-4-hydroxytamoxifen (*E*/*Z*)	Mass transition	374 -> 58 *m/z*
	Collision energy	47 V
	Declustering potential	81 V
	Collision exit potential	10 V
N-desmethyl-4′-hydroxytamoxifen	Mass transition	374 -> 58 *m/z*
	Collision energy	47 V
	Declustering potential	81 V
	Collision exit potential	10 V
4-Hydroxytamoxifen	Mass transition	388 -> 72 *m/z*
	Collision energy	45 V
	Declustering potential	91 V
	Collision exit potential	14 V
4′-Hydroxytamoxifen	Mass transition	388 -> 72 *m/z*
	Collision energy	45 V
	Declustering potential	91 V
	Collision exit potential	14 V
Tamoxifen-*d*5	Mass transition	377 -> 72 *m/z*
	Collision energy	45 V
	Declustering potential	70 V
	Collision exit potential	12 V
N-desmethyltamoxifen-*d*5	Mass transition	363 -> 58 *m/z*
	Collision energy	45 V
	Declustering potential	70 V
	Collision exit potential	12 V
N-desmethyl-4-hydroxytamoxifen -*d*5 (*E*/*Z*)	Mass transition	379 -> 58 *m/z*
	Collision energy	45 V
	Declustering potential	70 V
	Collision exit potential	12 V
4-Hydroxytamoxifen-*d*5	Mass transition	393 -> 72 *m/z*
	Collision energy	45 V
	Declustering potential	70 V
	Collision exit potential	12 V

Preparation of calibration standards, quality controls and internal standard solutions
Two separate stock solutions of all analytes (1 mg/mL) and internal standards (1 mg/mL) were prepared. Approximately 1 mg was accurately weighed (Sartorius Micro MC5 balance, Sartorius Instrumenten BV, Nieuwegein, the Netherlands) and dissolved in 1 mL methanol. For the analytes, one stock solution was used for the preparation of calibration standards and the other stock solution was used for the preparation of quality control (QC) standards. The preparation of the two stock solutions for each compound was checked and in all cases deviations were less than ±5%. The *E*/*Z*-ratio of the reference standard of *N*-desmethyl-4-hydroxytamoxifen was determined by liquid chromatography with ultraviolet absorption detection. The ratio was determined to be 1.0. The stock solutions were further diluted with methanol to obtain separate working solutions, each containing all of the six analytes at a 200-fold concentration of the corresponding serum samples. Calibration samples were prepared freshly for every run by spiking a volume of 10 µL working solution to 2.0 mL of control human serum. QC samples were prepared in batches and stored at -70˚C until analysis.
A mixture of internal standard stock solutions was prepared and diluted with acetonitrile to obtain a working solution that was used for sample pretreatment. This internal standard working solution contained: tamoxifen-*d5*, *N*-desmethyltamoxifen-*d5*, *N*-desmethyl-4-hydroxytamoxifen-*d5* (1:1 *E*/*Z* mixture) and 4-hydroxytamoxifen-*d5* at concentrations of 75, 75, 16.5 and 4 ng/mL, respectively.

Sample preparation
A volume of 150 µL internal standard working solution in acetonitrile was added to a 50 µL serum aliquot. The mixture was vortex mixed for 10 seconds followed by centrifugation for 10 min at 11,300 *g*. An aliquot of 120 µL of the clear supernatant was transferred to another 1.5 mL amber colored eppendorf tube and evaporated at 30˚C under a gentle stream of nitrogen to form a dried extract. This extract was subsequently reconstituted in 60 µL acetonitrile-4 mM ammonium formate buffer pH 3.5 (3:7, *v/v*) by vortex mixing for 15 s to form the final extract. The sample was transferred to an amber colored autosampler vial and stored at 2-8˚C until analysis.

Validation procedures

A full validation of the assay was performed according to the FDA guidelines [18].

Regression models

Eight non-zero calibration standards were prepared freshly in duplicate for each run and analyzed in three independent runs. For *N*-desmethyl-4-hydroxytamoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen linear least-squares regression was applied (area ratio of the internal standard versus the nominal concentration). The calibration standard data for tamoxifen and *N*-desmethyltamoxifen were fitted quadratic. For all analytes, the reciprocal of the squared concentration ($1/x^2$) was used as a weighting factor. Deviations from the mean calculated concentrations over three runs should be within 85-115% of nominal concentrations. At the LLOQ, a deviation of 20% was permitted and the response of the analyte should be at least five times higher than the response of a blank sample. For four of the six analyzed compounds, deuterated internal standards were commercially available. The stable isotope labelled internal standards 4-hydroxytamoxifen-*d5* and (*Z*)-*N*-desmethyl-4-hydroxytamoxifen-*d5* were used to correct for signal fluctuations of 4'-hydroxytamoxifen and *N*-desmethyl-4'-hydroxytamoxifen, respectively.

Accuracy and precision

Intra- and inter-assay accuracies and precisions of the method were determined by assaying five replicates of each of the QC samples at the LLOQ, low, mid and high concentration level in three separate runs. The concentration of each QC sample was calculated using the calibration standards that were analyzed in duplicate in the same run. The differences between the nominal and the measured concentration were used to calculate the accuracies. The accuracy should be within 85-115% except at the LLOQ, where an accuracy of 80-120% is allowed. The precision should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20%. The ability to dilute samples with an analyte concentration originally above the upper limit of quantification (ULOQ) was demonstrated by analyzing QC samples containing 10 times the concentration of the high QC sample. These samples were prepared in five-fold and analyzed after a 10-fold dilution in control human serum.

Matrix factor and carry-over

To determine the matrix factor, the following samples were prepared in triplicate: i) a QC sample at QC mid concentration level in serum processed to final extract according to the procedure described in the sample preparation section, ii) a sample in acetonitrile-4 mM ammonium formate buffer pH 3.5 (3:7, *v/v*) at QC mid concentration level processed to final extract according to the procedure described in the sample preparation section, iii) a blank serum sample processed to dried extract according to

the procedure described in the sample preparation section, followed by reconstitution with a processed sample obtained at ii). Carry-over was determined by injecting a processed control human serum sample after an ULOQ sample. Areas of peaks in the blank processed sample should be less than 20% of the peak area of the LLOQ sample.

Specificity and selectivity

Six individual batches of control human serum were used to assess the specificity and selectivity of the method. To determine whether endogenous constituents interfere with the assay, a double blank and a sample spiked at the LLOQ of each batch were processed. The samples were subsequently analyzed according to the procedures described above. Areas of peaks co-eluting with analytes should be less than 20% of the peak area of the LLOQ sample in each of the six batches. Areas of peaks in the double blanks co-eluting with the internal standards should be less than 5% of the peak area of the mean IS response. For the LLOQ, sample accuracies should be within ±20% of the nominal concentration in four out of six samples. To ensure that other tamoxifen phase I metabolites would not interfere, the chromatographic behaviour of tamoxifen metabolites with equal precursor and product masses (e.g. 3- and α-hydroxytamoxifen, *N*-desmethyl-3- and *N*-desmethyl-α-hydroxytamoxifen) was studied. These metabolites were all baseline separated from the metabolites quantified in this assay.

Stability

The stability of the analytes was investigated in the stock solutions at ambient temperature. Furthermore, stability was tested in human serum for 6 h at room temperature, during one month of storage at -70˚C and after three freeze (-70˚C)-thaw cycles with a minimum interval of 24 h. In-process (dried extract) and final extract stability was investigated during 4 days and 7 days of storage at 2-8˚C, respectively. The QC stability samples were quantified based on a freshly prepared calibration curve. Re-injection reproducibility was determined in the final extract after 24 h at nominally 2-8˚C. All stability experiments were performed in triplicate. Analytes are considered stable in stock and working solutions when 95-105% of the original concentration is found. Analytes are considered stable in the biological matrix or extract when 85-115% of the initial concentration is recovered. Stability of the deuterated internal standards was assumed to be equal to the corresponding undeuterated analytes. Isotopic purity of deuterated analytes (i.e. internal standard) was investigated during each analytical run by spiking control human serum with internal standard working solution. The peak area in the non-deuterated analyte window should be less than 20% of the peak area of the analytes at their LLOQ level.

Results and discussion

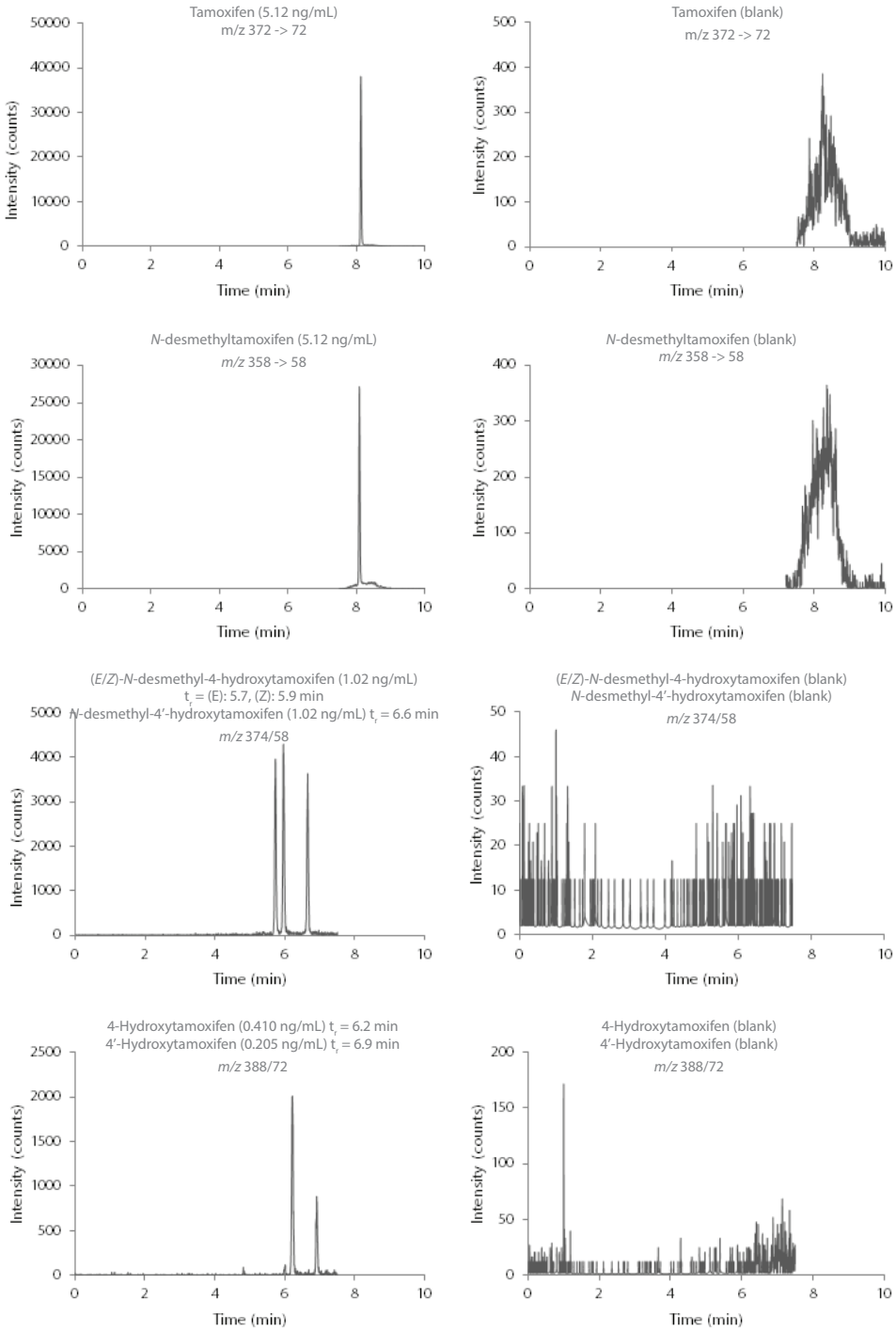
HPLC-MS/MS

The previously developed assay for the analysis of tamoxifen and its metabolites required improvements with regard to the overall time of analysis and sensitivity [8]. A Kinetex C18 column was capable of providing fast, high resolution separations. Thereby, the total run time could be reduced drastically from 19 to 10 minutes. This Kinetex C18 column was superior over other tested HPLC columns (C18, Synergi Hydro (Phenomenex, Torrance, CA, USA)) in terms of resolution and speed. The high column efficiencies are due to the halo-pellicular particles in the Kinetex column. As various tamoxifen metabolites have the same precursor and product mass, baseline separation of these metabolites was required. Reference standards of *N*-desmethyl-4-hydroxytamoxifen were provided as racemic mixtures of entgegen (*E*) and zusammen (*Z*) isomers. As tamoxifen is administered to patients as a pure *Z*-enantiomer [19], only the (*Z*)-form of metabolites is formed *in vivo*. Therefore, (*E*)- and (*Z*)-*N*-desmethyl-4-hydroxytamoxifen had to be baseline separated for an accurate and precise quantification of the separate isomers. The Kinetex HPLC column consists of stationary phase particles with a size of 2.6 µm. The relatively high flow rate of 400 µL/min at which the column is operated resulted in a backpressure of ±350 bar. By increasing the column temperature to 60˚C, the backpressure was reduced to ±300 bar, while the resolution increased as a result of increased mass transfer rate [20]. An eluent consisting of acetonitrile provided superior peak symmetry compared to methanol. The separation was further optimized by buffering the eluent at pH 3.5. Protonated molecules ($[M+ H]^+$) were used as precursor ions to generate product-ion spectra. The most intense product-ions were determined and used as MRM transitions to ensure high sensitivity and selectivity. In Table 1 and 3, the optimized mass transitions and proposed fragmentation pathways are presented for tamoxifen and its metabolites. The peak width of eluting metabolites was very small (±6 s) and required fast scanning of the mass spectrometer to ensure enough (>20) data points over the peak. To facilitate sufficient data collection per peak, the chromatogram was divided in two sections of 7.5 and 2.5 minutes, respectively. The measured mass transitions per section were defined based on the analytes and internal standards eluting therein.

Regression models

All calibration curves were constructed using a weighting factor of $1/x^2$ and fitted either linearly or quadratically. In the previously developed assay, electrospray ionization (ESI) was used to promote the liquid-gas conversion [8]. To improve the sensitivity

Figure 2 MRM chromatograms of QC samples at the lower limit of quantification of the analytes and their respective blanks



of the current method, a different mass spectrometer was used (API 4000 (AB Sciex, Foster City, CA, USA) instead of a Quantum Ultra (Thermo Fisher Scientific, Waltham, MA, USA)). When using an API 4000 equipped with an ESI source, quadratically fitted calibration curves for the majority of analytes were obtained. This is most likely due to the differences in source configuration between both types of mass spectrometers, which might result in e.g. saturation of the electrospray above a specific analyte concentration. Quadratically fitted calibration curves result in a high risk of deviations in the upper concentration region. To improve the linearity of the calibration curves, the ESI source was replaced by an APCI source. This resulted in linear calibration curves for the majority of the compounds, with the exception of tamoxifen and *N*-desmethyltamoxifen. However, the calibration curves of these compounds show only a minor deflection in the upper concentration region. The CVs were in all cases less than 15%. Accuracies were in all cases within 85-115%. Mean correlation coefficients (r^2) were ≥0.996.

Table 4 Assay performance data (n = 5 per run, n = 15 for total)

Analyte (concentration range)	Nominal concentration (ng/mL)	Inter-assay accuracy	Inter-assay precision (%CV)
Tamoxifen	5.12	0.117	8.92
(5.00-1000 ng/mL)	12.8	-2.34	7.13
	400	-3.90	4.75
	800	1.15	3.67
N-desmethyltamoxifen	5.12	-2.40	12.0
(5.00-1000 ng/mL)	12.8	-0.420	6.08
	400	-1.20	3.85
	800	-0.110	4.17
N-desmethyl-4-hydroxytamoxifen	1.02	-4.46	11.1
(1.00-200 ng/mL)	2.56	-1.61	7.94
	80.0	-0.120	4.97
	160	-1.42	5.01
N-desmethyl-4-hydroxytamoxifen	1.02	0.360	11.1
(1.00-200 ng/mL)	2.56	4.01	8.61
	80.0	0.670	4.62
	160	3.46	5.56
4-Hydroxytamoxifen	0.410	-1.01	6.50
(0.40-80 ng/mL)	1.02	-0.950	7.13
	32.0	-1.71	4.70
	64.0	-1.02	5.78
4'-Hydroxytamoxifen	0.205	-2.70	10.5
(0.20-40 ng/mL)	0.512	-0.810	9.62
	16.0	-5.00	4.40
	32.0	0.0200	6.33

CV: coefficient of variation.

Accuracy and precision

Assay performance data (intra-assay and inter-assay accuracy and precision) of all analyzed compounds are summarized in Table 4. Intra-assay and inter-assay accuracies were within 85-115% and precisions were less than 15% for all compounds.

Matrix factor and carry-over

The total recovery of all analytes was determined at one concentration level and was in all cases near 100%. The total recovery is defined as the analyte recovery after sample pretreatment plus the effect of the matrix on the measured MS signal. As the total recovery is near 100%, the effect of the matrix is negligible. Carry-over was determined by injecting a processed control human serum sample after an ULOQ sample. Areas of peaks in the blank processed sample were less than 20% of the peak area of the LLOQ sample.

Specificity and selectivity

MRM chromatograms of six batches of control human serum contained no co-eluting peaks larger than 20% of the area at the LLOQ level of all analytes, and no co-eluting peaks larger than 5% of the area of all internal standards. The influence of different control human serum batches on the accuracy and precision at LLOQ level was investigated. The accuracies of analytes at LLOQ level were in all six batches of control human serum within ±20% boundaries of the nominal concentration.

Stability

The results of the investigated stability parameters are presented in Table 5. *N*-desmethyltamoxifen is sensitive to light. To prevent possible degradation under the influence of light and temperature, all stock solutions and samples were stored at nominally -70˚C and samples were processed in amber colored vials. Stock solutions in methanol appeared to be stable for at least two hours at room temperature, sufficient for the preparation of working solutions and subsequent spiking of serum. Serum samples are stable for at least 6 hours at room temperature and for 1 month at -70˚C. The calibration standards and QC samples were aliquoted, stored at -70˚C and thawed directly before processing to keep the number of freeze/thaw cycles to a minimum. However, patient samples may require re-analysis, which results in extra freeze/thaw cycles. Stability of serum samples is guaranteed during at least three freeze (-70˚C)/thaw cycles. Dried extracts obtained during processing of the serum samples appeared to be stable for at least 4 days when stored at 2-8˚C in amber colored vials. Final extracts were stable for a minimum of 7 days when stored at 2-8˚C in amber colored vials.

When required, a full analytical run consisting of calibration standards, QCs and patient samples can be re-injected after 24 h. After one month of storage of an internal standard working solution at -70˚C, peak areas of analytes in a processed control human serum sample spiked with the internal standard working solution were less than 20% of the peak areas of the analytes in an LLOQ sample (data not shown).

Application of the method

The applicability of the assay was demonstrated by the analysis of steady-state serum concentrations of patients receiving the regular prescribed dose in breast cancer treatment of 20 mg tamoxifen once daily. The measured concentrations are shown in Table 6. The serum concentrations of tamoxifen, *N*-desmethyltamoxifen, *N*-desmethyl-4-hydroxytamoxifen, *N*-desmethyl-4′-hydroxytamoxifen, 4-hydroxytamoxifen and 4′-hydroxytamoxifen were all within the validated range of the developed assay.

Table 6 Steady-state concentrations of tamoxifen and five of its metabolites in serum collected from ten breast cancer patients receiving 20 mg tamoxifen once daily

Patient	Tamoxifen	*N*-desmethyl tamoxifen	*N*-desmethyl -4-hydroxy tamoxifen	*N*-desmethyl -4′-hydroxy tamoxifen	4-Hydroxy tamoxifen	4′-Hydroxy tamoxifen
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)
1	123	175	14.5	4.58	1.94	2.23
2	183	336	8.74	9.51	1.61	3.07
3	147	400	11.5	23.0	2.42	6.74
4	82.7	133	7.15	4.06	1.36	1.74
5	144	307	6.22	19.3	1.23	5.21
6	136	197	12.8	5.64	2.32	2.78
7	155	256	15.0	8.15	2.30	3.06
8	85.4	124	11.7	4,93	1.79	2.07
9	104	192	10.2	7.33	1.34	2.99
10	76.2	130	5.81	4.75	1.04	1.66
Mean	**124**	**225**	**10.4**	**9.13**	**1.74**	**3.16**
RSD (%)	**28.8**	**42.6**	**31.9**	**72.7**	**28.7**	**51.2**

Table 5 Results of the stability experiments (n=3)

Matrix	Conditions	Compound	Nominal conc. (ng/mL)	Dev (%)	CV (%)
Methanol	15-25˚C	Tamoxifen	400	-3.55	3.53
	2 hours	*N*-desmethyltamoxifen	400	-0.123	1.68
		N-desmethyl-4-hydroxytamoxifen	80.0	-2.21	2.27
		N-desmethyl-4′-hydroxytamoxifen	80.0	0.661	0.661
		4-Hydroxytamoxifen	32.0	-0.135	2.62
		4′-Hydroxytamoxifen	16.0	11.0	2.11
Serum	15-25˚C	Tamoxifen	12.8	-7.03	4.45
	6 hours		800	-1.25	2.34
		N-desmethyltamoxifen	12.8	-4.17	2.62
			800	-1.38	1.43
		N-desmethyl-4-hydroxytamoxifen	2.56	-9.11	2.37
			160	-4.58	0.378
		N-desmethyl-4′-hydroxytamoxifen	2.56	-12.2	1.85
			160	-0.625	1.66
		4-Hydroxytamoxifen	1.02	-13.4	4.62
			64.0	-4.69	1.43
		4′-Hydroxytamoxifen	0.512	-13.0	0.934
			32.0	-5.94	0.879
Serum	-70˚C	Tamoxifen	12.8	-7.81	3.06
	1 month		800	7.17	8.05
		N-desmethyltamoxifen	12.8	-8.07	5.46
			800	9.79	9.42
		N-desmethyl-4-hydroxytamoxifen	2.56	-14.5	2.85
			160	4.79	6.99
		N-desmethyl-4′-hydroxytamoxifen	2.56	-8.85	4.70
			160	13.3	9.40
		4-Hydroxytamoxifen	1.02	-11.5	2.84
			64.0	5.63	9.78
		4′-Hydroxytamoxifen	0.512	-13.8	2.63
			32.0	5.00	8.58
Serum	3 Freeze (-70˚C) - thaw cycles	Tamoxifen	12.8	-5.73	3.74
			800	-1.38	2.25
		N-desmethyltamoxifen	12.8	-1.82	5.30
			800	-0.0833	1.39
		N-desmethyl-4-hydroxytamoxifen	2.56	-5.99	3.33
			160	-4.17	4.72
		N-desmethyl-4′-hydroxytamoxifen	2.56	-0.781	2.19
			160	0.833	2.79
		4-Hydroxytamoxifen	1.02	-8.92	0.812
			64.0	-5.10	2.24
		4′-Hydroxytamoxifen	0.512	-4.75	0.427
			32.0	-3.96	0.939

Table 5 Results of the stability experiments (n=3) (continued)

Matrix	Conditions	Compound	Nominal conc. (ng/mL)	Dev (%)	CV (%)
Dried extract	2-8˚C 4 days	Tamoxifen	12.8	-8.85	1.31
			800	-2.38	1.66
		N-desmethyltamoxifen	12.8	-2.34	1.39
			800	-3.00	0.670
		N-desmethyl-4-hydroxytamoxifen	2.56	-13.3	3.40
			160	-10.4	0.403
		N-desmethyl-4′-hydroxytamoxifen	2.56	-0.521	3.81
			160	-1.25	0.00
		4-Hydroxytamoxifen	1.02	-5.63	4.09
			64.0	-7.08	0.541
		4′-Hydroxytamoxifen	0.512	-6.12	4.56
			32.0	-5.52	1.56
Final extract	2-8˚C 7 days	Tamoxifen	12.8	-4.95	3.32
			800	3.50	0.483
		N-desmethyltamoxifen	12.8	-5.47	3.79
			800	4.92	0.793
		N-desmethyl-4-hydroxytamoxifen	2.56	-13.9	2.50
			160	-8.54	1.97
		N-desmethyl-4′-hydroxytamoxifen	2.56	-1.17	2.09
			160	2.92	1.53
		4-Hydroxytamoxifen	1.02	-6.45	5.23
			64.0	-5.94	0.879
		4′-Hydroxytamoxifen	0.512	-4.43	1.94
			32.0	-4.48	1.36
Final extract	2-8˚C 4 days	Tamoxifen	12.8	-11.3	0.881
			400	-1.63	1.10
			800	-6.46	0.772
		N-desmethyltamoxifen	12.8	-4.95	6.64
			400	-1.00	1.16
			800	-0.417	1.34
		N-desmethyl-4-hydroxytamoxifen	2.56	-12.0	3.27
			80.0	0.792	3.29
			160	-3.96	0.376
		N-desmethyl-4′-hydroxytamoxifen	2.56	-7.94	1.91
			80.0	-0.458	2.64
			160	-1.67	1.32
		4-Hydroxytamoxifen	1.02	-7.16	2.00
			32.0	0.417	0.359
			64.0	-1.46	2.64
		4′-Hydroxytamoxifen	0.512	-12.8	3.18
			16.0	-2.29	2.06
			32.0	-5.94	3.45

conc.: concentration, Dev: deviation from the nomal concentration, CV: coefficient of variation.

Conclusions

An HPLC-MS/MS assay has been developed and validated for the simultaneous analysis of tamoxifen, *N*-desmethyltamoxifen, *N*-desmethyl-4-hydroxytamoxifen (endoxifen), *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen in human serum. The assay shows clear improvements with regard to the previously developed assay [8] in terms of time of analysis, sensitivity, resolution and the number of tamoxifen metabolites included in the assay. The LLOQ of tamoxifen and its metabolites is well below the lowest concentrations measured in patients at steady-state who receive the commonly prescribed dose of 20 mg tamoxifen once daily. The method is now successfully used to support clinical studies in which patient-specific dose-optimization is performed based on serum concentrations of tamoxifen and its metabolites.

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Importance of highly selective LC-MS/MS analysis for the accurate quantification of tamoxifen and its metabolites: focus on endoxifen and 4-hydroxytamoxifen

1.2

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Abstract

Background. The antiestrogenic effect of tamoxifen is mainly attributable to the active metabolites endoxifen and 4-hydroxytamoxifen. This effect is assumed to be concentration dependent and therefore quantitative analysis of tamoxifen and metabolites for clinical studies and therapeutic drug monitoring is increasing.

Methods. We investigated the large discrepancies in reported mean endoxifen and 4-hydroxytamoxifen concentrations. Two published LC-MS/MS methods are used to analyse a set of 75 serum samples from patients treated with tamoxifen.

Results. The method from Teunissen et al. (J Chrom B, 2011) was shown to separate endoxifen and 4-hydroxytamoxifen from other tamoxifen metabolites with similar masses and fragmentation patterns. The second method, published by Gjerde et al. (J Chrom A, 2005) however lacks selectivity, resulting in a factor 2 to 3 overestimation of the endoxifen and 4-hydroxytamoxifen levels, respectively. We emphasize the use of highly selective LC-MS/MS methods for the quantification of tamoxifen and its metabolites in biological samples.

Introduction

Tamoxifen is widely administered in the treatment and chemoprevention of estrogen receptor positive breast cancer, which accounts for about 60-70% of all breast cancers [1–3]. Tamoxifen is considered to be a prodrug that is converted into many metabolites. The most therapeutically active metabolites are *N*-desmethyl-4-hydroxytamoxifen (endoxifen) and 4-hydroxytamoxifen, being 30 to 100-fold more potent than tamoxifen itself. The antiestrogenic activities of endoxifen and 4-hydroxytamoxifen are similar, although endoxifen, unlike 4-hydroxytamoxifen, is also a potent inhibitor of aromatase and is present at a higher steady-state concentration in patients than 4-hydroxytamoxifen. [4–7]

The steady-state levels of the active tamoxifen metabolites are proposed predictors of the clinical outcomes of tamoxifen treatment; it is suggested that there is a minimum concentration threshold above which endoxifen is effective against the recurrence of breast cancer. [8] It is well known from the literature that there is a considerable inter-patient variability in steady-state levels of tamoxifen and its metabolites [5,8–10]. However, the mean levels reported by recent studies [8,10–14], that all included patients using 20 mg tamoxifen per day and analyzed patient samples with liquid chromatography – tandem mass spectrometry (LC-MS/MS), differ more than expected purely based on the inter-patient variability. Three of these studies report mean endoxifen concentrations between 7.10 and 14.5 ng/mL and mean 4-hydroxytamoxifen levels between 1.55 and 2.25 ng/mL [9–11], similar to the levels we find in our laboratory, whereas another recent study reports concentrations twice as high [14]. Two studies from Norway, both using the LC-MS/MS assay developed by Gjerde et al. [15], report even higher concentrations; median concentrations of around 50 ng/mL for endoxifen and around 5.75 ng/mL for 4-hydroxytamoxifen [12,13].

In this article we describe the investigation of these discrepancies, by analyzing a set of 75 patient samples with the assay published by Gjerde et al. [15] and with an assay developed in our laboratory [16].

Methods

Patient samples

Serum samples were obtained in the period between December 2010 and September 2011 from patients treated with tamoxifen in the Netherlands Cancer Institute, Amsterdam, the Netherlands. The samples were collected in serum gel tubes and blood was allowed to coagulate for 30 minutes at room temperature. After coagulation, serum gel tubes were centrifuged for 10 minutes at 2,500-3,000 *g* (temperature was allowed to range from 4°C to ambient temperature). Serum was transferred into polypropylene tubes, which were stored at -70°C until the time of analysis.

Extraction and measurement of tamoxifen and metabolites

Tamoxifen and its metabolites were analyzed in 75 patient samples. All patient samples, 20 calibration standards and 6 quality control samples were handled according to the method described by Teunissen et al. [16]. A volume of 50 µL human serum was processed. Sample pre-treatment involved protein precipitation with acetonitrile. After mixing, samples were centrifuged and the clear supernatant was evaporated to dryness under a gentle stream of nitrogen (30°C). The extracts were reconstituted in acetonitrile - 4 mM ammonium formate buffer pH 3.5 (3:7, *v/v*). The final extracts were analyzed by two different LC-MS/MS assays, method 1 from Teunissen et al. [16] and method 2 from Gjerde et al. [15], during consecutive days.

Method 1

The assay for the determination of tamoxifen (5 – 500 ng/mL), *N*-desmethyltamoxifen (10 – 1000 ng/mL), (*E*)-endoxifen (1 – 100 ng/mL), (*Z*)-endoxifen (1 – 100 ng/mL), *N*-desmethyl-4'-hydroxytamoxifen (1 – 100 ng/mL), 4-hydroxytamoxifen (0.4 – 40 ng/mL) and 4'-hydroxytamoxifen (0.4 – 40 ng/mL), from Teunissen et al. [16] was used with slight modifications. A volume of 5 µL of the final extract was injected onto a Kinetex C18 100 Å column (150 x 2.1 mm ID) and detection was performed on a triple-quadrupole MS/MS detector with an electrospray ionization source (API4000, AB Sciex, Foster City, USA) operating in the positive ion mode. A partial validation was executed and all requirements for acceptance, as defined in the FDA and EMA guidelines on Bioanalytical Method Validation [17,18] were fulfilled.

Method 2

The assay for the determination of tamoxifen (5 – 500 ng/mL), *N*-desmethyltamoxifen (10 – 1000 ng/mL), endoxifen (1 – 100 ng/mL), and 4-hydroxytamoxifen (0.4– 40 ng/mL)

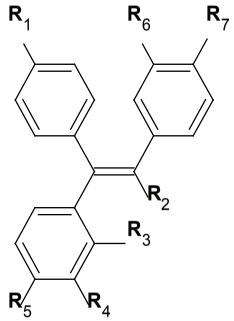
from Gjerde et al. [15] was used.
Online extraction was not executed in order to analyze the identical final extracts that were used when method 1 was applied. The flow rate was set at 0.8 mL/min, to obtain comparable retention times.
A volume of 5 µL of each sample was injected onto a Chromolith Performance RP 18-e column (100 x 4.6 mm ID) and a gradient elution similar to the separation mode of the method used by Gjerde et al. was applied. The separation was performed at room temperature and the autosampler was thermostatted at 7°C. Detection was performed on a triple-quadrupole MS/MS detector with an electrospray ionization source (API4000, AB Sciex, Foster City, USA) operating in the positive ion mode.

Quantification of tamoxifen and metabolites
Tamoxifen, *N*-desmethyltamoxifen-HCl, *N*-desmethyl-4-hydroxytamoxifen (endoxifen, *E*/*Z* mixture 1:1), *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen, 4'-hydroxytamoxifen, tamoxifen-*N*-oxide, tamoxifen-*d5*, *N*-desmethyltamoxifen-*d5*, *N*-desmethyl-4-hydroxytamoxifen-*d5* (endoxifen-*d5*, *E*/*Z* mixture 1:1) and 4-hydroxytamoxifen-*d5* were purchased from Toronto Research Chemicals (North York, ON, Canada). The chemical structures of the analytes are shown in Table 1. Characterization of the peaks in patient samples was based on comparison with the retention times and MS fragmentation patterns of the reference standards. When no reference standard was available, identification was based on MS fragmentation and data found in the literature [10,19]. The reference standard of endoxifen was a racemic mixture (1:1), resulting in baseline separated peaks when using method 1, but in a single peak when using method 2. For the quantification of (*Z*)-endoxifen in patient samples analyzed with method 2, the analyte peak area of the calibration standards and quality control samples was divided by a factor 2.
Multiple reaction monitoring chromatograms were acquired at unit resolution (0.7 Da) for quantification.

Results and discussion

There are large differences in reported mean steady-state concentrations of the therapeutically active tamoxifen metabolites, endoxifen and 4-hydroxytamoxifen. These discrepancies can only partly be assigned to inter-patient variability in the biotransformation of tamoxifen. For this article, we investigated the bioanalytical

Table 1 Trivial names, chemical structures and retention times of tamoxifen and metabolites with molecular mass 371.5, 357.5, 373.5 or 387.5 (reprinted and adjusted from Teunissen et al [5], used with permission).



	Trivial name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Formula	Mol. Mass	Transition (m/z)	RT_1 (min)	RT_2 (min)
1	Tamoxifen	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_3	H	H	H	H	H	$C_{26}H_{29}NO$	371.5	372/72	8.00	2.99
2	*N*-desmethyltamoxifen	O-CH_2-CH_2-NH-CH_3	CH_2-CH_3	H	H	H	H	H	$C_{25}H_{27}NO$	357.5	358/58	7.91	2.96
3	*N*-desmethyl-α-hydroxytamoxifen *	O-CH_2-CH_2-NH-CH_3	CH(OH)-CH_3	H	H	H	H	H	$C_{25}H_{27}NO_2$	373.5	374/58	6.65	2.81
4	*N*-desmethyl-4-hydroxytamoxifen (Endoxifen)	O-CH_2-CH_2-NH-CH_3	CH_2-CH_3	H	H	OH	H	H	$C_{25}H_{27}NO_2$	373.5	374/58	5.79	2.81
5	*N*-desmethyl-3-hydroxytamoxifen *	O-CH_2-CH_2-NH-CH_3	CH_2-CH_3	H	OH	H	H	H	$C_{25}H_{27}NO_2$	373.5	374/58	5.85	2.81
6	*N*-desmethyl-4'-hydroxytamoxifen	O-CH_2-CH_2-NH-CH_3	CH_2-CH_3	H	H	H	H	OH	$C_{25}H_{27}NO_2$	373.5	374/58	6.41	2.81
7	α-hydroxytamoxifen *	O-CH_2-CH_2-N(CH_3)$_2$	CH(OH)-CH_3	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	3.91	2.84
8	4-hydroxytamoxifen	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_3	H	H	OH	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	6.03	2.84
9	3-hydroxytamoxifen *	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_3	H	OH	H	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	6.17	2.84
10	4'-hydroxytamoxifen	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_3	H	H	H	H	OH	$C_{26}H_{29}NO_2$	387.5	388/72	6.66	2.84
11	Tamoxifen-*N*-oxide	O-CH_2-CH_2-NO(CH_3)$_2$	CH_2-CH_3	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	8.32	3.06
12	β-hydroxytamoxifen **	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_2-OH	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	-	-
13	2-hydroxytamoxifen **	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_3	OH	H	H	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	-	-
14	1,2-epoxytamoxifen **	O-CH_2-CH_2-N(CH_3)$_2$	CH_2-CH_3	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5	388/72	-	-

RT_1: Retention time obtained with method 1, based on the method developed by Teunissen et al [16]. RT_2: Retention time obtained with method 2, based on the method developed by Gjerde et al [15]. * No reference standard available, identification was based MS fragmentation and data found in the literature [10,19] ** The levels of these metabolites were below the lower limit of detection of current LC-MS platforms [11,20], therefore retention times are unknown

variability by analyzing a set of 75 patient samples with two different LC-MS/MS methods; method 1 from Teunissen et al. [16] and method 2 from Gjerde et al. [15]. The bioanalytical data were accepted for both methods, since the back-calculated concentrations of the calibration standards and quality control samples were all within ±15%. The results are presented in Table 2.

Tamoxifen and *N*-desmethyltamoxifen

The measured concentration of tamoxifen and *N*-desmethyltamoxifen in each serum sample was very similar for both methods (Figure 2), resulting in comparable mean concentrations (Table 2). There are no tamoxifen metabolites described in the literature [6,20] that have molecular masses similar to tamoxifen or *N*-desmethyltamoxifen, therefore co-elution of tamoxifen analogues with fragmentation patterns similar to tamoxifen or N-desmethyltamoxifen is not expected.

Endoxifen (*m/z* 374 -> 58)

There are several metabolites with close resemblance in molecular structure to endoxifen (Table 1). These compounds also have similar molecular masses and fragmentation patterns, making chromatographic separation of crucial importance for selective analysis. The chromatogram obtained with the method from Teunissen et al. [16] (Figure 1a) shows separate peaks for the metabolites with mass transition 374/58, whereas the chromatogram obtained with the method from Gjerde et al. [15] (Figure 1b) shows only a single peak, consisting of *N*-desmethyl-α-hydroxytamoxifen, endoxifen, *N*-desmethyl-3-hydroxytamoxifen and *N*-desmethyl-4'-hydroxytamoxifen eluting at the same retention time. This lack of selectivity leads to a consequent overestimation of the endoxifen level of around a factor 2 (Figure 2 and Table 2). Furthermore, method 1 separates (*E*)-endoxifen from the therapeutically active (*Z*)-endoxifen, whereas method 2 does not separate these isoforms. However, for all 75 patient samples the (*E*)-endoxifen level was below the lower limit of quantitation (1.0 ng/mL), which is in agreement with the literature [10,19].

Table 2 Mean concentrations of tamoxifen and three of its metabolites analysed with the two described methods, from serum samples of 75 patients treated with tamoxifen

	Mean concentration (ng/mL)	
Analyte	Method 1 [16] ± s.d.	Method 2 [15] ± s.d.
Tamoxifen	99.7 ± 39.3	103.3 ± 40.4
N-desmethyltamoxifen	184.0 ± 74.7	187.1 ± 77.9
Endoxifen	9.0 ± 4.5	18.1 ± 6.4
4-Hydroxytamoxifen	1.7 ± 0.7	4.6 ± 1.7

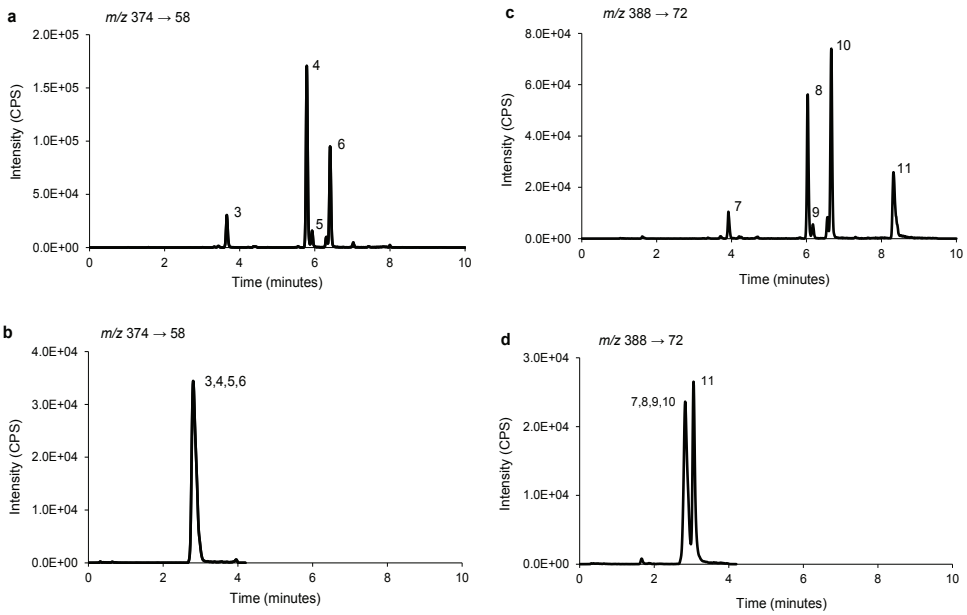


Figure 1 Representative LC-MS/MS chromatograms obtained from a study patient sample. Chromatograms a and b were obtained with method 1, from Teunissen et al. [16], and method 2, from Gjerde et al. [15], respectively, when *m/z* 374 -> 58 was monitored. Chromatograms c and d were obtained with method 1 and 2, respectively, when *m/z* 388 ->72 was monitored. Peak numbers correspond with metabolite numbers in Table 1

4-Hydroxytamoxifen (*m/z* 388 -> 72)

As shown in Table 1, there are at least seven tamoxifen metabolites with masses and fragmentation patterns similar to 4-hydroxytamoxifen. From these metabolites, the levels of *β*-hydroxytamoxifen, 2-hydroxytamoxifen and 1,2-epoxytamoxifen are below the lower limit of detection (LLOD) of current LC-MS platforms (±0.05 ng/mL) [11,20]. The chromatogram obtained with method 1 (Figure 1c) shows separate peaks for the other four metabolites with mass transition 388/72, whereas the chromatogram obtained with method 2 (Figure 1d) shows only two separated peaks. Tamoxifen-*N*-oxide elutes at 3.06 minutes and α-hydroxytamoxifen, 4-hydroxytamoxifen, 3-hydroxytamoxifen and 4′-hydroxytamoxifen are co-eluting at 2.84 minutes. This co-elution leads to a consequent overestimation of the 4-hydroxytamoxifen levels of around a factor 3 (Figure 2) and therefore the mean 4-hydroxytamoxifen concentration obtained with method 2 is a factor 3 higher (Table 2). The results obtained with method 1 are in good agreement with the levels reported by three recent published studies. The analytical methods used in these studies all separated endoxifen and 4-hydroxytamoxifen from compounds with similar masses and fragmentation patterns [8,10,11].

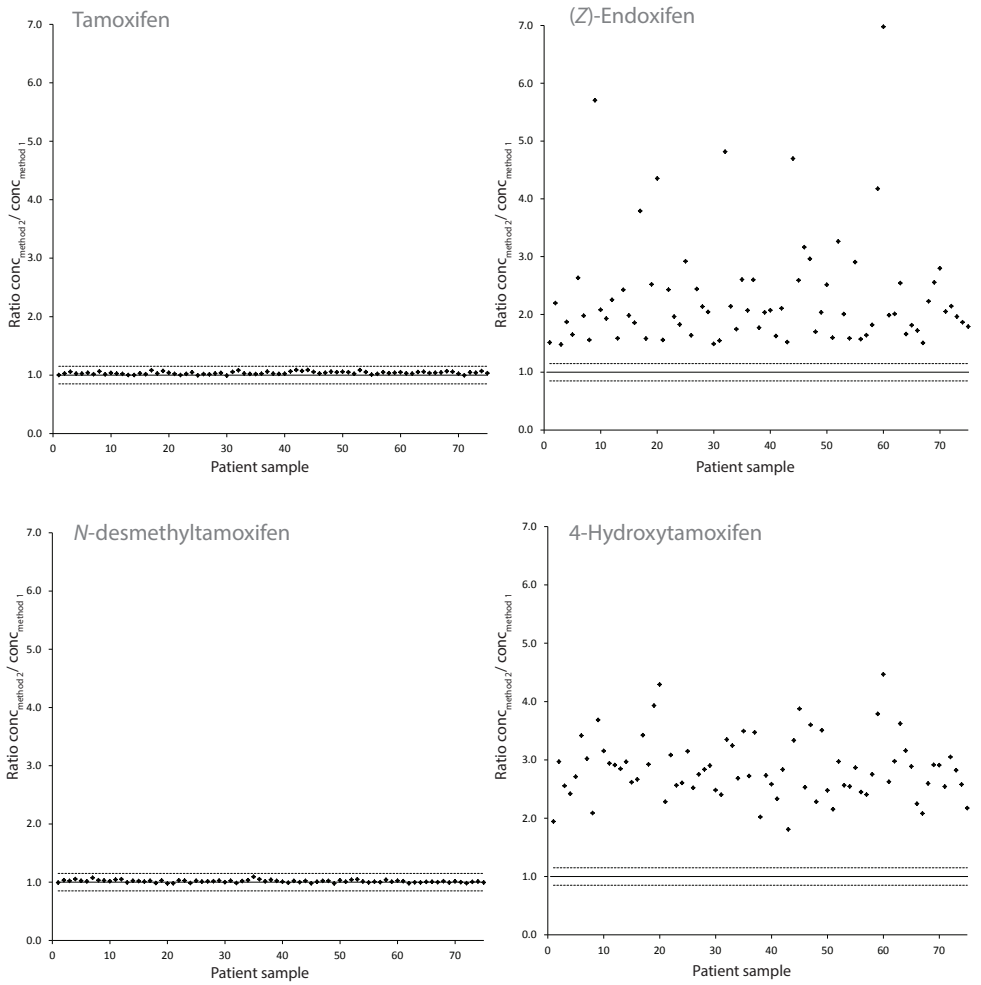


Figure 2 Ratio of the measured concentrations obtained with method 1 ($conc_{method\ 1}$) and method 2 ($conc_{method\ 2}$) in 75 patient samples for tamoxifen, *N*-desmethyltamoxifen, (*Z*)-endoxifen and 4-hydroxytamoxifen. The solid line represents a ratio of 1.0 (i.e. equal measured concentrations) and the dotted lines represent the (bioanalytically accepted) ±15% deviation from method 2 in comparison with method 1

When investigating correlations between the levels of the active tamoxifen metabolites and efficacy and toxicity parameters, it is crucial to distinguish between the active metabolites and the 4'-hydroxylated metabolites, which are about ten times less active than 4-hydroxytamoxifen and endoxifen [10,11]. Also, for therapeutic drug monitoring based on reaching a sufficient endoxifen level, it is important to use a highly selective analysis in order to accurately quantify endoxifen in the patient sample.

Conclusions

This article demonstrates that high selectivity is of major importance for the analysis of tamoxifen metabolites, some of which show marked resemblance in molecular structure and have similar masses and fragmentation patterns. Lack of high selectivity results in an overestimation of the concentration of the therapeutically active metabolites, endoxifen and 4-hydroxytamoxifen, in patient samples.

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Procedures and practices for the validation of bioanalytical methods using dried blood spots: a review

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Abstract

Dried blood spot (DBS) sampling, the collection of whole blood samples on paper, is an emerging technique used for bioanalytical methods. Several analytical challenges, such as possible effects of spotting volume, haematocrit and spot inhomogeneity are identified for these methods, however, no regulatory-based guidelines for the specific validation of DBS-based assays are available hitherto.

To date, 68 validation reports concerning methods for the quantitative determination of drugs in human DBS could be traced in the literature, with large differences in the extensiveness of the reported validations. This review aims to present an overview of these published validations. Additionally, the different challenges of DBS-based assays are discussed and recommendations on how to perform validation tests addressing these challenges are provided.

Introduction

Dried blood spot (DBS) sampling dates back to the 1960s, when it was developed to measure phenylalanine for the detection of phenylketonuria in newborns. This novel approach of blood collection led to the population screening of newborns for several inherited metabolic disorders. [1] Some years ago, the application of DBS sampling was extended to the determination of drug concentrations for clinical and preclinical studies, therapeutic drug monitoring and for use in toxicology studies. Moreover, dried blood spot analysis is determined to be feasible for analytes representing a wide range of physico-chemical as well as pharmacokinetic parameters [2–4].

DBS sampling is considered to be a simple and minimally invasive sampling method. To obtain a DBS, the clean skin of the fingertip or heel is punctured with a lancing device and the drops of blood are collected on a DBS card. The obtained blood spots should be allowed to dry at room temperature for several hours (depending upon the DBS card type) and stored in a foil bag containing a desiccant package. In general, a fixed diameter punch with a size smaller than the size of the DBS is used, so a part of the DBS sample is punched out and used for the extraction procedure. The punch is transferred to a clean tube and subsequently the analytes are extracted from the DBS with a suitable extraction solvent (depending on the analyte of interest).

The collection of whole blood samples as DBS offers a number of advantages over conventional serum or plasma sampling. First, it is a less invasive sampling method; a finger or heel prick, rather than a venapuncture. Also, the DBS sample can be shipped and stored without refrigeration, resulting in easier and lower-cost logistics. Third, DBS sampling requires a smaller blood volume, making this method particularly suitable for the collection of samples in animal and pediatric studies. Finally, with adequate training, patients can self-sample at home and send the samples to the laboratory by regular mail service. In the last decade, numerous reports have been published describing the validation of a bioanalytical method using DBS. The Food and Drug Administration (FDA) [5] and the European Medicines Agency (EMA) [6] both reported extended guidelines for the validation of bioanalytical methods, to provide assistance for method validation in order to obtain reliable and completely validated bioanalytical methods. However, these guidelines apply for quantitative analyses in biological matrices such as blood, serum, plasma, urine or saliva. A dried blood spot, a solid matrix consisting of dried whole blood on a paper card, is a different type of matrix than the liquid matrices mentioned in the FDA and EMA guidelines. This difference in matrix type between DBS and more conventional sampling matrices makes the FDA and EMA guidelines insufficient for the complete validation of DBS-based assays. There are no specific

regulatory-based guidelines for the validation of the quantitative determination of drugs in DBS. Yet, in recent years several review and recommendation papers have been published, describing a number of challenges of DBS-based methods and providing recommendations for the development and validation of these assays [7–12]. This review aims to present an overview of publications describing the validation of bioanalytical assays developed for the quantitative determination of drugs in human dried blood spots. Additionally, the different challenges of DBS assays identified in the literature are discussed and recommendations on how to perform validation tests addressing these challenges are provided.

Methods

Analysis of the literature was performed using the PubMed database with the search term (((dried blood spot[title] OR dried blood spots[title]) OR dry blood spot[title]) OR dry blood spots[title]) AND (((((((validation[title/abstract] OR assay[title]) OR analysis[title]) OR determination[title]) OR quantification[title]) OR method[title]) OR measurement[title]) OR quantitation[title]) NOT ((((((((((((immunoassay[Title/Abstract] OR biomarker[Title/Abstract]) OR biomarkers[Title/Abstract]) OR newborn screening[Title/Abstract]) OR vitamin[Title/Abstract]) OR antibody[Title/Abstract]) OR antigen[Title/Abstract]) OR genotype[Title/Abstract]) OR genotyping[Title/Abstract]) OR DNA[Title/Abstract]) OR RNA[Title/Abstract]) OR virological testing[Title/Abstract]) OR review[Title/Abstract]) OR amino acid[Title/Abstract]). The last search was performed on April 22nd 2014. All publications written in English where the validation of a bioanalytical assay for the quantitative determination of drugs in human DBS was described were included.

Specific considerations for dried blood spot assays

A number of factors have been identified as having a potential effect on the quantitative outcomes obtained from DBS analysis. Some of these parameters are only applicable to DBS-based assays, whereas other parameters also apply to regular bioanalytical assays but require special attention in DBS analysis. Here, we briefly discuss the different factors that are reported to possibly affect the performance of a DBS-based assay.

Internal standard addition

Common practice for internal standard addition in DBS methods is to add the internal standard to the extraction solvent. By this process, the internal standard will not compensate for potential variations in extraction recovery. Other procedures, such as pretreatment of the DBS card with internal standard before spotting or adding the internal standard to the spotted sample have been investigated [13,14]. However, all proposed procedures appear to have their analytical and logistical pros and cons and the most suitable method seems to be analyte-dependent.

Spot volume

Calibration standards and quality control samples are usually prepared by spotting a pre-defined volume of whole blood on the DBS card. However, for clinical samples obtained from a finger prick, this volume is not controlled and thus will vary. Before extraction of clinical DBS samples, the DBS should be visually inspected to ensure that i) the DBS is larger than the punch that will be taken out from the DBS, ii) the blood is symmetrically spread around the center and iii) there is an even dark red color on both sides of the DBS card [12]. In general, it is described that spotting precise volumes of blood is not necessary to obtain accurate results [8,10] when the DBS samples meet the above mentioned criteria. However, minor effects are observed and shown to be dependent on the card type and analyte of interest [15].

Haematocrit

Haematocrit (Hct), the relative volume of red blood cells in whole blood, influences the viscosity and thus spreadability of whole blood on DBS cards [9,12,16]. A high Hct value leads to a high viscosity of the blood, resulting in a poor spreadability and the formation of a relatively small blood spot. When a fixed diameter subpunch is used, a high Hct value will result in a higher analyzed concentration, since the punched out disc will contain a larger volume of blood [15–18]. Hct is usually between 41 and 53% for men and between 36 and 46% for women [19]. However, the Hct value can deviate from these ranges in special populations, for example in neonates and patients with physiological conditions such as anemia, cancer or renal impairment, or patients under medical treatment affecting the haematocrit. In the more recent review and recommendation papers, the impact of haematocrit on the accuracy and precision of the assay is the main focus, stating this is the most important parameter influencing DBS assay performance [11].

Spot homogeneity

Unlike liquid samples, for which sample homogeneity can be assured by thorough vortexing, the analytes in the DBS sample distribute directly after the blood is spotted on the card and re-homogenizing is not possible. When using a fixed diameter subpunch from within the DBS sample rather than using the whole spot, it is of importance that the analyte in the blood samples distributes evenly and reproducible on the paper card, so a fixed spot size equals a fixed volume. Although spot homogeneity usually does not seem to be an issue in DBS analysis, there are some reports describing a difference between punches taken from the center or from the periphery of the DBS. This effect seems to be dependent on the card type and the analyte of interest. [20,21] A possible solution for non-homogenous spots could be the use of a large enough punch and taking punches from the same location in a spot [10].

Spot-to-spot carry-over

Spot-to-spot carry-over is a possible source of error caused by the punching device used to punch out the DBS. This type of carry-over can possibly be eliminated by punching blank DBS cards between punching DBS samples. [10]

The following parameters are also described in FDA and EMA guidelines for bioanalytical method validation, but require special attention when validating a DBS-based assay.

Recovery

The FDA guidelines describe the requirements for recovery in bioanalytical assays; recovery should be consistent, precise and reproducible, and should therefore be determined at three concentration levels [5]. These requirements can also be applied to DBS-based assays. However, in DBS-based assays the internal standard is usually added to the extraction solvent and may therefore not compensate for potential variations in extraction efficiency. It is reported that aging of DBS could influence the extraction recovery [16,22]. Also, Hct can have an effect on the extraction recovery of some analytes [16,23].

Matrix effects

The EMA guidelines describe the requirements for matrix effects in mass spectrometric bioanalytical assays, being that the matrix effect should be consistent and reproducible and therefore should be determined in at least six batches of blank matrix, obtained from six individual donors, at low and high concentration levels [6]. This approach is also applicable for DBS assays, where matrix effects can be expected not only from whole

blood, but also from DBS cards. It is reported that especially coated DBS cards can be a source of matrix effects. For instance, sodium dodecyl sulfate, a substituent of the coating of DMPK-A DBS cards, can cause ion suppression [22,24].

Stability under transport and storage conditions

As mentioned in the FDA and EMA guidelines, stability testing should be carried out to ensure that every step taken after sampling, during sample preparation and analysis, as well as storage conditions, does not affect the concentration of the analyte [5,6]. A large advantage of DBS-based assays is that there is, in general, no need to refrigerate the samples during transportation. This results, however, in varying transport conditions, which should be considered during the stability experiments. When DBS samples are mailed by regular post service, which implies that the samples will be kept in mailboxes for some hours up to several days. [12] Temperature in mailboxes will vary considerably, depending on the geographic location and the season. Especially in summer very high temperatures are measured in mailboxes, up to 20˚C above the outside temperature [25]. On the contrary, in winter temperatures can decrease to far below zero. Also, temperatures in the cargo holding of airplanes can drop below zero [26].

Sample dilution above the upper limit of quantitation

Dilution integrity should be taken into account, covering the concentration range that is expected in clinical samples [6]. Dilution of a sample is normally performed with corresponding blank matrix before addition of the internal standard and subsequent processing of the diluted sample. However, due to its solid format, dilution of a DBS sample following the regular dilution procedure is not possible. Two main approaches to overcome this are described in the literature. The first is to extract the DBS sample in the same way as the other samples and to dilute the final extract with blank DBS final extract containing the internal standard [8,10]. The second approach is the 'internal standard tracked dilution method'; extract the DBS sample with extraction solvent containing a multiplied concentration of internal standard and dilute the final extract with blank final extract (dilution factor is identical to the factor used to multiply the internal standard concentration in the extraction solvent), not containing the internal standard [27].

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Koal T, et al. 2005 [64]	Amprenavir Nelfinavir Indinavir Lopinavir Saquinavir Ritonavir Atazanavir Nevirapine Efavirenz	Testcards 76x108 mm Schleicher &Schuell	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects	Not performed	Not performed	1 patient sample n=5	Not performed	5 days, RT 1 conc n=1	Not performed
Aburuz S, et al. 2006 [65]	Metformin	Whatman 903	HPLC-UV	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	3 months, -70˚C 2 conc n=6	Not performed
Lejeune D, et al. 2007 [66]	Chloroquine Proguanil	Whatman 903	HPLC-UV	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	20 days, -20˚C, 4˚C, RT, 50˚C 3 conc n=3	Not performed
Hoogtanders K, et al. 2007 [7]	Tacrolimus	10535097 Schleicher &Schuell	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	10 – 30 µL Spots weighted 1 conc n=6	Not performed	Not performed	Not performed	31 days, 4˚C 9 days, RT 7 days, 37˚C, -20˚C 1 day, 70˚C 5 conc n=1	Not performed
ter Heine R, et al. 2008 [37]	Atazanavir Darunavir Lopinavir Ritonavir Efavirenz Nevirapine	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects	2 – 60 µL 2 conc n=3	Not performed	Not performed	Not performed	7 days, 30˚C 2 conc n=5	Not performed

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Garcia Boy R, et al. 2008 [67]	Morphine 6-Acetyl morphine	BFC 180 Whatman	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects Instr carry-over	Not performed	Not performed	Not performed	Not performed	7 days, 4˚C, -20˚C 5 days, 40˚C 3 conc n=5	Not performed
ter Heine R, et al. 2009 [68]	Etravirine	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	7 days, 30˚C 2 conc n=5	Not performed
ter Heine R, et al. 2009 [69]	Raltegravir	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	7 days, RT, 30˚C 1 conc n=3	Not performed
Spooner N, et al. 2009 [8]	Acetaminophen	Whatman FTA (DMPK-A)	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	10 – 20 µL 2 conc n=6	Not performed	Not performed	Not performed	113 days, RT 2 conc n=6	[8,10]
van der Heijden J, et al. 2009 [70]	Everolimus	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	34 days, RT, 32˚C 30 days, 4˚C 4 days, 60˚C 1 conc n=6	Not performed
Liang X, et al. 2009 [71]	Dextromethorphan	Whatman FTA elute (DMPK-B)	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	10 – 50 µL 3 conc n=3	Not performed	Periphery and center 4 conc n=6	Not performed	107 days, RT 2 conc n=6	Not performed

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Wilhelm AJ, et al. 2009 [41]	Cyclosporin A	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Recovery Matrix effects	Not performed	20 – 72% 2 conc n=2	Not performed	Not performed	45 days, 4˚C 17 days, RT 3 conc n=2	Not performed
Damen CWN, et al. 2009 [55]	Vincristine Actinomycin-D	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	20 – 60 µL 2 conc n=3	Not performed	Not performed	Not performed	3 months, 40-45˚C, RT 2 conc n=3	[8,10]
Wilhelm AJ, et al. 2009 [42]	Mycophenolic acid	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery	Not performed	22 – 45% 2 conc n=6	Not performed	Not performed	26 days, 4˚C 3 conc n=3	Not performed
Déglon J, et al. 2010 [32]	Fluoxetine Norfluoxetine Reboxetine Paroxetine	105355097, Whatman	GC-NICI-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	Not performed	Not performed	Not performed	Not performed	1 month, -20˚C, 4˚C, RT, 40˚C 1 conc n=3	Not performed
Suyagh MF, et al. 2010 [72]	Canrenone	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	1 month, -20˚C 4 conc n=3	Not performed
Suyagh MF, et al. 2010 [73]	Metronidazole	Whatman 903	HPLC-UV	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	4 weeks, -20˚C 3 conc n=3	Not performed

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Meesters RJW, et al. 2010 [74]	Lopinavir Ritonavir	Whatman FTA (DMPK-A)	MALDI-QqQ-MS-MS	Calibration model Sensitivity Accuracy&precision	Not performed	Not performed	Not performed	Not performed	20 days, -20˚C 3 conc n=10	Not performed
Ingels ASME, et al. 2010 [31]	Gamma hydroxybutyric acid	Whatman 903	GC-MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Instr carry-over	Whole spot used	Whole spot used	Whole spot used	Not performed	14 days, -20˚C 7 days, RT 2 conc n=3	Final extract diluted with ethylacetate
Mercolini L, et al. 2010 [33]	Cocaine and metabolites	Whatman 903	HPLC-spectrofluorimetry	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Precision capillary used	Not performed	Not performed	Not performed	Not performed	Not performed
Patel P, et al. 2010 [75]	Dexamethasone	Whatman 903	HPLC-MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	25 – 35 µL 2 conc n=5	30 – 40% 3 conc n=5	Not performed	Not performed	28 days, 4˚C 7 days, RT 2 conc n=2	Not performed
Lad R. 2010 [76]	Caffeine Flurbiprofen Midazolam Omeprazole Rosiglitazone	Whatman DMPK-B	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Incurred sample reanalysis	10 – 20 µL 2 conc n=6	Not performed	Not performed	Not performed	5 days, RT 1 conc n=6	Not performed
Michi M, et al. 2010 [77]	Zatebradine	Whatman FTA (DMPK-A)	HPLC- MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	10 – 20 µL 2 conc n=6	Not performed	Not performed	Not performed	Not performed	[8,10]

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Ia Marca G et al. 2011 [78]	Rufinamide	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Range nm n=14 patients	Not performed	Not performed	1 month, -20˚C, 4˚C, RT 3 conc n=2	Not performed
Ewles MF, et al. 2011 [56]	Ramoplanin	Whatman DMPK-B	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	15 – 25 µL 1 conc n=6	Not performed	Not performed	Not performed	9 days, RT 3 conc n=6	[8,10]
Saracino MA, et al. 2011 [28]	Clozapine	Whatman Type 41	HPLC-coulometry	Calibration model Sensitivit Accuracy&precision Selectivity&specificity Recovery	Accurately pipetted venous blood	Whole spot used	Whole spot used	Not performed	50 days, RT 4 conc n=3	Not performed
Clavijo CF, et al. 2011 [79]	Morphine and metabolites	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects Instr carry-over	Not performed	Not performed	Not performed	Not performed	3 days, RT 5 conc n=6	Not performed
Swales JG, et al. 2011 [80]	Metformin Sitagliptin	Whatman DMPK-C	LDTD-MS/MS	Accuracy&precision Selectivity&specificity	Not performed	Not performed	Not performed	Not performed	40 days, RT 3 conc n=3	Not performed
Vu DH, et al. 2011 [15]	Moxifloxacin	Whatman 31 ET CHR	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	30 – 100 µL 3 conc n=5	20 – 50% 3 conc n=5	Not performed	Not performed	4 weeks, -80˚C, RT, 50˚C 2 conc n=5	[8,10]

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Hooff GP, et al. 2011 [34]	Oseltamivir Oseltamivir-carboxylate	Schleicher& Schuell 2992	UPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	7 days, RT, 4°C 2 conc n=4	Not performed
Li W, et al. 2011 [43]	NIM811	Whatman FTA (DMPK-A)	HPLC- MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	10 – 40 µL 3 conc n=3	20 – 70% 3 conc n=3	Periphery and center 3 conc n=3	Not performed	62 days, ≤-60˚C, 2-8˚C, RT 3 conc n=3	[8,10]
Römsing S, et al. 2011 [53]	Tafenoquine	31ET chr Whatman	HPLC-fluorescence	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	90 days, -17˚C, 4˚C 60 days, 22˚C, 37˚C 2 conc n=3	Not performed
Yakkundi S, et al. 2011 [81]	Ranitidine	Whatman 903	HPLC MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	6 months, -20˚C, RT 3 conc n=3	Not performed
Ingels ASME, et al. 2011 [57]	Gamma hydroxybutyric acid	Whatman 903	GC-MS	Calibration model Sensitivity Accuracy&precision	20 – 50 µL 2 conc n=5	34 – 56% 2 conc n=5	Periphery and center 2 conc n=5	Not performed	148 days, RT 2 conc n=6	Final extract diluted with ethylacetate
Reddy TM, et al. 2011 [38]	Posaconazole	Ahlstrom Ahl-226	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects	10 – 35 µL 2 conc n=1	25 – 65% 2 conc n=6	Periphery and center 2 conc n=3	Not performed	13 days, RT 2 conc n=6	[8,10]

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Li Y, et al. 2011 [58]	Guanfacine	Whatman FTA DMPK-C	HPLC-MS/MS	Accuracy&precision Selectivity&specificity Recovery Matrix effects	15 – 25 µL 2 conc n=5	Not performed	Periphery and center 4 conc n=5	Not performed	76 days, RT 4 conc n=5	Smaller puncher
Hinchliffe E, et al. 2012 [82]	Cyclosporin A	Whatman 903	UPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	14 days, RT 10 conc n=1	Not performed
Ia Marca G, et al. 2012 [54]	Ertapenem	Whatman 903	UPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects	Not performed	Range nm n=3 patients	Not performed	Not performed	1 month, -20˚C, 4˚C, RT, 37˚C 4 conc n=1	Not performed
Ganz N, et al. 2012 [83]	Bosentan and metabolites	Whatman DMPK-A	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	20 – 30 µL 2 conc n=6	35 – 65% 2 conc n=6	Not performed	Not performed	3.5 months, RT 2 conc n=6	Not performed
Thomas A, et al. 2012 [84]	26 model compounds for doping control	Sartorius TFN card	UPLC-MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	7 days, 2–8°C 1 conc n=1	Not performed
Saussereau E, et al. 2012 [85]	Illicit drugs	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	6 months, -20˚C, 4˚C 2 conc n=5	Not performed

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Lawson G, et al. 2012 [86]	Atenolol	Whatman 903	LC-HRMS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	20 – 40 µL 3 conc n=6	Not performed	Not performed	Not performed	10 weeks, RT 3 conc n=6	Not performed
Ia Marca G, et al. 2012 [51]	Linezolid	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery	Not performed	Range nm n=9 patients	Periphery and center 1 conc n=1	Not performed	1 month, -20˚C, 4˚C, RT, 37˚C 3 conc n=3	Not performed
Ansari M, et al. 2012 [29]	Busulfan	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	Whole spot used	Whole spot used	Whole spot used	Not performed	1 month, -20˚C, 4˚C, RT 3 conc n=2	Not performed
Saracino MA, et al. 2012 [35]	Methadone	Whatman FTA (DMPK-A)	HPLC-coulometric	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	1 month, RT 3 conc n=3	Not performed
Déglon J, et al. 2012 [87]	15 benzodiazepines	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects Instr carry-over	Not performed	Not performed	Not performed	Not performed	30 days, -20˚C, RT 4 conc n=3	Not performed
Li W, et al. 2012 [88]	Acetaminophen and metabolites	Ahlstrom Ahl-226	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects Instr carry-over Incurred sample reanalysis	10 – 40 µL 2 conc n=3	Not performed	Not performed	Not performed	27 days, RT 2 conc n=3	[8,10]

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Kralj E, et al. 2012 [30]	Imatinib Nilotinib Dasatinib	Agilent cards	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Whole spot used	30 – 60% 3 conc n= nm	Whole spot used	Not performed	28 days, RT 3 days, -20˚C, 40˚C conc nm n= nm	Not performed
den Burger JCG, et al. 2012 [18]	Cyclosporin A Tacrolimus Sirolimus Everolimus	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects	20 – 100 µL 2 conc n=3	22 – 44% 2 conc n=1	Periphery and center 2 conc n=6	Not performed	168 days, 2-8˚C 6 conc n=1	Not performed
Vu DH, et al. 2012 [44]	Linezolid	Whatman 31 ET CHR paper	HPLC-MS/MS	Calibration model Accuracy&precision Recovery Matrix effects	30 – 90 µL 3 conc n=5	20 – 50% 3 conc n=5	Not performed	Not performed	2 months, RT, 37˚C 1 week, 50˚C 2 conc n=5	Not performed
Xu Y, et al. 2012 [45]	MK-1775	Whatman DMPK-A	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Incurred sampler reanalysis	30 – 50 µL 7 conc n=3	16 – 85% 2 conc n=4	Periphery and center 7 conc n=3	Not performed	14 months, RT 6 months, -20˚C 8 days, 40˚C 3 conc n=5	[8,10]
Mercolini L, et al. 2013 [36]	Δ9-tetrahydro-cannabinol and metabolites	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	3 months, RT 3 conc n=6	Not performed
Della Bona ML, et al. 2013 [89]	Propranolol	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects	Not performed	23 – 43% n=7 patients	Not performed	Not performed	1 month, -20˚C, 4˚C, RT 4 conc n=3	Not performed

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Sadilkova K, et al. 2013 [46]	Cyclosporin Tacrolimus Sirolimus	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Matrix effects Instr carry-over	25 – 100 µL 2 conc n=3	20 – 45% n=179 patients	21 punches from 1 spot	Not performed	5 days, -20˚C, RT, 37˚C, 60°C 5 conc n=1	Increase volume extraction solvent
Shah NM, et al. 2013 [90]	Levetiracetam Lamotrigine Phenobarbital Carbamazepine	Whatman 903	HPLC-UV	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	20 – 50 µL 1 conc n=3	30 – 55% 1 conc n=3	Not performed	Not performed	6 weeks, -80˚C, RT 3 days, 40˚C 1 conc n=3	Not performed
Hoffman JT, et al. 2013 [59]	Efavirenz	Whatman 903	HPLC-UV	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	35 – 48% n=31 patients	Not performed	Not performed	1 year, -20˚C 1 month, -70˚C, 4˚C, RT, 37˚C,45˚C 3 conc n=3	Increase volume extraction solvent
Vnučec Popov T, et al. 2013 [50]	Topiramate	Whatman DMPK-C FTA	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	15 – 35 µL 1 conc n=3	34 – 45% 1 conc n=1	Not performed	[10]	194 days, RT 2 conc n=8	[8,10]
Lawson G, et al. 2013 [91]	Bisoprolol Ramipril Simvastatin	Whatman 903	LC-HRMS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	20 – 40 µL 2 conc n=6	Not performed	Not performed	Not performed	12 weeks, RT 3 conc n=6	Not performed
Taylor RR, et al. 2013 [92]	Acetaminophen	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects Instr carry-over	Not performed	Not performed	Not performed	Not performed	1 month, -80˚C 1 week, 4˚C 1 day, RT 3 conc n=1	Not performed

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
van der Elst KCM, et al. 2013 [47]	Voriconazole Fluconazole Posaconazole	Whatman DMPK-C FTA	HPLC-MS/MS	Calibration model Accuracy&precision Recovery Matrix effects	30 – 90 µL 2 conc n=nm	25 – 45% 2 conc n=nm	Not performed	Not performed	12 days, -80˚C, RT, 37˚C, 50˚C 2 conc n=nm	Not performed
Hatami M, et al. 2013 [93]	Salmeterol	Whatman filter paper type 41 55 mm	HPLC-fluorescence	Calibration model Sensitivity Accuracy&precision Recovery	Not performed	Not performed	Not performed	Not performed	Not performed	Not performed
Koster RA, et al. 2013 [23]	Cyclosporin Tacrolimus Sirolimus Everolimus	Whatman 31 ET CHR paper	HPLC-MS/MS	Calibration model Accuracy&precision Recovery Matrix effects	30 – 90 µL 2 conc n=nm	20 – 50% 2 conc n=nm	Not performed	Not performed	7 days, -80˚C, RT 2 days, 37˚C 2 conc n=5	Not performed
Antelo-Domínguez Á, et al. 2013 [94]	Cocaine	Whatman 2012-10	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery	Not performed	Not performed	Not performed	Not performed	Not performed	Not performed
Zheng JH, et al. 2014 [39]	Tenofovir Emtricitabine	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	5 – 50 µL 2 conc n=3	10 – 77% 2 conc n=3	Periphery and center 2 conc n=3	Not performed	7 days, RT 2 conc n=3	[27]
Jimmerson L, et al. 2014 [40]	Ribavirin	Whatman 903	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	5 – 60 µL 2 conc n=3	10 – 60% 2 conc n=3	Periphery and center 2 conc n=3	Not performed	415 days, -20˚C, RT 2 conc n=5	[27]

Table 1 Published reports describing the validation of an assay for the quantitative determination of drugs in human dried blood spots (continued)

Report	Analyte	Card type	Method	Regular validation parameters	Dried blood spot specific validation parameters					
					Spot volume	Haematocrit (%)	Spot homogeneity	Spot-to-spot carry-over	Long-term stability	Dilution integrity
Geditz MCK, et al. 2014 [52]	Mefloquine	Whatman 903	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Selectivity&specificity Recovery Matrix effects	Not performed	Not performed	Not performed	Not performed	Not performed	Not performed
Berm EJJ, et al. 2014 [48]	Venlafaxine O-desmethyl venlafaxine	Whatman DMPK-C	HPLC-MS/MS	Calibration model Sensitivity Accuracy&precision Recovery Matrix effects Instr carry-over	20 – 100 µL 2 conc n=3	25 – 50% 2 conc n=3	Periphery and center 2 conc n=3	Not performed	Not performed	Not performed
Vu D, et al. 2014 [49]	Rifampicin Clarithromycin	Whatman 31 ET CHR	HPLC-MS/MS	Calibration model Accuracy&precision Selectivity&specificity Recovery Matrix effects Instr carry-over	30 – 100 µL 3 conc n=5	20 – 50% 3 conc n=5	Not performed	Not performed	60 days, RT 30 days, 37°C 15 days, 50°C 2 conc n=5	[8,10]

Instr: instrumental, conc: concentration, RT: room temperature, nm: not mentioned.

Procedures and practices

In total 68, validated DBS-based assays for the quantitative determination of drugs in human whole blood have been published in the literature over the past decade. An overview of these reports is provided in Table 1.

Spotting and sampling procedures

Most reports describe the preparation of calibration and quality control samples from freshly drawn human whole blood and the use of a spot volume of 20 to 50 µL. Subsequently, a fixed diameter subpunch (usually 3-8 mm) is taken from the DBS and extraction solvent containing the internal standard is added to the punched sample. However, in four papers the use of the whole spot instead of a fixed diameter subpunch is described; for three assays venous blood is obtained and subsequently spotted onto the card with an accurate pipette [28–30], one assay uses a precision capillary to transfer blood from a fingerprick to the card [31]. With this approach, the spotting volume and spot homogeneity will not be an issue. Also, Hct will be of less influence on assay performance when the entire spot is used, since the difference in spot size caused by the Hct will not change the volume of blood used for the analysis. However, accurate spiking diminishes a large advantage of DBS sampling, being easy sampling and the option for the patient to self-sample at home. Additionally, six reports describe applying the internal standard to the DBS card instead of adding the internal standard to the extraction solvent in order to correct for possible variations in extraction recovery [31–36].

Validation practices

Spot volume. The first DBS specific validation parameter that is examined during assay validation is the investigation of the effect of spot volume, in 2007 by Hoogtanders et al. [7] and in 2008 by ter Heine et al. [37]. In total, 30 out of 68 (44%) reviewed validation papers describe the effect of spot volume on assay performance. The tested spot volumes differ per assay, for some assays only a volume range of 10 to 20 µL was tested, whereas for other assays a wide range of 10 to 100 µL was investigated. For most assays reporting the investigation of the effect of spotted volume, this was performed at two concentration levels using at least three replicates per concentration level. The majority of these reports describe no effect of spot volume on assay performance. The few articles that reported an effect of spot volume; a low spot volume resulted in a lower analyzed concentration, attributed this effect to the fact that the obtained DBS was too small for the punching device that was used [18,38–40].

Haematocrit. The potential influence of haematocrit on DBS assay accuracy and precision is described in review and recommendation papers since 2009 [12]. The first validation report describing the investigation of Hct effect was published in 2009, by Wilhelm et al. [41], although it took until 2013 before at least half of the validation reports included the investigation of Hct effects. In total, 27 of the 68 published validation reports (40%) described the investigation of possible effect of Hct on the performance of the DBS method. There are large differences in the extent to which the influence of Hct is investigated. Especially the more recent papers describe the investigation of a broad range of Hct values, i.e. at least between 20 and 50% [15,18,23,39–49], usually evaluated at two concentration levels. The majority of the reports investigating the effect of Hct on assay performance describe a trend that Hct value is positively related to measured analyte concentration. Consequently, in several validation reports where a very wide range of Hct values is analyzed, the low (≤20%) and high (≥50%) Hct samples fall outside the acceptable ranges.

Spot homogeneity. The (in)homogeneity of DBS samples and its possible effect on assay performance is described in 13 out of 68 reviewed reports (19%). Most of these papers describe punching from the center and the periphery of the spot, usually at low and high concentration level, and comparing the analysis results. None of the reviewed reports described the occurrence of inhomogeneity of DBS.

Spot-to-spot carry-over. Investigation of the spot-to-spot carry-over is only described in a single report [50].

Recovery. In a majority of the papers (61; 90%) the examination of the recovery of the analytes from the DBS is described, where most papers follow the FDA guidelines. For most assays, a high recovery (≥85%) is reported, however for a few methods the recovery is low (≤60%) [51–53]. For one of these assays, the recovery increased when the cellulose based card was treated with tartaric acid before spotting a blood sample on the card [53]. Only one report described the effect of haematocrit on recovery [23], none of the reports described the effect of aging on recovery.

Matrix effects. Matrix effects are studied in the majority of the 58 validated methods using mass spectrometry (48 out of 58; 83%). Yet, only in 15 out of 48 papers describing the matrix effects the EMA guidelines were followed, i.e. six batches of blank matrix from individual donors were examined. During most validations no matrix effect was observed, however ion suppression was observed in 4 reports [23,40,43,54] and ion enhancement in 2 reports [55,56]. For five assays using the coated Whatman DMPK-A cards the matrix effects were investigated, and one of these assays showed ion suppression [43]. Possible ion suppression caused by DMPK-A cards is previously described to be attributable to sodium dodecyl sulfate, one of the constituents of the

coating [22,24]. For one of the two assays using coated Whatman DMPK-B cards and where matrix effects were investigated, ion enhancement was reported [56].

Stability under transport and storage conditions. The long-term stability of analytes in DBS samples is described in most published assays (63 out of 68; 93%), with large variation in the extent to which this is investigated. Some papers only describe the stability for 2 days at room temperature, where other researchers mimic the possible situation where DBS samples are collected and/or transported at low or high temperature, analyzing DBS samples that were stored for at least 24 hours at a temperature from 2-8˚C to above 20˚C and for a period of several months at room temperature. Most analytes appeared to be stable in the DBS matrix when stored at room temperature for several months.

Sample dilution above the upper limit of quantitation. The dilution integrity is described in 18 papers (26%). Most researchers use the approach of diluting the sample final extract with blank final extract containing the internal standard. In two recent published methods [39,40] the 'internal standard tracked dilution method' of Liu et al. [27] is utilized. Two papers describe the dilution of final extract with ethyl acetate [31,57]. Other reports describe the use of a smaller punch from the DBS sample. The actual concentration of these samples is calculated using the area of the punches [38,56,58]. One of these reports takes the decreased amount of matrix into account by using the 'doughnut punch method'. With this method, a blank doughnut punch, a punch with the central part of the size of the small punch cut out, is added to the extraction solvent with the small sample punch [56]. In two articles it is described that a larger volume of extraction solvent was added to the punch [46,59]. In all papers examining the dilution integrity, analysis results were within the predefined limits.

Table 2 The inclusion of dried blood spot specific parameters in method validation

Year of publication	Spot volume	Haematocrit	Spot homogeneity	Spot-to-spot carry-over
2005, n=1	0 (0%)	0 (0%)	1 (100%)	0 (0%)
2006, n-1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2007, n=2	1 (50%)	0 (0%)	0 (0%)	0 (0%)
2008, n=2	1(50%)	0 (0%)	0 (0%)	0 (0%)
2009, n=7	3 (43%)	2 (29%)	1 (14%)	0 (0%)
2010, n=9	3 (33%)	2 (22%)	0 (0%)	0 (0%)
2011, n=13	6 (46%)	5 (38%)	4 (31%)	0 (0%)
2012, n=15	6 (40%)	7 (47%)	3 (20%)	0 (0%)
2013, n=13	6 (46%)	7 (54%)	1 (8%)	0 (0%)
2014*, n=5	4 (80%)	4 (80%)	3 (60%)	0 (0%)
Overall, n=68	**30 (44%)**	**27 (40%)**	**13 (19%)**	**1 (1%)**

* Until April 22nd.

Discussion

In the past decade, an increasing number of DBS-based assays has been validated and reported. A wide variability in the extensiveness of these validations is observed; the earliest reported DBS-based assay validations only cover the general bioanalytical validation parameters as described in the FDA and EMA guidelines, the more recently published validations also include some DBS specific parameters. Several review and recommendation papers describing DBS specific validation parameters are published in the literature, starting in 2007 [7], 2009 [8,12] and 2010 [9]. These first review and recommendation papers mainly describe the possible effect of the volume of blood spotted. This is in agreement with the finding that this is the DBS specific parameter most frequently included in validation reports (44%). The European Bioanalysis Forum published an extensive recommendation paper in 2011 [10], with an update and more in-depth research backing up these recommendations in 2013 [11]. In these reports, haematocrit is identified as the most important parameter possibly affecting DBS assay performance. This is in line with the finding that the recent papers more frequently included this parameter in the validation. In total, 40% of the validation reports included this parameter. The investigation of the homogeneity of DBS is mentioned in several recommendation papers [10–12], but in none of these papers is this issue addressed extensively. This lack of attention to the possible effects of non-homogenous DBS could possibly explain the low percentage of validation papers describing this issue (19%). Also, spot-to-spot carry-over is very marginally described in the publications; in only a single recommendation paper [10] and validation report [50].

It has become clear that the dried blood spot technology is promising and has multiple advantages, but it also has some challenges. The fast growing experience with this technology leads to a better understanding of some of the challenges, but also raises questions. The possible impact of blood volume, haematocrit and spot homogeneity on assay performance is known for many years, but the possible effect of aged samples on recovery is a more recently reported issue. Although recommendations for the development and validation of DBS methods are described quite extensively in the literature, there were 27 (40%) published DBS assays not describing any DBS specific validation parameter; spot volume, haematocrit, spot homogeneity and spot-to-spot carry-over, at all. The hurdles of DBS analysis are known to have an impact on assay performance, so it is highly recommended to implement these tests in the validation. However, since there is no regulatory-based guideline for the validation of DBS assays, it is the responsibility of each single researcher to look into the literature to find the different recommendation papers and come up with an adequate and complete

validation plan. In this situation, it is not surprising that not all reported DBS methods include a full validation according to the different published recommendation papers. Also, high variability in the extent to which different parameters are investigated is observed.

Recommendations for the validation of bioanalytical methods using dried blood spots

In this section, we aim to provide concrete recommendations for the validation of DBS-based assays, by incorporating and combining the knowledge that is gained by different research groups so far.
Upon arrival in the laboratory, visual inspection of the clinical DBS samples is recommended to ensure that i) the DBS is larger than the punch that will be taken out from the DBS, ii) the blood is symmetrically spread around the center and iii) there is an even dark red color on both sides of the DBS card.
For all parameters, acceptance criteria should be preset and should be in alignment with acceptance criteria applied in regulated bioanalysis, i.e. accuracy and precision should be within ±15%.

Spot volume
A spot volume range of at least 15 to 40 µL should be investigated during the validation. The different spotting volumes should be tested at a minimum of two concentration levels (low and high), in triplicate. In all cases, the spot volume for calibration standards and quality control samples should be equal to the expected spot volume of the clinical samples. When these tests show that the effect of spot volume falls within acceptable ranges, there is no need to use accurately spotted volumes for the clinical application. Conversely, when deviated analysis results are obtained, the source of this difference should be investigated. A possible cause for influence of spot volume on assay performance is a too small spot, i.e. not covering the whole punch. These DBS samples can be identified during the visual inspection upon arrival in the laboratory and should be excluded from analysis.

Haematocrit
The possible influence of different Hct values on analysis results is an important parameter in DBS assay performance and seems to be analyte- and card type

dependent. It is recommended to identify the expected range of Hct values in the target population, based on gender, age and possible medical conditions and co-medication. For a target population consisting of male and female adults without medical conditions affecting haematocrit, this range should be at least 30 to 55%, since the normal range is between 41 and 53% for men and between 36 and 46% for women [19]. However, when deviations of these values can be expected, for instance in oncology or anemic patients, a wider range should be investigated, starting from 20% Hct. Additionally, when an assay is intended to be used for neonates, higher Hct values should be validated, since their reference range is 42 to 65% [60].

The predefined range should be validated by preparing batches with different Hct values and spiking these batches at a minimum of two concentration levels (low and high). The spiked blood should be spotted in triplicate. When the target Hct range results in analyte concentrations outside the acceptable range, an adequate solution for this problem should be reported. For example, the measurement of Hct values in all patients, thus a correction of the analysis result can be made based on the individual haematocrit. This is not an ideal situation, since venous sampling is necessary to obtain the Hct value of a patient, which diminishes the large advantage of DBS sampling, being the easier sampling and option to self-sample at home. Recently, a method for the determination of Hct values in DBS samples is reported, where the Hct value can be predicted by potassium measurement [61]. This is a promising development and could be a good option for target populations with a large Hct range. A drawback of this procedure is the extra actions that are needed to extract and analyze the DBS sample to obtain the potassium level. Another approach is to try another DBS card type, since it is reported that different card types result in different impact of Hct [15].

Spot homogeneity

Possible differences between punches taken from the center or from the periphery of the spot should be investigated during DBS method development and validation, by preparing DBS at a minimum of two concentration levels (low and high) in triplicate, and punching from the center and the periphery of the DBS. When deviated analysis results are observed for the peripheral punches, a bigger puncher or another DBS card type could be tested in order to overcome this problem.

Spot-to-spot carry-over

Additional to the instrumental carry-over that is described in the EMA guidelines, spot-to-spot carry-over should be investigated by punching out two subsequent blank DBS after punching out an ULOQ sample. In alignment with the EMA guidelines, carry-over

should not be greater than 20% of the lower limit of quantification. Spot-to-spot carry-over caused by the punching device can possibly be eliminated by punching blank DBS cards between punching DBS samples. Punching through a clean DBS card was shown to be a faster and less labor-intensive cleaning procedure than cleaning the punching device with ethanol and subsequent drying of the device [62].

Extraction recovery

It is recommended to develop an assay where the extraction recovery of the analytes from the DBS cards is high (≥85%), constant and reproducible (CV <15%) since the internal standard, when present in the extraction solvent, will not compensate for variations in extraction recovery. The recovery should be investigated under the circumstances that are expected during the targeted (pre)clinical application (i.e. conditions and time of storage, expected Hct range). In alignment with the experiments required to determine the matrix effects, it is recommended to determine the recovery in at least six different batches, with different Hct values, at a minimum of two concentration levels (low and high). [11] Performing a recovery experiment for DBS is not straightforward; when a subpunch is used for the analysis, the exact volume of blood punched out with this subpunch is unknown. Therefore, for the recovery experiments we suggest to use an accurately pipette aliquot of whole blood that results in a spot slightly smaller than the size of the puncher that is used, so the whole spot is extracted, and to prepare and analyze three differently prepared samples: (A) DBS sample extracts at QC low and high level; (B) a neat solution at QC low and high level prepared in mobile phase, representing 100% recovery, and (C) blank DBS sample extracts reconstituted with neat solution B. The sample pretreatment recovery can be calculated as the detector response (analyte peak area) of A/C*100% [24].

Matrix effects

Matrix effects should be consistent and reproducible (CV <15%). Therefore, it is recommended that the matrix effects are determined in at least six different batches of whole blood, at a minimum of two concentration levels (low and high). [11] This experiment can be performed by preparing and analyzing two different samples: (A) a neat solution at QC low and high level prepared in mobile phase, representing 100% recovery, and (B) blank DBS sample extracts reconstituted with neat solution B. The matrix factor can be calculated as the detector response (analyte peak area) of B/A.

Stability under transport and storage conditions

There will be a wide variability in intended transport conditions and storage times between the assays, so the extensiveness of the stability experiments should be evaluated on a case-by-case basis. Of consideration are the temperature ranges in mailboxes, that can increase up to 20˚C above the outside temperature in summer [25]. In winter, temperatures in mailboxes can decrease to far below zero, depending on the geographic location. Also, temperatures in the cargo holding of airplanes can drop to at least -3˚C [26]. For the stability experiments, the same type of storage bags and desiccant packages as intended for the (pre)clinical application, should be used. The stability should be investigated at a minimum of two concentration levels (low and high), in triplicate.

Sample dilution above the upper limit of quantification

The dilution integrity should be investigated at concentration levels that are foreseen for the (pre)clinical application of the assay. Two main approaches on how to perform these experiments are published in the literature; dilute the final extract with blank final extract containing the internal standard [10] or process the sample using a multiplied concentration of internal standard, and diluting the final extract with blank final extract not containing the internal standard, called the internal standard track dilution method [27]. Either method can be used, as long as it is demonstrated that it does not affect the performance of the assay. Other approaches such as using a smaller spot or adding a larger volume of extraction solvent are not recommended, since for both these methods less matrix will be present in the extracts of these samples compared to regular samples. In alignment with the EMA guidelines, five determinations per dilution factor should be tested.

Incurred sample reanalysis

For the incurred sample reanalysis, a DBS sample can be obtained as a second punch from the same DBS as the initial sample, or from the second spot of the same sample [10,63]. It is recommended to reanalyze 10% of the (pre)clinical samples in case the number of samples is less than 1000 and 5% when the number of samples exceeds 1000. The analysis results obtained for the initial analysis and the reanalysis should be within 20% of their mean for at least 67% of the repeats [6].

Conclusions

In the last decade, 68 reports have been published describing the validation of bioanalytical methods for the quantitative determination of drugs in human dried blood spots. Large differences between these validations are observed. The earliest reported DBS-based assay validations only cover the general bioanalytical validation parameters mentioned by the FDA and the EMA, the more recently published validations also cover some of the aspects discussed in DBS recommendation articles. The parameters mainly described in these recommendation articles are spot homogeneity, influence of haematocrit and the influence of spot volume. The parameter that is most frequently included in the validation reports is spot volume (44%), followed by haematocrit effect (40%), spot homogeneity (19%) and spot-to-spot carry-over (1%). In the most recent recommendation papers the possible effect of aging of DBS and haematocrit of the spots on recovery is described, although these parameters are not yet incorporated in the validations performed. In as many as 40% of the published DBS assays not one DBS specific validation parameter was described.

Future perspectives

Dried blood spot sampling is a promising technology, having several advantages over conventional venous sampling. This type of sampling, that is shown to be suitable for a wide variability of compounds, can simplify and lower the costs of the logistics of (pre) clinical trials. Also, this method could make TDM more patient-friendly, especially for patients that are not admitted to the hospital, but who need to obtain a sample on a regular basis.

However, a number of pitfalls of this type of DBS analysis is described. Therefore, a complete validation where parameters mentioned in FDA and EMA guidelines on method validation are combined with DBS specific validation parameters as mentioned in this paper, should be executed. With this report we hope to encourage the field to more extensively investigate the different aspects of dried blood spot analysis, in order to support (pre)clinical studies with robust and reliable DBS-based assays.

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Determination of tamoxifen and endoxifen in dried blood spots using LC-MS/MS and the effect of coated DBS cards on recovery and matrix effects

1.4

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Abstract

Background. We developed an HPLC-MS/MS method to quantify tamoxifen (2.5-250 ng/mL) and its metabolite (*Z*)-endoxifen (0.5-50 ng/mL) in dried blood spots.

Results. Extraction recovery of both analytes from Whatman DMPK-A cards was 100% and consistent over time, however, recovery of (*Z*)-endoxifen from Whatman 903 cards was incomplete and increased upon storage. When SDS, a constituent of the DMPK-A coating, was present during the extraction, recovery improved. The method using DMPK-A cards was validated using bioanalytical guidelines. Additionally, influence of haematocrit (0.29-0.48 L/L), spot volume (20-50 µL) and homogeneity was within limits and both analytes were stable in DBS for at least four months.

Conclusion. The method for the quantification of tamoxifen and (*Z*)-endoxifen in DBS collected on DMPK-A cards was successfully validated.

Introduction

Tamoxifen is widely administered in the treatment of estrogen receptor (ER) positive breast cancer. It undergoes extensive metabolism leading to the formation of at least 22 phase I metabolites in humans [1,2]. These metabolites are primarily present in the *Z* (zusammen) form, since tamoxifen is administered as a pure *Z*-isomer [3]. There are large differences in the pharmacological activity between the metabolites, where *N*-desmethyl-4-hydroxytamoxifen (endoxifen) is suggested to be the most therapeutically active [2,4,5]. In vitro studies show that the anti-estrogenic effects of endoxifen are concentration dependent [4,6,7]. Also, a recent clinical trial with 1370 ER positive breast cancer patients showed a significant correlation between endoxifen serum levels and breast cancer outcome. The results of this study show that the patients who had an endoxifen serum concentration higher than 5.9 ng/mL had a 26% lower recurrence rate than patients with a lower endoxifen serum concentration. [8] These findings encourage optimization of tamoxifen treatment by individual dosing in order to reach target endoxifen levels. Current practice for (*Z*)-endoxifen quantification is by analysis of patient serum samples. In recent years, patient friendly, simple and cost-effective alternatives have been explored, of which dried blood spot (DBS) sampling appears to be a good option. With DBS sampling, patients can easily self-sample at home and the samples can be sent to the laboratory by regular mail service, since no special conditions for transport and storage of DBS samples are required. [9,10]
This report presents the development and validation of a method to quantify tamoxifen and its active metabolite (*Z*)-endoxifen in dried blood spots using high performance liquid chromatography (HPLC) with MS detection. The validation was performed based on FDA and EMA guidelines [11,12] and EBF recommendations [13,14].

Materials and methods

Chemicals

Tamoxifen, tamoxifen-*d5*, *N*-desmethyl-4-hydroxytamoxifen (endoxifen, 1:1 *E*/*Z* mixture) and *N*-desmethyl-4-hydroxytamoxifen-*d5* (endoxifen-*d5*, 1:1 *E*/*Z* mixture) were purchased from Toronto Research Chemicals (North York, ON, Canada). Acetonitrile and methanol (both HPLC grade) were obtained from Biosolve BV (Valkenswaard, the Netherlands). Ammonium formate and Trizma® hydrochloride (tris, trometamol) were purchased from Sigma-Aldrich BV (St. Louis, USA). Formic acid, sodium dodecyl

sulfate (SDS) and water for chromatography (Lichrosolv) were purchased from Merck (Darmstadt, Germany).

Materials

For collection of DBS, pure cellulose based cards (Whatman 903 Protein Saver cards), Whatman FTATM DMPK-A DBS cards (cellulose with a coating containing 3-5% SDS and 1-5% trometamol (tris(hydroxymethyl)aminomethane) [15]), Whatman foil bags and desiccant packages for storage of DBS samples were purchased from GE Healthcare (Buckinghamshire, UK). Freshly drawn drug-free whole blood with EDTA as anticoagulant was obtained from healthy volunteers in the Slotervaart Hospital (Amsterdam, the Netherlands) using 3 and 10 mL BD Vacutainer® EDTA sampling tubes provided by Becton, Dickinson and Company (Plymouth, UK).

Preparation of calibration standards and QC samples

The isomeric (*E*/*Z*) ratio of the reference standard of endoxifen was determined by liquid chromatography with ultraviolet absorption detection and found to be 1.0. Separate stock solutions of each analyte were prepared from independent weightings in methanol at a concentration of 2 mg/mL tamoxifen and 2 mg/mL endoxifen (corresponding to 1 mg/mL (*E*)-endoxifen and 1 mg/mL (*Z*)-endoxifen). The stock solutions were diluted with acetonitrile to combined working solutions. One set of working solutions was used for the preparation of calibration standards (CALs), the other set was used for the preparation of quality control (QC) samples. For the internal standards tamoxifen-*d5* and endoxifen-*d5* stock solutions of 1 mg/mL were prepared in methanol. All stock and working solutions were stored at -70°C.

Calibrations standards were prepared by diluting a volume of 10 µL working solution containing both analytes with 2.0 mL whole blood. The CALs contained (*E*)- and (*Z*)-endoxifen in a range of 0.5 to 50 ng/mL and tamoxifen in a range of 2.5 to 250 ng/mL. In a similar way, QC samples at four concentrations (lower limit of quantification (LLOQ), low, mid and high level) were prepared from an independent set of working solutions. The QCs contained 0.50, 1.4, 5.6 and 40 ng/mL (*E*)- and (*Z*)-endoxifen and 2.5, 7.0, 28 and 200 ng/mL tamoxifen. For Whatman 903 cards, 40 µL spiked whole blood was spotted and for Whatman DMPK-A cards 30 µL spiked whole blood was spotted, unless stated otherwise.

A mixture of internal standard stock solutions was prepared and diluted with methanol to obtain the internal standard solution, containing 5.0 ng/mL tamoxifen-*d5* and 2.5 ng/mL endoxifen-*d5* (corresponding to 1.25 ng/mL (*E*)-endoxifen-*d5* and 1.25 ng/mL (*Z*)-endoxifen-*d5*). The internal standard solution was stored at -20°C.

Sample pretreatment

DBS were prepared from freshly drawn whole blood, harvested the same day, and allowed to dry overnight. A 6 mm subpunch was taken out of the dried blood spot and placed in an amber colored polypropylene Eppendorf tube (2.0 mL capacity). For the extraction of the analytes from the DBS, a 200 µL aliquot of methanol containing tamoxifen-*d5* and endoxifen-*d5* was added to the DBS and the tube was vortexed and sonicated for 45 minutes. Subsequently, the sample mixture was centrifuged for 10 minutes at 10,500 g and 140 µL of the supernatant was transferred to another tube. The extract was dried under a gentle stream of nitrogen (30˚C) and the dried extract was dissolved in 120 µL ACN-4 mM ammonium formate buffer solution pH 3.5 (3:7, *v/v*). The final extract was transferred to an amber colored autosampler vial and 10 µL was injected onto the HPLC column.

LC-MS/MS

An LC-MS/MS method previously reported by our laboratory [16,17] was used for the quantification of tamoxifen and endoxifen in DBS. Positive electrospray ionization was applied and the precursor/product transition was set at *m/z* 372 ->129 for tamoxifen and for (*Z*)-endoxifen *m/z* 374 -> 58 was measured.

Validation of the assay for the quantification of tamoxifen and (*Z*)-endoxifen in DBS

Assay validation was performed according to the FDA and EMA guidelines for validation of bio-analytical assays [11,12]. Additionally, experiments were conducted to determine the effects of volume of blood used to prepare the DBS, the effects of different haematocrit values and the effects of DBS homogeneity on the accuracy and precision of the method, according to EBF recommendations [13,14].

Results and discussion

Development

Development of the DBS sample pretreatment method

During method development, two different types of commonly used DBS cards; Whatman 903 and Whatman DMPK-A, were tested in combination with 200 µL of the following extraction solvents: 100% methanol, 100% acetonitrile, methanol:water (1:1, *v/v*), acetonitrile:water (1:1, *v/v*) and methanol:acetonitrile (1:1, *v/v*).

The detector response obtained with the different extraction solvents, normalized to

the response obtained when extracting with 100% methanol, is depicted in Figure 1. Either 100% methanol or a 1:1 (*v/v*) mixture of methanol and acetonitrile resulted in the highest detector response and lowest standard deviation. Since extraction with methanol yielded the highest response for (*Z*)-endoxifen and this analyte is present in much lower concentrations in patient samples than tamoxifen and thus a lower LLOQ is required, 100% methanol was selected as the extraction solvent for this assay. Furthermore, the effect of the sonication time of the sample mixture on the extraction of tamoxifen and (*Z*)-endoxifen was investigated, by sonicating the sample mixtures for 15, 30 and 45 minutes. Sonication for 45 minutes resulted in the highest recovery and the lowest standard deviation.

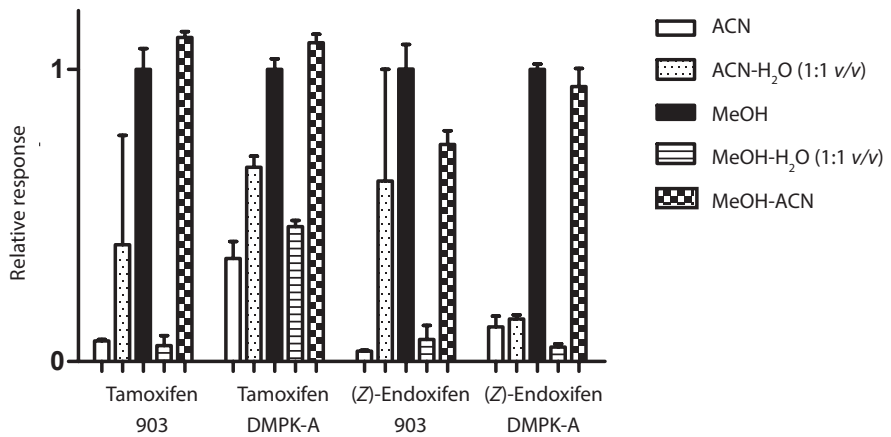

Figure 1 Detector response, normalized to the response obtained when extracting with methanol, obtained after extraction of tamoxifen and (*Z*)-endoxifen from Whatman 903 and Whatman DMPK-A cards (n=3), using different extraction solvents.
ACN: acetonitrile, MeOH: methanol. Error bars indicate the standard deviation.

Recovery and matrix factors using different types of DBS cards

The sample pretreatment recovery of tamoxifen and (*Z*)-endoxifen from Whatman 903 and Whatman DMPK-A cards was investigated. Five replicates of three differently prepared samples were analyzed: (A) DBS sample extracts at QC mid level; (B) a neat solution at QC mid level prepared in mobile phase, representing 100% recovery, and (C) blank DBS sample extracts reconstituted with neat solution B. Aliquots of 20 µL whole blood were used to prepare the DBS samples which resulted in DBS slightly bigger than the 6 mm subpunch, however we choose this volume since we were able to pipette reproducible spots using this volume. The whole spot was cut out manually.
The sample pretreatment recovery was calculated as the detector response (analyte

peak area) of (A/C)*100%. For tamoxifen, the sample pretreatment recovery from both card types was around 100%. For (*Z*)-endoxifen, the sample pretreatment recovery was around 100% when using the DMPK-A cards, but when using 903 cards only 43% was recovered.
The matrix factor was calculated as the detector response (analyte peak area) of C/B. The absolute matrix factor was 1.0 for both analytes when using Whatman 903 cards, but substantially lower when DMPK-A cards were used; around 0.7 for tamoxifen and around 0.6 for (*Z*)-endoxifen.

Influence of DMPK-A coating on extraction recovery and matrix factors

To assess the influence of the substituents of the coating of DMPK-A cards (3-5% *w/w* SDS and 1-5% *w/w* trometamol) on the recovery and matrix factor of the analytes, SDS, trometamol or both chemicals were added to the extraction solvent. Additionally, the effect of a physical coating was assessed by manual application of the DMPK-A coating on the 903 cards.
To calculate the amounts of SDS and trometamol for this experiment, a piece of a Whatman 903 card (without sample) of the size of a 20 µL DBS was cut out in triplicate and determined to be 26.6 mg. Therefore 0.78 – 1.33 mg SDS and 0.27 – 1.33 mg trometamol per spot was needed to mimic the DMPK-A coating.
For the investigation of the influence of the presence of DMPK-A coating substituents, replicates of three differently prepared samples were analyzed; (A) DBS sample extracts at QC mid level from Whatman 903 cards; (B) a neat solution at QC mid level prepared in mobile phase, representing 100% recovery, and (C) blank DBS sample extracts from Whatman 903 cards reconstituted with neat solution B. Aliquots of 20 µL whole blood were used to prepare the DBS samples and the whole spot was cut out manually.
The extraction of the spots prepared at (A) and (C) was carried out with 200 µL SDS in methanol (n=5, 1.06 mg absolute, resembling 4% *w/w* on the card), 200 µL trometamol in methanol (n=5, 0.80 mg absolute, resembling 3% *w/w* on the card) or 200 µL SDS and trometamol in methanol (n=5). The sample pretreatment recovery and matrix factor were determined as described before.
To investigate the effect of a physical coating on recovery and matrix effects, this coating was mimicked on Whatman 903 cards. Therefore, 1.06 mg SDS (20 µL of a solution containing 53 mg/mL in water, resembling 4% *w/w* on the card) and 0.80 mg trometamol (20 µL of a solution containing 40 mg/mL in water, resembling 3% *w/w* on the card) were spotted on a Whatman 903 card separately or as a mixture. The cards were allowed to dry for 24 hours before blood samples were spotted on the cards. Replicates of three differently prepared samples were analyzed; (A) DBS sample extracts

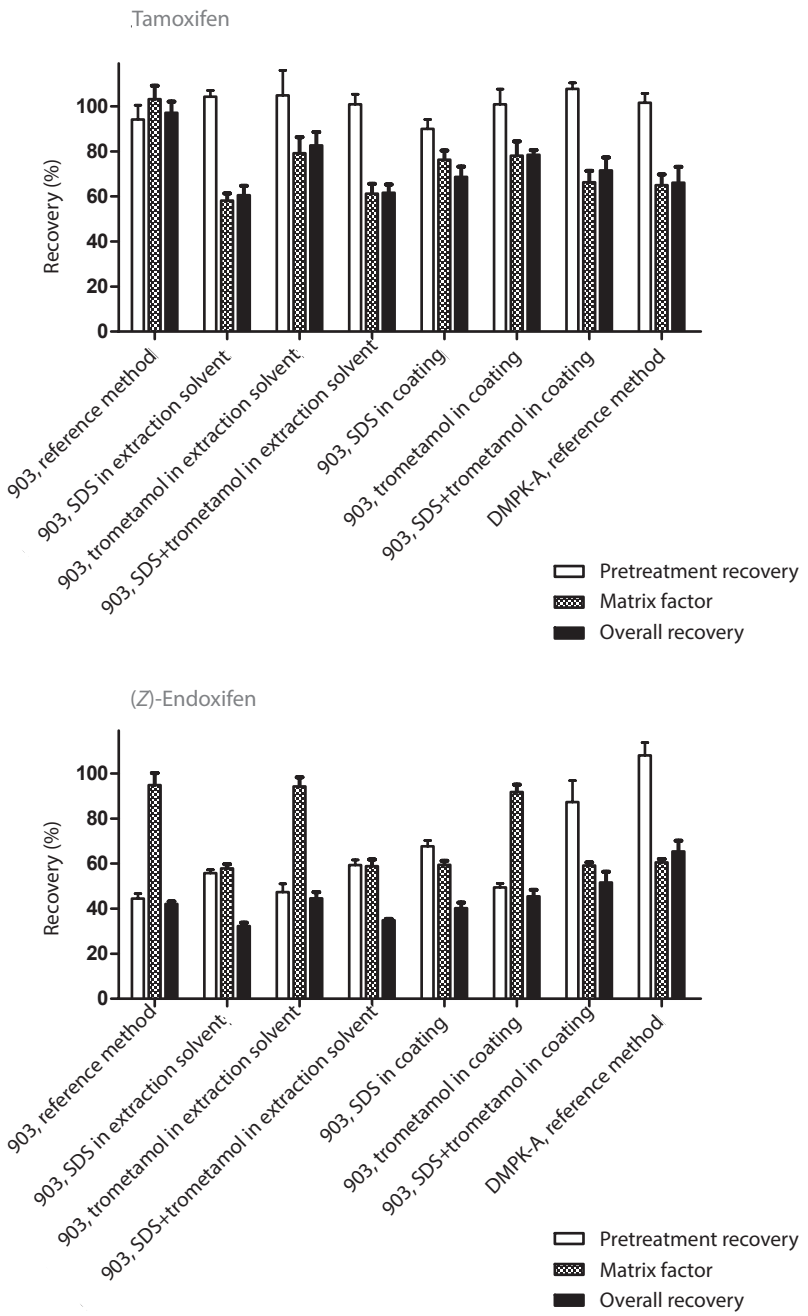

Figure 2 Sample pretreatment recovery, matrix factor and overall recovery for both analytes on the conventional Whatman 903 card, extracted with pure methanol and pure methanol spiked with either SDS, trometamol or both and the recovery from the coated cards; the manually impregnated 903 cards and the DMPK-A cards (n=5). Error bars indicate the standard deviation.

at QC mid level from Whatman 903 cards coated with SDS (n=5), trometamol (n=5) or both (n=5); (B) a neat solution at QC mid level prepared in mobile phase, representing 100% recovery, and (C) blank DBS sample extracts from Whatman 903 cards coated with SDS (n=5), trometamol (n=5) or both (n=5), reconstituted with neat solution B. Aliquots of 20 µL whole blood were used to prepare the DBS samples and the whole spot was cut out manually. Extraction was performed using pure methanol, as described in the sample pretreatment. The recovery of the sample pretreatment and matrix factor were determined as described before.

Figure 2 shows the results of these experiments. It is shown that for tamoxifen, the sample pretreatment recovery is above 90% for all cards and extraction methods used. The sample pretreatment recovery of (*Z*)-endoxifen from 903 cards is low, but increases when SDS is added to the extraction solvent, either alone or in combination with trometamol. Trometamol alone does not seem to influence the pretreatment recovery. Presumably SDS, a surfactant, enhances the solubility of (*Z*)-endoxifen in the extraction solvent and therefore the presence of SDS during the extraction increases the sample pretreatment recovery of (*Z*)-endoxifen.

When the DMPK-A coating was manually applied to 903 cards, a further increase of the sample pretreatment recovery of (*Z*)-endoxifen was observed, especially when SDS was present in the coating. A possible explanation for this further increase in extraction recovery could be the function of the coating as a physical barrier between (*Z*)-endoxifen and the DBS card, weakening the bond between the analyte and the cellulose 903 card. The sample pretreatment recovery of (*Z*)-endoxifen was shown to be the most optimal when DMPK-A cards are used.

The absolute matrix factor was around 1.0 for both tamoxifen and (*Z*)-endoxifen when using the 903 cards, but decreased when SDS was present, either in the extraction solvent or in the coating. This ionization suppression caused by SDS is also reported for other compounds [18]. The IS normalized matrix factor was between 0.972 and 1.03 for both analytes and all performed tests, indicating that the matrix effect is identical for the analyte and its internal standard. This demonstrates that the stable isotope labeled standards were most effective to compensate for the observed matrix effects.

Impact of card storage on extraction recovery from Whatman 903 and DMPK-A cards

The impact of card storage on the extraction recovery of tamoxifen and (*Z*)-endoxifen from both card types was investigated. For this purpose, four replicates of three differently prepared samples were analyzed at 4 time points; (A) DBS sample extracts at QC mid level from Whatman 903 and DMPK-A cards, (B) a neat solution at QC mid level prepared in mobile phase, representing 100% recovery, and (C) blank DBS sample

extracts from Whatman 903 and DMPK-A, reconstituted with neat solution B. Aliquots of 20 µL whole blood were used to prepare the DBS samples and the whole spot was cut out manually. The DBS samples were stored at room temperature in an aluminum bag containing a desiccant package. The extraction recovery was determined after 0, 1, 1.5 and 3 months. The extraction recovery was calculated as the normalized detector response (area ratio) of (A/C)*100%.

The results are depicted in Figure 3. No change in extraction recovery during the storage period was observed for tamoxifen on either of the DBS cards. For (*Z*)-endoxifen, the extraction recovery from the DMPK-A cards did not change upon storage. However, the extraction recovery from the 903 cards increased from 50 to 80% after three months. Changes in extraction recovery upon storage are also mentioned in the EBF white paper [14] and demonstrated in a recent paper, however in this paper the extraction recovery decreased over time [19]. A possible explanation for the increase in recovery observed in our experiments might be a change in binding strength of (*Z*)-endoxifen to the cellulose 903 card upon aging of the DBS. The high and consistent extraction recovery of tamoxifen and (*Z*)-endoxifen from DMPK-A cards indicates that the analytes are stable in the DMPK-A DBS matrix and that the extraction process is consistent in time.

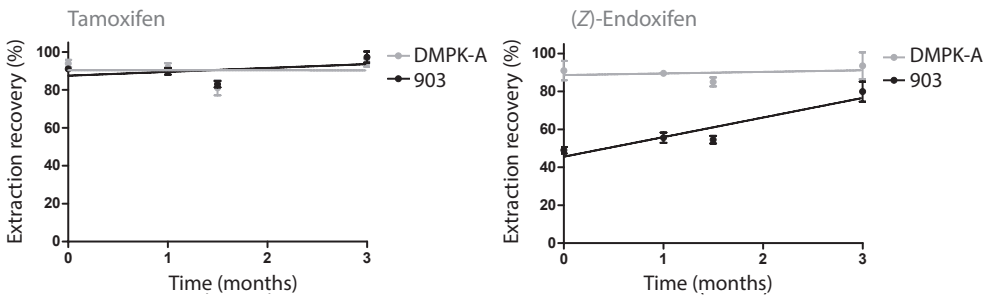

Figure 3 Extraction recovery of tamoxifen and (*Z*)-endoxifen from Whatman 903 and DMPK-A cards upon storage in an aluminum bag with desiccant at room temperature for up to 3 months (n=4)

Validation of the assay for the quantification of tamoxifen and (*Z*)-endoxifen in DBS on DMPK-A cards

Calibration model

The calibration model was assessed by preparing and analyzing two sets of 8 calibration standards, on three separate days. For tamoxifen, the calibration model was quadratic when using *m/z* 372 -> 72. In order to obtain linear calibration models, a suboptimal mass transition was determined at *m/z* 372 ->129. With this mass transition, the detector response was substantially lower, however, linear calibration models were obtained.

For both analytes, calibration curves of analyte/internal standard peak area ratio versus the nominal concentration of tamoxifen and (*Z*)-endoxifen in DBS were constructed and linear regression with a weighting factor of $1/x^2$ was applied to the data. Linear responses were observed for tamoxifen in a range of 2.5 – 250 ng/mL, represented by a correlation coefficient (R^2) of ≥ 0.9981 for all three runs. Linear responses for (*Z*)-endoxifen were observed in a range of 0.50 – 50 ng/mL, with an R^2 of ≥ 0.9963 for all three runs.

Accuracy and precision

The accuracy and precision of the method were determined by assaying five replicates of freshly prepared and processed QC samples at the LLOQ, low, mid and high concentration level in three separate runs.
Intra-assay and inter-assay accuracies were within 86.4 and 103% for tamoxifen and within 87.7 and 108% for (*Z*)-endoxifen. Precisions were less than 12.9% for tamoxifen and less than 9.44% for (*Z*)-endoxifen and were therefore found to be acceptable.

Dilution integrity

The ability to dilute samples above the upper limit of quantification (ULOQ) was demonstrated by preparing five replicates of DBS containing 10 times the concentration of the high QC sample. These DBS were processed as described in the sample pretreatment section. The final extract was diluted 10-fold with the final extract of processed blank DBS containing the internal standard [13,20]. The intra-assay bias and within-run precision were -5.47% and 1.83% for tamoxifen and 3.88% and 1.83% for (*Z*)-endoxifen, respectively.

Specificity and selectivity

To determine whether endogenous constituents interfere with the assay, a double blank and a sample spiked at the LLOQ level were prepared for six different batches of whole blood, obtained from six healthy donors. The DBS samples were allowed to dry overnight and processed and analyzed according to the procedure described before.
For the LLOQ samples, the accuracies were within ±20% for both analytes in all six batches. No interferences with an analyte area above 20% of the peak area of the LLOQ samples and/or above 5% of the internal standard peak area were observed in any of the six blank DBS samples for both analytes and internal standards.

Cross analyte interference

To investigate possible cross interference between the analytes and internal standards,

DBS samples with either tamoxifen, tamoxifen-*d5*, endoxifen or endoxifen-*d5* at ULOQ level were prepared and processed without addition of internal standards. No interferences with an analyte area above 20% of the peak area of the LLOQ samples and/or above 5% of the internal standard peak area were observed in any of the cross analyte interference DBS samples.

Carry-over

Two types of possible carry-over were evaluated; carry-over caused by the HPLC-MS instrumentation and carry-over originating from the punching device [13]. Instrumental carry-over was tested by the injection of two double blank processed DBS samples, without internal standard, sequentially directly after the injection of a processed ULOQ sample. Analysis of the two double blank final extracts resulted in a response of ≤6% of the mean response of an LLOQ sample for both analytes and below 1% of the mean response of the internal standards. The punching device carry-over was examined by punching two double blank DBS out of the card directly after punching out a DBS at ULOQ level and processing and analyzing the samples according to the method described in the sample pretreatment section. Analysis of the double blank samples resulted in a response of ≤8% of the mean response of an LLOQ sample for both analytes, and below 1% of the mean response of the internal standards.
Since all double blank samples resulted in a response less than 20% of the mean response of a LLOQ sample and less than 5% of the mean response of the internal standard, the carry-over was found to be acceptable.

Influence of spotted blood volume

Aliquots of 20 µL and 50 µL spiked whole blood at QC low and QC high level were spotted in triplicate. The DBS were processed and analyzed as described in the sample pretreatment section and quantified on the calibration curve obtained with DBS prepared by spotting 30 µL of whole blood.
For tamoxifen, the intra-assay bias and precision were within ±8.71% and ±7.25% for the 20 µL samples and within ±11.2% and ±2.65% for the 50 µL samples, respectively. For (*Z*)-endoxifen, the intra-assay bias and precision were within ±2.88% and ±12.1% for the 20 µL samples and within ±6.71% and ±7.76% for the 50 µL samples, respectively. These results indicate that a spotted blood volume between 20 and 50 µL does not result in a notably different concentration of either of the analytes when quantified on a calibration curve derived from 30 µL DBS. The majority of reports describing the investigation of the effect of spot volume on assay performance also report that spotting accurate volumes is not necessary to obtain acceptable results [13,20,21], in line with our findings.

Dried blood spot homogeneity

The spot homogeneity was evaluated by punching out the disc from the periphery of the DBS. Blood spots at QC low and QC high level were prepared in triplicate. The obtained DBS were processed and analyzed as described in the sample pretreatment section.

For tamoxifen, the intra-assay bias and precision were -7.65% and 4.93%, for the QC low sample and -5.70% and 3.55% for the QC high sample, respectively. For (*Z*)-endoxifen, the intra-assay bias and precision were -9.59% and 3.59%, for the QC low sample and 8.79% and 3.84% for the QC high sample, respectively. These results indicate that punching from the middle or the periphery of the spot does not result in a notably different concentration of either of the analytes and therefore it can be concluded that the dried blood spots were homogenous.

Influence of the haematocrit value

Haematocrit (Hct), the relative volume of red blood cells in whole blood, can influence the viscosity and thus spreadability of whole blood on DBS cards. Generally, this results in the formation of smaller spots when Hct is high and larger spots when Hct is low, possibly leading to deviations in analysis results when a fixed diameter subpunch of the DBS is used. However, the extent to which this influences assay performance is dependent on the analyte and DBS card type used. [9,10,14,19,21–23] Hct is usually between 0.41 and 0.53 L/L for males and between 0.36 and 0.46 L/L for women [24]. However, the Hct value can deviate from these values in special populations, for example in neonates and patients with physiological conditions such as anemia, cancer or renal impairment, or patients under medical treatment affecting the haematocrit. The target population for the application of this assay consists of adult females, cured from breast cancer and receiving adjuvant tamoxifen treatment. This results in a homogenous group of patients, since they are all adult females and physiological conditions having a major effect on Hct values are not expected. Therefore, the target population is assumed to have an Hct range between 0.36 and 0.46 L/L.

In order to examine the effect of Hct values on the quantification of tamoxifen and (*Z*)-endoxifen in dried blood spots, five whole blood batches with various levels of Hct, resulting in a clinically relevant range for our target population, were prepared. The batches with different Hct values were created by centrifuging a batch of fresh whole blood, separating the blood cell compartment from the plasma compartment and combining these compartments in different quantities. This resulted in Hct levels of 0.29, 0.34, 0.38, 0.42 and 0.48 L/L, analyzed using an automated haematology analyzer Cell-Dyn 4000 (Abbott Laboratories, Abbott Park, IL, USA). The obtained batches of blood

with different Hct values were spiked with tamoxifen and (*Z*)-endoxifen at QC low and QC high level. DBS samples prepared from the spiked blood (n=4 per concentration level) were processed and analyzed as described before and quantified on a calibration curve with Hct 0.41 L/L.

The results, shown in Table 1, show a trend that low Hct values lead to a lower measured concentration, probably due to an increased spreadability of the blood. However, for all tested Hct values the bias was within 12.2% for both analytes at both concentration levels. These results indicate that adequate concentration levels for both analytes can be measured in DBS with Hct values above 29%.

Table 1 Influence of haematocrit on the precision and accuracy at QC low and QC high level (n=4)

Analyte	Haematocrit (L/L)	Nominal conc. (ng/mL)	Mean conc. (ng/mL)	Accuracy (% bias)	Precision (% CV)
Tamoxifen	0.29	7.00	7.41	6.35	4.68
	0.34	7.00	7.21	3.37	4.80
	0.38	7.00	7.51	7.71	1.60
	0.42	7.00	7.46	7.03	5.38
	0.48	7.00	7.36	5.52	6.07
	0.29	200	188	-5.78	2.37
	0.34	200	183	-8.17	0.935
	0.38	200	209	4.77	2.02
	0.42	200	206	3.52	1.98
	0.48	200	214	7.66	2.30
(*Z*)-Endoxifen	0.29	1.40	1.32	-5.40	4.41
	0.34	1.40	1.40	0.540	3.80
	0.38	1.40	1.47	5.76	4.34
	0.42	1.40	1.44	3.60	5.44
	0.48	1.40	1.53	10.1	4.86
	0.29	40.0	35.8	-10.1	1.42
	0.34	40.0	34.9	-12.2	1.66
	0.38	40.0	39.4	-0.817	2.28
	0.42	40.0	38.5	-3.39	2.90
	0.48	40.0	40.4	1.51	1.07

conc.: concentration, CV: coefficient of variation.

Recovery and matrix factor

Six batches of whole blood obtained from six different healthy donors were used to prepare three different types of samples; (A) DBS sample extracts at QC low (n=6) and QC high level (n=6); (B) neat solutions at QC low and QC high level prepared in mobile phase, representing 100% recovery, and (C) blank DBS sample extracts reconstituted with neat solutions B, resulting in extracts at QC low (n=6) and extracts at QC high level (n=6). Aliquots of 20 µL whole blood were used to prepare the DBS samples and the

whole spot was cut out manually. The sample pretreatment recovery was calculated as the detector response (analyte peak area) of A/C*100%, the matrix factor was calculated as the detector response of C/B. Table 2 shows the sample pretreatment recovery and matrix factor data obtained for both analytes at two concentration levels. The sample pretreatment recovery for both tamoxifen and (*Z*)-endoxifen was above 90%. The absolute matrix factor was 0.4 for (*Z*)-endoxifen and 0.8 for tamoxifen, the relative matrix factor was around 1.0 for both analytes. Coefficients of variation were below 15% for both analytes. Consistent recovery values at low and high concentration levels indicate that the extraction process is acceptable across this concentration range. Also, these results show that both analytes are fully recovered from the DBS matrix.

Table 2 Recovery and matrix factor data for tamoxifen and (*Z*)-endoxifen at two concentration levels (n=6)

Analyte	Nominal conc. (ng/mL)	Sample pretreatment recovery (%)	Absolute matrix factor	Relative matrix factor
Tamoxifen	7.00	102	0.785	1.03
	2.00	91.2	0.768	1.02
(*Z*)-Endoxifen	1.40	96.6	0.386	0.928
	40.0	93.9	0.450	1.06

conc.: concentration, CV: coefficient of variation.

Stability of the analytes in dried blood spot samples

For the clinical application of this method, the DBS samples will be sent by regular mail service and stored at the laboratory. Therefore, the stability is investigated at room temperature, and also for 24 hours at low (2-8˚C) and high (37˚C) temperatures in order to mimic possible transport conditions. DBS were prepared at QC low and QC high level according to the regular procedure and allowed to dry overnight. The next day, DBS cards were placed in a sealed aluminum bag with a desiccant package and stored at room temperature, 2-8˚C or 37˚C. The 2-8˚C and 37˚C samples were processed in triplicate and analyzed together with a freshly prepared calibration curve after 24 hours of storage, the room temperature samples after four months of storage. For the samples stored at 2-8˚C for 24 hours, the intra-assay bias and precision was 0.861% and 6.16% for tamoxifen at low concentration level and 7.04% and 1.41% at high concentration level. For (*Z*)-endoxifen, the intra-assay bias and precision was 1.20% and 3.65% at low concentration level and 2.01% and 2.19% at high concentration level. For the samples stored at 37˚C for 24 hours, the intra-assay bias and precision were -0.622% and 4.71% for tamoxifen at low concentration level and 0.00% and 1.33% at high concentration level. For (*Z*)-endoxifen, the intra-assay bias and precision were 9.83% and 6.23% for (*Z*)-

endoxifen at low concentration level and -1.01% and 3.55% at high concentration level. Storage for 4 months at room temperature resulted in an intra-assay bias and precision of 2.58% and 1.78%, for the tamoxifen QC low sample and -12.4% and 2.59% for the tamoxifen QC high sample, respectively. For *(Z)*-endoxifen, the intra-assay bias and precision were 1.44% and 7.20% for the QC low sample and 4.69% and 6.12% for the QC high sample, respectively. Both analytes were proven to be stable in the DBS matrix for at least 24 hours at 2-8˚C or 37˚C and four months at room temperature, when stored in an aluminum bag containing a desiccant package.

Application of the method

The applicability of the method was demonstrated by the analysis of steady-state DBS samples of eight patients receiving 20 mg tamoxifen per day, according to standard of care. The measured concentrations were in a range of 73.6 to 120 ng/mL for tamoxifen and 3.71 to 12.2 ng/mL for (*Z*)-endoxifen. These concentrations were all well within the validated range of the developed assay.

Conclusions

The method developed for the quantification of tamoxifen and its active metabolite (*Z*)-endoxifen in DBS collected on Whatman DMPK-A cards was successfully validated according to FDA and EMA guidelines and EBF recommendations. Whatman 903 cards were shown to be unsuitable for this application, due to an increasing extraction recovery over time for (*Z*)-endoxifen. We demonstrated that the difference in extraction recovery between the two types of DBS cards is mainly attributable to the presence of SDS, one of the constituents of the DMPK-A coating. Also, the presence of a physical coating further increased the extraction recovery of (*Z*)-endoxifen. Although SDS caused matrix effects, the stable isotope labeled internal standards were most effective to compensate for these effects. The DBS samples were shown to be stable when stored at room temperature for at least four months, indicating that this method is suitable for the support of clinical trials or therapeutic drug monitoring. Also, the analytes were stable in DBS at the temperature range expected during transport, 2-8˚C to 37˚C, for 24 hours, indicating that the DBS samples can be sent by regular mail service.

Future perspectives

In the last decade, numerous DBS assays were validated and implemented in clinical practice. However, the knowledge about DBS is limited and the pitfalls of this type of analysis are not fully understood. Also, there is no consensus guideline for the validation of DBS methods. With this report, we hope to encourage the field to more extensively investigate the different aspects of dried blood spot analysis. Also, we postulate the need for a specific guideline for the validation of DBS analyses.

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Clinical application of the bioanalysis of tamoxifen and its metabolites

2

Tamoxifen dose and serum concentrations of tamoxifen and six of its metabolites in routine clinical outpatient care

2.1

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Abstract

Background. A sensitive and selective HPLC-MS/MS assay was used to analyze steady-state serum concentrations of tamoxifen, N-desmethyltamoxifen, (*E*)-endoxifen, (*Z*)-endoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen to support therapeutic drug monitoring (TDM) in patients treated with tamoxifen according to standard of care.

Methods. When the (*Z*)-endoxifen serum concentration was below the predefined therapeutic threshold concentration of 5.9 ng/mL, the clinician was advised to increase the tamoxifen dose and to collect another serum sample. Paired serum samples from patients at one dose level at different time points during the tamoxifen treatment were used to assess the intra-patient variability.

Results. A total of 251 serum samples was analyzed, obtained from 205 patients. Of these patients, 197 used 20 mg tamoxifen per day and 8 patients used 10 mg per day. There was wide variability in tamoxifen and metabolite concentrations within the dosing groups. The threshold concentration for (*Z*)-endoxifen was reached in one patient (12%) in the 10 mg group, in 153 patients (78%) in the 20 mg group and in 26 (96%) of the patients who received a dose increase to 30 or 40 mg per day. Dose increase from 20 mg to 30 or 40 mg per day resulted in a significant increase in the mean serum concentrations of all analytes ($p<0.001$). The mean intra-patient variability was between 10 and 20% for all analytes.

Conclusions. These results support the suitability of TDM for optimizing tamoxifen treatment. It is shown that tamoxifen dose is related to (*Z*)-endoxifen exposure and increasing this dose leads to a higher serum concentration of tamoxifen and its metabolites. The low intra-patient variability suggests that only one serum sample is needed for TDM, making this a relatively non-invasive way to optimize the patient's treatment.

Introduction

Tamoxifen, a selective estrogen receptor modulator, is widely administered in the treatment of estrogen receptor (ER) positive breast cancer, which accounts for ~70% of all breast cancers. It has been used for over 40 years and has dramatically reduced the recurrence and mortality rates of ER positive breast cancer patients. Unfortunately, not all patients benefit from tamoxifen as in about one third of the patients the disease recurs despite adjuvant treatment with tamoxifen. [1–3]

There is great interest in identifying predictive factors associated with tamoxifen efficacy [4]. However, underlying mechanisms of the variable response to tamoxifen treatment remain unclear. Early attempts to link the clinical outcome of tamoxifen treatment to tamoxifen serum concentrations did not identify a significant advantage for the patients with higher tamoxifen serum levels. [5] Davies et al. [3] reported a significant test for trend for recurrence reduction with higher tamoxifen doses, however, studies that investigated whether a dose of 30 or 40 mg tamoxifen per day for an unselected group of ER positive breast cancer patients would lead to an improved survival compared to the standard dose of 20 mg per day did not give significant results. [3,5,6]

It is now known that tamoxifen undergoes extensive metabolism, especially by Cytochrome P450 (CYP) enzymes, leading to the formation of at least 22 phase I metabolites in humans, some of which are much more active than tamoxifen itself. [7,8] The major metabolic pathway involves demethylation of tamoxifen, mainly by CYP3A4/5, to N-desmethyltamoxifen, which is next hydroxylated by CYP2D6 to form *N*-desmethyl-4-hydroxytamoxifen (endoxifen). To a smaller extent tamoxifen is hydroxylated, mainly by CYP2D6, to form 4-hydroxytamoxifen, which is subsequently demethylated by CYP3A4/5 to form endoxifen. The biotransformation of tamoxifen is depicted in Figure 1. Tamoxifen is administered as a pure *Z* (zusammen) isomer [9] and the metabolites formed are primarily in the *Z*-form. To a much smaller extent, *E* (entgegen) isomers are formed. For some of the tamoxifen metabolites, especially *N*-desmethyltamoxifen, (*Z*)-endoxifen and 4-hydroxytamoxifen, the pharmacological effects are investigated, whereas for other metabolites no data or only uniform activity tests are reported in the literature. There are large differences in the pharmacological activity of the tamoxifen metabolites, whereby the *E*-isomers seem to be much less potent than their *Z*-isomers. [10–13] (*Z*)-endoxifen and 4-hydroxytamoxifen have a comparable anti-estrogenic activity, being 30 to 100-fold more potent towards the estrogen receptor than *N*-desmethyltamoxifen and tamoxifen itself. [14,15] Recent reports suggest (*Z*)-endoxifen to be the most important metabolite, considering it is also an inhibitor of aromatase [16] and causes concentration-dependent degradation of the ER [17]. Also,

(*Z*)-endoxifen is present at a much higher steady-state serum concentration in patients using tamoxifen than 4-hydroxytamoxifen [7]. Two other metabolites, *N*-desmethyl-4'-hydroxytamoxifen and 4'-hydroxytamoxifen, are very similar in structure to endoxifen and 4-hydroxytamoxifen, respectively, although their inhibitory effect on the ER is significantly lower. [8]

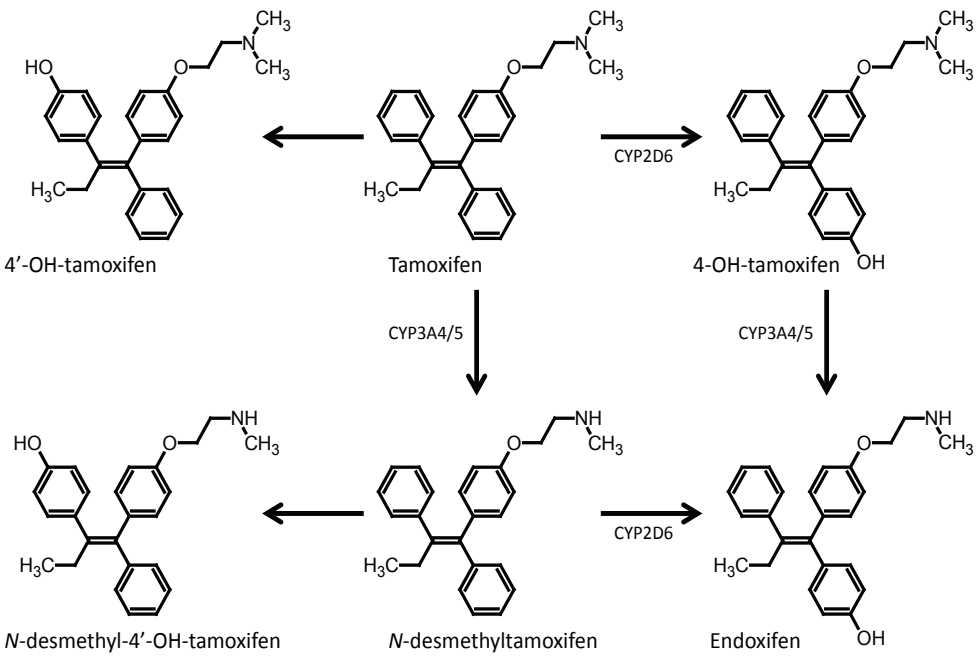

Figure 1 Part of the biotransformation of tamoxifen, showing the metabolites described in this report

The finding that (*Z*)-endoxifen is much more pharmacologically active than tamoxifen itself can possibly explain the earlier negative findings of a correlation between serum levels and outcome, since these studies were focused on the serum concentration of tamoxifen itself, not on the concentration of its active metabolite. In vitro studies show that the anti-estrogenic effects of (*Z*)-endoxifen are concentration dependent. [17–19] Also, a recent clinical trial with 1370 ER positive breast cancer patients reported by Madlensky et al., showed a significant correlation between endoxifen serum levels and breast cancer outcome. The results of this study show that the patients with an endoxifen serum concentration higher than 5.9 ng/mL had a 26% lower recurrence rate than patients with a lower endoxifen serum concentration. [20] Clearly, more studies are required to confirm the finding of a significant relationship between the serum endoxifen concentration and tamoxifen treatment outcome. Also, the suggested threshold concentration of 5.9 ng/mL remains an uncertain value at this

point in time, since this is the result of a single study. Before this threshold value can be used as a decision tool in daily clinical practice, it needs to be investigated more extensively and confirmed by other studies. However, these results suggest a potential role for therapeutic drug monitoring (TDM) in optimizing the patients' tamoxifen treatment based on endoxifen serum concentrations. The goal of TDM is to use drug concentrations to manage a patient's dosing regimen and thereby personalizing and optimizing their treatment. Wide inter-individual differences in systemic exposure of a drug in combination with a positive dose-exposure relationship and a positive exposure-efficacy relationship form a rationale for TDM of a drug. It is well known from the literature that there is considerable inter-patient variability in steady-state levels of tamoxifen and its metabolites [7,8,20–22]. Part of this variability can be explained by the use of non-selective analysis methods [23], however, even when using a highly selective HPLC-MS/MS assay, there is substantial variability in steady-state serum concentrations of tamoxifen and metabolites.

A sensitive and selective HPLC-MS/MS assay was developed in our laboratory to support TDM in the tamoxifen treatment of breast cancer patients. This assay was used to analyze the serum concentrations of tamoxifen and six of its metabolites: *N*-desmethyltamoxifen, (*E*)-endoxifen, (*Z*)-endoxifen, N-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen in a large group of patients treated with tamoxifen. Additionally, we used this method to identify patients with a low (*Z*)-endoxifen serum concentration, thereby using the literature as a guidance, and evaluated the changes in serum concentrations of tamoxifen and its metabolites after a dose increase in these patients. Also, the intra-patient variability was assessed by drawing more than one serum sample from the same patient in one dose level at different time points during the tamoxifen treatment.

Patients and methods

Patients

At the Netherlands Cancer Institute, Amsterdam, the Netherlands, a service is offered to patients who receive tamoxifen to determine exposure levels of this drug and its metabolites, to enable dose individualization. Serum samples were obtained from patients in the period between January 2009 and July 2013. All patients had taken tamoxifen at a dose of 10 mg or 20 mg per day for at least two months, to ensure steady-state concentrations. Blood samples were drawn at random time points during the day,

given the long half life of tamoxifen and its metabolites [24–26] we do not expect a wide variability in serum concentrations of tamoxifen and its metabolites at steady-state as a result of differences in time between the intake of tamoxifen and blood sampling.
Before the report of Madlensky et al. [20] was published (2011) the threshold concentration of (*Z*)-endoxifen was arbitrarily set at 7.0 ng/mL, based on the knowledge at that time about (*Z*)-endoxifen concentrations. After 2011, the report of Madlensky et al. was used as a guidance and (*Z*)-endoxifen serum concentrations above 5.9 ng/mL were accepted as above threshold.
When the (*Z*)-endoxifen serum concentration was below the predefined threshold concentration, the clinician was advised to discuss with the patient the option to increase the tamoxifen dose and obtain another serum sample after at least two months. In some cases, clinical decision-making resulted in the request of drawing a second or third blood sample while the patient did not receive a dose increase. These paired samples were used to assess the intra-patient variability, expressed as the coefficient of variation: CV (%) = (SD/mean value) * 100%.

Bioanalysis

The serum samples were collected in serum gel tubes and stored at -70°C until the time of analysis, usually within six weeks after the sample was drawn. Patient samples, calibration standards and quality control samples were handled according to the method described by Teunissen et al. [27]. The liquid chromatography – tandem mass spectrometry (LC-MS/MS) method developed by Teunissen et al. [27] was slightly modified and used for the determination of tamoxifen (5 to 500 ng/mL), *N*-desmethyltamoxifen (10 to 1000 ng/mL), (*E*)-endoxifen (1 to 100 ng/mL), (*Z*)-endoxifen (1 to 100 ng/mL), *N*-desmethyl-4'-hydroxytamoxifen (1 to 100 ng/mL), 4-hydroxytamoxifen (0.4 to 40 ng/mL) and 4'-hydroxytamoxifen (0.4 to 40 ng/mL). Detection was performed on a triple-quadrupole MS/MS detector with an electrospray ionization source (API4000, AB Sciex, Foster City, USA) operating in the positive ion mode. [23] A partial validation was executed and all requirements for acceptance, as defined in the FDA and EMA guidelines on bioanalytical method validation [28,29] were fulfilled.

Statistical methods

Pairwise student's t-test was used to test the null hypothesis that the change in tamoxifen and metabolite concentrations between a tamoxifen daily dose of 20 mg and a daily dose of 30 or 40 mg equals zero.

Results

Patients

A total of 251 serum samples were analyzed, obtained from 205 patients (median age 50 years, range 21-77). The median time period between the start of the tamoxifen treatment and the first drawn serum sample was 8 months (range 2 – 174). Of these patients, 197 used 20 mg tamoxifen per day according to standard practice; the other 8 patients used 10 mg per day. Of the patients using 10 mg tamoxifen per day, 6 were afraid of the side effects of tamoxifen and requested this lower dose, for 1 patient the pharmacy misread the prescription and delivered 10 mg tablets, for 1 patient the reason was unknown.

Steady state serum concentrations of tamoxifen and its metabolites

Wide variability was observed in the serum concentrations of tamoxifen and its metabolites, however there was a clear difference in mean serum concentrations between the two start dose levels (10 or 20 mg). These results are shown in Table 1. For fifteen patients a second or third blood sample was drawn on the same dose level, only the first result is included in the table.

For tamoxifen, *N*-desmethyltamoxifen, (*Z*)-endoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen, the concentrations in all serum samples were above the lower limit of quantification (LLOQ). For (*E*)-endoxifen, 203 out of 205 samples resulted in a concentration below the LLOQ of 1.0 ng/mL.

Table 1 Mean steady state serum concentration of tamoxifen and its metabolites in the 10 mg and 20 mg per day dosing groups. For repeated samples at the same dose level, only the first analysis result was used for this table

Analyte	Mean serum concentration (range)	
	10 mg/day (n=8)	20 mg/day (n=197)
Tamoxifen	51.6 (21.3 - 76.8)	103 (39.7 - 259)
N-desmethyltamoxifen	109 (55.1 - 169)	187 (82.3 - 426)
(*E*)-endoxifen	<1.00	<1.00 (<1.00 - 1.81)
(*Z*)-endoxifen	4.13 (2.43 - 7.99)	9.72 (1.73 - 30.8)
N-desmethyl-4'-hydroxytamoxifen	3.52 (2.11 - 5.41)	6.87 (2.35 - 15.5)
4-Hydroxytamoxifen	0.865 (0.528 - 1.56)	1.74 (0.481 - 4.04)
4'-Hydroxytamoxifen	1.15 (0.639 - 1.70)	2.33 (0.874 - 4.24)

The relationship between the serum concentrations of (*Z*)-endoxifen and the prescribed tamoxifen dose is depicted in Figure 2. For seven patients (88%) in the 10 mg group and 44 patients (22%) in the 20 mg group, the (*Z*)-endoxifen serum concentration was below the predefined threshold concentration of 5.9 ng/mL and clinicians were advised to discuss with their patients the option to increase the tamoxifen dose. Of the patients in the 10 mg group, three patients received a dose increase to 20 mg per day, including the patient who received 10 mg tablets instead of 20 mg due to an unclear prescription that was misread by the pharmacy. This mistake became clear when the low (*Z*)-endoxifen concentration was reported to the clinician and the patient was informed about this very low serum concentration. One patient switched to an aromatase inhibitor and the other three patients refused a higher dose because of the fear of more frequent and severe side effects, especially hot flashes.

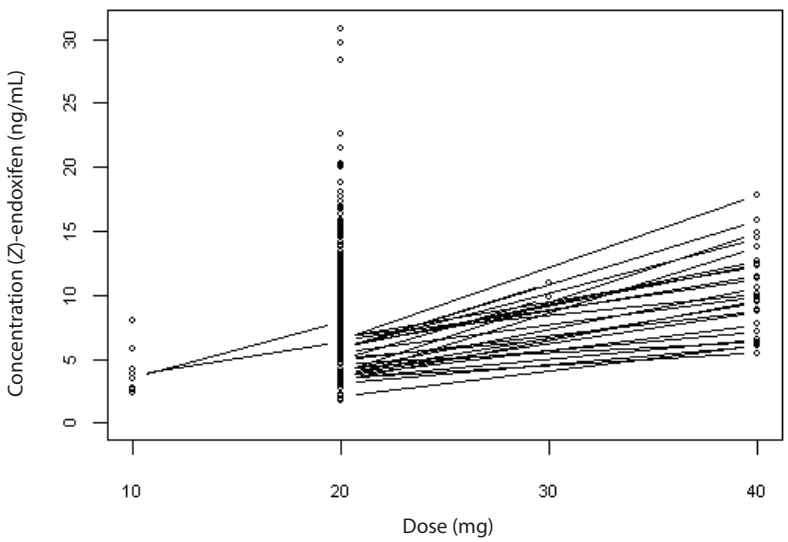

Figure 2 Relationship between the prescribed tamoxifen dose and the serum concentrations of (*Z*)-endoxifen. Each line represents a dose increase in a single patient. For repeated samples at the same dose level, only the first analysis result was used for this figure

Of the 44 patients with a low (*Z*)-endoxifen serum concentration using 20 mg tamoxifen, the clinician implemented the advice to increase the tamoxifen dose to 30 or 40 mg per day in 21 patients (48%). Six patients switched to an aromatase inhibitor and six patients refused a higher dose because of the fear of more severe and frequent side effects. For 11 patients, no action was taken.

Intra-individual variability

For fifteen patients, the clinician requested drawing a second or third blood sample without adjusting the tamoxifen dose. The main reasons were doubt whether action should be taken when the (*Z*)-endoxifen serum concentration of the first sample was just below the threshold level and interest in the potential change in serum concentrations after a period of about a year. The mean intra-patient variability for tamoxifen was 15.5% (range 4.68 – 46.1), for *N*-desmethyltamoxifen 18.7% (7.48 – 38.9), for (*Z*)-endoxifen 11.1% (range 3.88 – 21.4), for *N*-desmethyl-4'-hydroxytamoxifen 10.5% (range 2.33 – 23.1), for 4-hydroxytamoxifen 12.9% (range 3.08 – 34.5) and for 4'-hydroxytamoxifen the mean intra-patient variability was 14.4% with a range of 0.353 – 19.7%. The mean time period between the analyses was 9 months, range 2 – 20 months.

Dose adjustment

For three patients the tamoxifen dose was increased from 10 to 20 mg per day and for two of these patients a second sample was drawn. Both resulted in a (*Z*)-endoxifen serum concentration above 5.9 ng/mL. For 27 patients, the tamoxifen dose was increased from 20 to 30 mg (n=3) or 40 mg (n=24) per day. Initial serum samples of 21 of these patients resulted in a (*Z*)-endoxifen serum concentration below 5.9 ng/mL, for the other six patients the (*Z*)-endoxifen level was above 5.9 ng/mL but below the former threshold of 7.0 ng/mL, set at our clinic before publication of the report of Madlensky et al. [20]. The dose increase from 20 mg to 30 or 40 mg per day resulted in a significant increase in the mean serum concentrations of tamoxifen and its metabolites ($p<0.001$ for all analytes). Data are presented in Table 2. After the dose increase, for 26 patients (96%) the (*Z*)-endoxifen serum concentration was above the predefined threshold of 5.9 ng/mL. The serum samples of two patients in the 40 mg group resulted in an (*E*)-endoxifen level above the LLOQ of 1.0 ng/mL. The individual increases in (*Z*)-endoxifen serum concentration after dose adjustments are shown in Figure 2.

Table 2 Mean steady state serum concentrations of tamoxifen and metabolites for the patients who received a dose increase from 20 mg to 30 or 40 mg tamoxifen per day

Analyte	Mean serum concentration (range)		
	20 mg/day (n=27)	30 mg/day (n=3)	40 mg/day (n=24)
Tamoxifen	96.3 (50.3 - 247)	133 (91.0 – 193)	200 (88.5 - 431)
N-desmethyltamoxifen	194 (91.3 - 397)	258 (187 – 329)	386 (213 - 674)
(*E*)-endoxifen	<1.00	<1.00	<1.00 (<1.00- 1.06)
(*Z*)-endoxifen	4.80 (2.86 – 6.78)	9.11 (6.59 – 10.9)	10.4 (5.47 - 17.8)
N-desmethyl-4'-hydroxytamoxifen	7.50 (4.42 - 12.2)	12.1 (11.0 – 13.2)	15.6 (6.85 - 24.9)
4-Hydroxytamoxifen	1.13 (0.607 - 1.93)	2.00 (1.25 – 2.41)	2.27 (1.10 - 3.85)
4'-Hydroxytamoxifen	2.22 (1.24 - 4.06)	3.74 (3.23 - 4.67)	4.70 (2.03 - 7.09)

Discussion

A sensitive and selective HPLC-MS/MS assay was used to analyze the concentrations of tamoxifen, *N*-desmethyltamoxifen, (*E*)-endoxifen, (*Z*)-endoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen in serum samples of patients treated with tamoxifen according to standard of care. Considerable inter-patient variability in the serum concentrations of tamoxifen and its metabolites was observed. The serum concentrations of tamoxifen, *N*-desmethyltamoxifen, (*Z*)-endoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen were above the lower limit of quantitation (LLOQ) for all patient samples. Although we optimized the assay to also quantify (*E*)-endoxifen levels in patient samples, only four serum samples (drawn from four different patients) resulted in an (E)-endoxifen concentration above the LLOQ of 1.0 ng/mL (two from the group of patients receiving 20 mg tamoxifen per day and two patients whose dose was increased to 40 mg per day). These four patients also had a relatively high (*Z*)-endoxifen level; 30.8 and 28.3 ng/mL for the two patients using 20 mg tamoxifen and 17.8 and 14.9 ng/mL for the two patients using 40 mg. The other 247 serum samples resulted in an (*E*)-endoxifen level below 1.0 ng/mL. The finding that the E-isomer is present in a very low concentration in patient serum is in line with a previously reported study [8]. To determine which patients were eligible for a dose increase, the minimum threshold (*Z*)-endoxifen concentration of 5.9 ng/mL suggested in the literature [20] was used as a guidance. This value should be interpreted with care, since this is the result of a single study. However, since several in vitro studies [17–19] support the finding of an association between (*Z*)-endoxifen exposure and tamoxifen treatment outcome and at this moment there is only one report suggesting a certain threshold, this is the value used for the TDM described in this report. Indirect evidence for a dose-effect relation comes from the Oxford Overview data, where a significant test for trend was found for improved recurrence-free survival with increasing doses of adjuvant tamoxifen [3].

Our results show that patients who used 10 mg tamoxifen per day had a much higher likelihood of having (*Z*)-endoxifen serum concentrations below the predefined threshold concentration compared to the patients who used the standard dose of 20 mg tamoxifen per day. The serum samples of 88% of the patients who used 10 mg per day resulted in a (*Z*)-endoxifen serum concentration below the predefined threshold. Increasing the tamoxifen dose from 10 mg to 20 mg per day resulted in a higher (*Z*)-endoxifen serum concentration. 22% of the serum samples of patients using 20 mg tamoxifen per day resulted in a (*Z*)-endoxifen level below the predefined threshold. The dose increase from 20 mg to 30 or 40 mg tamoxifen per day resulted in a statistically significant increase in

tamoxifen and metabolite levels. Most patients (96%) who received this dose increase were able to reach the endoxifen threshold concentration when using 30 or 40 mg tamoxifen per day. This finding was in line with the results of two previous studies examining the effect of tamoxifen dose increase on endoxifen serum concentrations. Irvin et al. [30] used CYP2D6 genotype-guided dosing, where intermediate and poor metabolizers (n=60) received a dose increase from 20 mg to 40 mg tamoxifen per day. This resulted in a significant increase in endoxifen levels. Barginear et al. [31] identified patients with a low endoxifen level and/or low CYP2D6 activity to receive a dose increase from 20 mg to 30 mg tamoxifen per day (n=24). The major limitation of this study was the lack of a selective LC-MS/MS method for the identification of the group of patients with low endoxifen levels [31]. This resulted in biased endoxifen levels, and thus an incorrectly composed group of patients eligible for the dose increase. However, the authors reanalyzed the patient samples after the study was finished and their results showed a significant increase in (*Z*)-endoxifen levels after the dose increase to 30 mg tamoxifen per day. Studies investigating tamoxifen at different dose levels (20 versus 30 or 40 mg per day) showed no significant difference in the frequency and severity of the experienced side effects [5,6], suggesting a dose increase to 30 or 40 mg tamoxifen per day is safe. However, these studies included a relatively limited number of patients and no data on long-term serious toxicity, such as endometrial cancer, was reported. One report suggests a slightly higher risk of endometrial cancer when using a higher tamoxifen dose [32]. More research is needed to confirm this finding and to investigate the possible association between serum concentrations and the risk of serious toxicity. Although serious toxicity is not common in tamoxifen-treated patients and the benefits of tamoxifen outweigh the risk of endometrial cancer, the risk should be minimized where possible. At this moment, we would suggest to only increase the tamoxifen dose in patients with low (*Z*)-endoxifen exposure. In our clinical dataset, the intra-patient variability was low. This was assessed using real-life data, with a different time period between the paired samples. Although we only have 15 paired samples in our database, these results suggest that the serum concentrations of tamoxifen and its metabolites are constant during the steady-state phase of tamoxifen treatment. This suggests that only one serum sample per patient is necessary to apply TDM for tamoxifen treatment, making this a relatively non-invasive way to optimize tamoxifen treatment.

Conclusions

These results support the suitability of TDM for optimizing and personalizing tamoxifen treatment of breast cancer patients. It is shown that the prescribed tamoxifen dose is related to (*Z*)-endoxifen exposure and increasing the tamoxifen dose leads to a significantly higher serum concentration of tamoxifen and its metabolites. The intra-patient variability in our clinical dataset was low, suggesting that during steady-state only one serum sample is needed for TDM, making this a relatively non-invasive way to optimize the patient's treatment. Also, these results show that tamoxifen dosing below the standard of care of 20 mg per day is not advised, since these patients had a much higher likelihood of having (*Z*)-endoxifen serum concentrations below the predefined threshold concentration compared to the patients who used the standard dose of 20 mg tamoxifen per day.

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Use of dried blood spots for the determination of serum concentrations of tamoxifen and endoxifen

2.2

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Abstract

Background. The anti-estrogenic effect of tamoxifen is suggested to be mainly attributable to its metabolite (*Z*)-endoxifen, and a minimum therapeutic threshold for (*Z*)-endoxifen in serum has been proposed. The objective of this research was to establish the relationship between dried blood spot (DBS) and serum concentrations of tamoxifen and (*Z*)-endoxifen to allow the use of DBS sampling, a simple and patient-friendly alternative to venous sampling, in clinical practice.

Methods. Paired DBS and serum samples were obtained from 50 patients using tamoxifen and analyzed using HPLC-MS/MS. Serum concentrations were calculated from DBS concentrations using the formula *calculated serum concentration = DBS concentration/([1-haematocrit (Hct)] + blood cell-to-serum ratio × Hct)*. The blood cell-to-serum ratio was determined ex vivo by incubating a batch of whole blood spiked with both analytes. The average Hct for female adults was imputed as a fixed value. Calculated and analyzed serum concentrations were compared using weighted Deming regression.

Results. Weighted Deming regression analysis comparing 44 matching pairs of DBS and serum samples showed a proportional bias for both analytes. Serum concentrations were calculated using $[Tamoxifen]_{serum,\ calculated} = [Tamoxifen]_{DBS}/0.779$ and $[(Z)\text{-}Endoxifen_{serum,\ calculated} = [(Z)\text{-}Endoxifen]_{DBS}/0.663$. Calculated serum concentrations were within 20 % of analyzed serum concentrations in 84 and 100 % of patient samples for tamoxifen and (*Z*)-endoxifen, respectively.

Conclusion. DBS concentrations of tamoxifen and (*Z*)-endoxifen were equal to serum concentrations after correction for Hct and blood cell-to-serum ratio. DBS sampling can be used in clinical practice.

Introduction

Tamoxifen is widely administered in the treatment of estrogen receptor (ER) positive breast cancer. It undergoes extensive metabolism leading to the formation of at least 22 phase I metabolites in humans [1,2]. These metabolites are primarily present in the *Z* (zusammen) form, since tamoxifen is administered as a pure *Z*-isomer [3]. There are large differences in pharmacological activity between the metabolites, where *N*-desmethyl-4-hydroxytamoxifen (endoxifen) and 4-hydroxytamoxifen are considered to be the most therapeutically active, being 30 to 100-fold more potent than tamoxifen itself. [4,5] Recent reports suggest (*Z*)-endoxifen to be the most important metabolite, considering that it is also an inhibitor of aromatase [6] and causes concentration-dependent degradation of the ER [7]. Also, endoxifen, when compared to 4-hydroxytamoxifen, is present at a much higher steady-state concentration in serum of patients using tamoxifen [1]. A recent clinical trial with 1370 ER positive breast cancer patients showed a significant correlation between endoxifen serum levels in patients and breast cancer outcome; patients who had an endoxifen serum concentration above 5.9 ng/mL had a 26% lower recurrence rate than patients with a lower endoxifen serum concentration. [8] These findings encourage optimization of tamoxifen treatment by individual dosing in order to reach target endoxifen levels. Current practice for (*Z*)-endoxifen quantification is by analysis of patient serum samples obtained in the clinic, via a venapuncture. In recent years, patient friendly and simple alternatives have been explored, of which dried blood spot (DBS) sampling appears to be a good option. DBS sampling consists of the collection of a whole blood sample, obtained by a fingerprick, on a paper DBS card. Patients can easily self-obtain the DBS samples at home and sent the DBS to the laboratory by regular mail service, since no special conditions for transport and storage of DBS samples are required [9,10]. However, threshold levels for (*Z*)-endoxifen have been determined in serum [8]. Therefore, we aimed to establish the ratio between serum and DBS concentrations of tamoxifen and (*Z*)-endoxifen, to allow the use of DBS sampling as a patient-friendly way to obtain tamoxifen and (*Z*)-endoxifen serum concentrations.

Patients and methods

Patients

For this study, 50 female patients who underwent surgical treatment for early, ER positive breast cancer and subsequently received adjuvant tamoxifen for at least 2 months were recruited from the outpatient clinic of the Netherlands Cancer Institute, Amsterdam, the Netherlands. This number was based upon the Guidelines for Method Comparison from the Clinical and Laboratory Standards Institute [11]. In our clinic, a service is offered to patients who receive tamoxifen treatment to determine steady-state exposure levels of tamoxifen and (*Z*)-endoxifen in serum, to enable dose individualization [12]. The patients who wished to use this service, were asked if they would be willing to participate in this study and, next to a venapuncture to obtain a serum sample, also would undergo a fingerprick to obtain a DBS sample. Patients were recruited between April and November 2013. This study (Dutch Trial Registry NTR4042) was approved by the ethics committee of the Netherlands Cancer Institute and informed consent was provided by each participant after full explanation of the purpose and nature of all procedures used and before sampling.

Sampling

The serum samples were collected in serum gel tubes and stored at -70°C until the time of analysis, within 6 weeks after the sample was drawn. DBS samples were obtained using a 1.8 mm contact-activating lancet (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) within 5 minutes after the venapuncture. The blood spots were collected on Whatman FTA™ DMPK-A DBS cards (Whatman, GE Healthcare, Buckinghamshire, UK) and allowed to dry for at least three hours at room temperature before they were stored at room temperature in an aluminum bag containing a desiccant package until the time of analysis, within 6 weeks after the sample was drawn.

Bioanalysis

On arrival in the laboratory, the blood spots were visually inspected for complete filling of a circle of at least 6 mm and dark red coloring on both sides of the paper. The approved DBS samples were extracted and analyzed together with DBS calibration standards and quality control samples, according to a previously published HPLC-MS/MS method [13].

The HPLC-MS/MS method developed by Teunissen et al. [14] for the determination of tamoxifen and metabolites in serum was slightly modified and used for the

determination of tamoxifen (5–500 ng/mL) and (*Z*)-endoxifen (1–100 ng/mL) in patient serum samples. Detection was performed on a triple-quadrupole MS/MS detector with an electrospray ionization source (API4000, AB Sciex, Foster City, USA) operating in the positive ion mode [12,15]. A partial validation was executed and all requirements for acceptance, as defined in the FDA and EMA guidelines on bioanalytical method validation [16,17], were fulfilled. Both DBS and serum samples were processed in duplicate according to the Guideline for Method Comparison of the Clinical and Laboratory Standards Institute [11].

Dried blood spot versus serum concentrations

Whole blood is composed of serum, red blood cells, white blood cells and platelets, where the fractional volume of white blood cells and platelets is very small. The concentration of an analyte in whole blood can be calculated by equation 1, where [Analyte]Blood is the analyte concentration in whole blood, $[Analyte]_{Serum}$ is the analyte concentration in serum, V_{Serum} is the serum volume, $[Analyte]_{BC}$ is the concentration in the blood cells, V_{BC} is the volume occupied by the blood cells and V_{Blood} is the volume of the whole blood.

$$[Analyte]_{Blood} = \frac{[Analyte]_{Serum} * V_{Serum} + [Analyte]_{BC} * V_{BC}}{V_{Blood}} \quad [1]$$

Equation 1 shows that concentrations determined in a DBS sample and in a plasma or serum sample in general will not be similar. The difference depends on the concentration of the analyte in blood cells and the volume of serum and blood cells in whole blood. The concentration of the analyte in blood cells relies upon the blood cell-to-serum partitioning ($K_{BC:Serum}$), which depends on the permeability of the cell membrane for the analyte and the affinity of the analyte for constituents within the serum and blood cells [18–21]. The concentration of an analyte in blood cells can be calculated by using equation 2. [20]

$$[Analyte]_{BC} = K_{BC:Serum} * [Analyte]_{Serum} \quad [2]$$

The volume of whole blood that is occupied by blood cells, the haematocrit (Hct), differs between individuals. When the Hct is known, both the volume of the blood cells and the volume of serum in a whole blood sample can be calculated, using equation 3 and 4.

$$V_{BC} = Hct * V_{Blood} \quad [3]$$

$$V_{Serum} = (1 - Hct) * V_{Blood} \quad [4]$$

When imputing equation 2-4 into equation 1, equation 5 is obtained. [20]

$$[Analyte]_{Blood} * V_{Blood} = [Analyte]_{Serum} * (1 - Hct) * V_{Blood} + K_{BC:Serum} * [Analyte]_{Serum} * Hct * V_{Blood} \quad [5]$$

Rearranging equation 5 gives equation 6, which can be used to convert analyte concentrations in whole blood (DBS) to calculated analyte concentrations in serum.

$$[Analyte]_{serum} = \frac{[Analyte]_{Blood}}{(1 - Hct) + K_{BC:Serum} * Hct} \quad [6]$$

Blood cell-to-plasma partitioning

The blood cell-to-plasma partitioning coefficient ($K_{BC:Plasma}$) and not the blood cell-to-serum partitioning coefficient was determined, since the blood would have coagulated when performing the incubation experiment when no anticoagulant is present. However, it is presumed that the blood cell-to-plasma partitioning coefficient is similar to the blood cell-to-serum partitioning coefficient. The $K_{BC:Plasma}$ was established *ex vivo* by using fresh human whole blood with a Hct value of 0.41 L/L, determined using an automated haematology analyzer Cell-Dyn 4000 (Abbott Laboratories, Abbott Park, IL, USA). An aliquot of 6 mL whole blood was spiked with both analytes at low, mid and high concentration level. The spiked blood samples were incubated for 1 hour at 37˚C. Subsequently, the blood samples were centrifuged for 10 minutes and the plasma was separated from the blood cells. Three replicates of 50 µL plasma per concentration level were handled and analyzed together with calibration standards and quality control samples in serum, according to the method described above. The concentration of the analytes in blood cells was calculated using equations 1-3. $K_{BC:Plasma}$ was calculated by dividing the calculated analyte concentration in blood cells by the analyte concentration measured in plasma, see equation 2.

Statistics

Analyzed DBS and serum concentrations were compared using weighted Deming regression. Additionally, serum concentrations were calculated from DBS concentrations

using equation 6, where $[Analyte]_{blood}$ refers to the analyte concentration measured in dried blood spots. The mean Hct value for the target population; adult females (0.41 L/L), and the obtained $K_{BC:Plasma}$ were imputed as fixed values in this equation. The serum concentrations calculated from DBS concentrations and the analyzed serum concentrations were compared using weighted Deming regression. Additionally, the agreement between the methods was presented using the Bland-Altman difference plot. All statistical analyses were conducted with Analyse-it® (Method evaluation edition, version 2.30; Analyse-it Software Ltd, Leeds, UK). Acceptation criteria for the agreement between calculated and analyzed serum concentrations were based on the guideline on Bioanalytical Method Validation of the EMA; the difference in concentration should be within ±20% of their mean for at least 67% of the samples [17].

Results

Patients

Of the 50 recruited patients, 44 patients (median age 53, range 25-76) provided at least one usable spot and were included in this study. For 40 of these patients, the fingerprick resulted in at least two usable DBS; for these patients two DBS were extracted and analyzed. The other 4 patients provided one usable DBS. For 6 patients, not one usable DBS was obtained. The DBS that were not usable for the analysis were all too small; i.e. the area of the 6 mm subpunch was not completely filled with blood on both sides of the card.

Of the included patients, 39 (89%) received a dose of 20 mg tamoxifen per day according to standard practice; one patient used 10 mg per day, two patients used 30 mg per day and two patients used 40 mg tamoxifen per day.

The analyte concentrations in serum samples ranged from 54.7 to 231 ng/mL (repeatability CV 2.7%) for tamoxifen and from 2.12 to 21.6 ng/mL (repeatability CV 4.7%) for (*Z*)-endoxifen. The analyte concentrations in DBS samples ranged from 51.0 to 176 ng/mL (repeatability CV 5.2%) for tamoxifen and from 1.61 to 12.2 ng/mL (repeatability CV 4.7%) for (*Z*)-endoxifen.

Blood cell-to-plasma partitioning

For both analytes, the analyzed concentration in plasma was higher than the spiked concentration in whole blood. The blood cell-to-plasma partitioning coefficient was determined to be 0.461 for tamoxifen and 0.179 for (*Z*)-endoxifen, see Table 1.

Table 1 Results from the blood-to-plasma partitioning experiment (n=3)

Analyte	Spiked conc. whole blood (ng/mL)	Analyzed conc. plasma (ng/mL)	Blood-to-plasma ratio	Calculated conc. BCs (ng/mL)	BC-to-plasma partitioning
Tamoxifen	12.4	16.3	0.763	6.86	0.422
	49.8	64.3	0.774	28.9	0.450
	398	498	0.800	255	0.512
Mean			**0.779**		**0.461**
(*Z*)-Endoxifen	2.49	3.78	0.660	0.641	0.170
	9.95	15.2	0.656	2.44	0.161
	79.6	118	0.675	24.3	0.206
Mean			**0.663**		**0.179**

conc.: concentration, BC: blood cell.

DBS versus serum concentrations

The relationship between tamoxifen and (*Z*)-endoxifen concentrations analyzed in DBS and serum samples using weighted Deming regression is plotted in Figure 1. The slope of the tamoxifen curve was 0.81 (95% CI, 0.69 – 0.92) and the intercept was 3.42 (95% CI, -9.10 – 15.9). For (*Z*)-endoxifen, the slope was 0.65 (95% CI, 0.61 – 0.99) and the intercept was 0.03 (95% CI, -0.21 – 0.27).

Serum concentrations were calculated from DBS concentrations using equation 6, where average Hct for adult females (0.41 L/L) and the obtained $K_{BC:serum}$ (0.461 for tamoxifen and 0.179 for (*Z*)-endoxifen) were imputed as fixed values. This results in equation 7 and 8, which can be used to calculate serum concentrations from DBS concentrations.

$$[\text{Tamoxifen}]_{\text{serum,calculated}} = \frac{[\text{Tamoxifen}]_{\text{DBS}}}{0.779} \quad [7]$$

$$[(\text{Z})\text{-Endoxifen}]_{\text{serum,calculated}} = \frac{[(\text{Z})\text{-Endoxifen}]_{\text{DBS}}}{0.663} \quad [8]$$

Weighted Deming regression was used to investigate the relationship between calculated and analyzed serum concentrations. This relationship is depicted in Figure 2. The slope and intercept were not significantly different from 1 and 0, respectively, for both analytes. For tamoxifen the slope was 1.04 (95% CI, 0.89 – 1.19) and the intercept was 4.39 (95% CI, -11.7 – 20.5) and for (*Z*)-endoxifen, the slope was 0.99 (95% CI, 0.93 – 1.05) and the intercept was 0.05 (95% CI, -0.31 – 0.41).

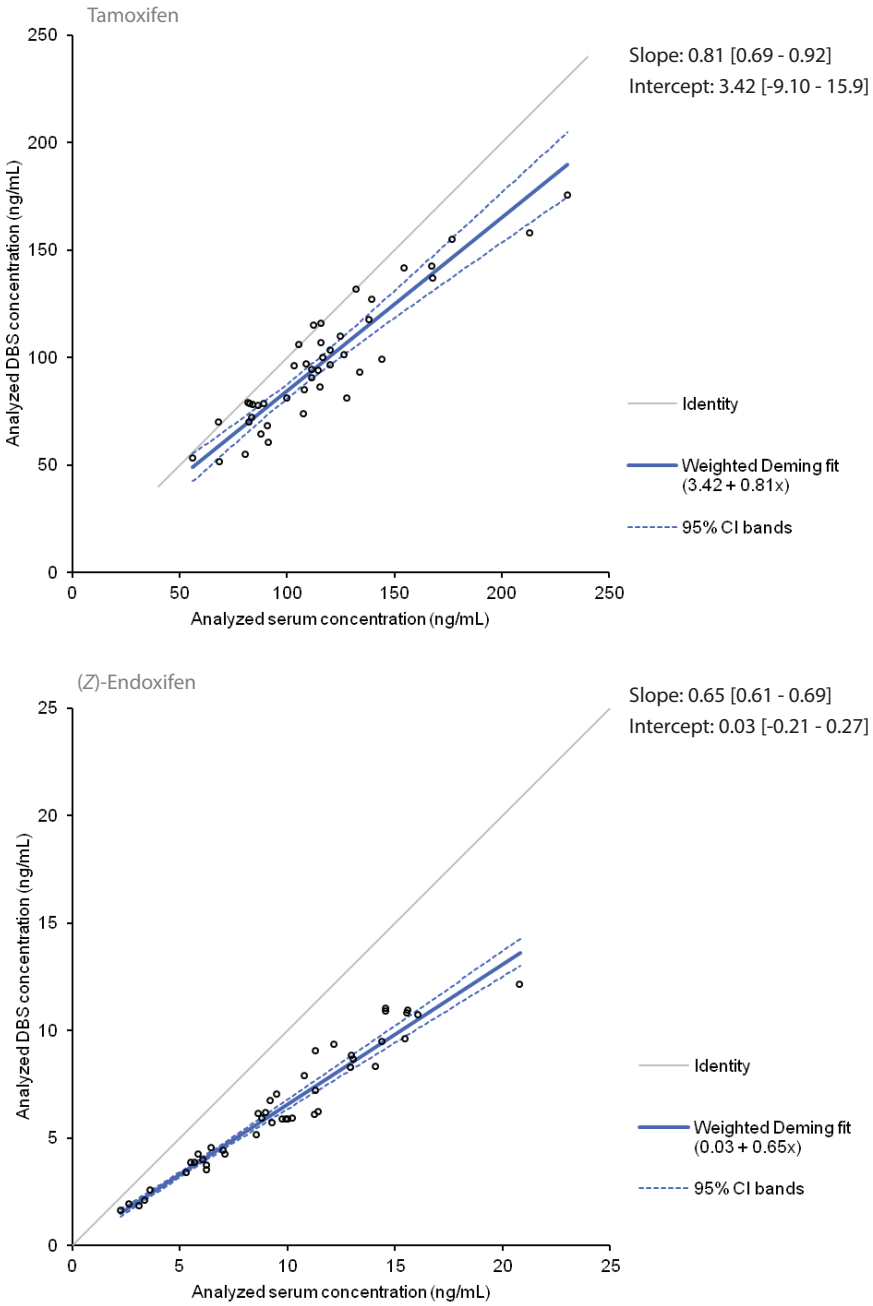

Figure 1 Analyzed DBS concentrations plotted against analyzed serum concentrations for tamoxifen and (*Z*)-endoxifen using weighted Deming regression analysis (n=44)

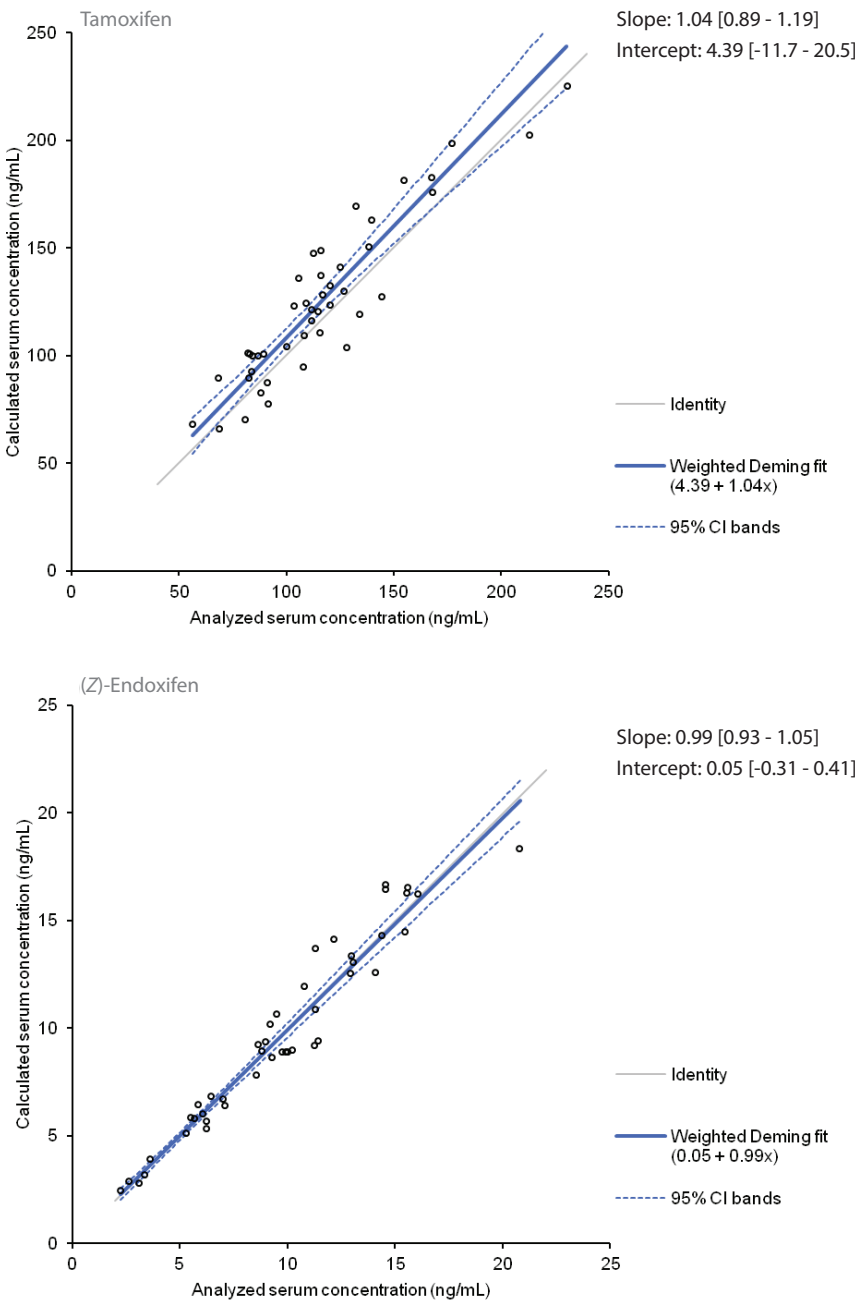

Figure 2 Calculated serum concentrations, based on analyzed DBS concentrations, plotted against analyzed serum concentrations for tamoxifen and (*Z*)-endoxifen using weighted Deming regression analysis (n=44)

The Bland-Altman difference plot (Figure 3) showed a small bias for the calculated serum concentrations of tamoxifen (8.72 ng/mL) and (*Z*)-endoxifen (0.042 ng/mL). Mean calculated serum concentrations were within ±20% of the mean of the analyzed serum concentrations in 84.1% of the patient samples for tamoxifen and in 100% of the patient samples for (*Z*)-endoxifen.

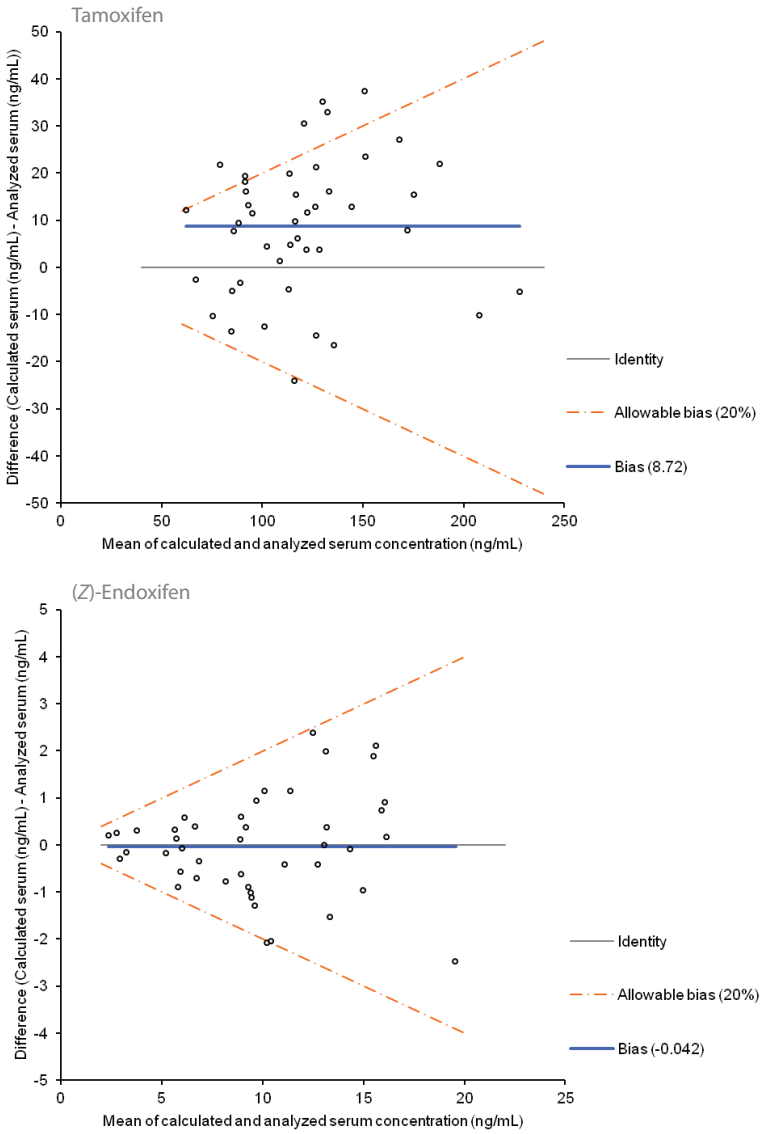

Figure 3 Bland-Altman plots for tamoxifen and (*Z*)-endoxifen using the calculated serum concentration, based on analyzed DBS concentrations, and the analyzed serum concentration. The line at difference=0 is the line of true identity, the bold line is the mean observed difference, whereas the dashed lines resemble a bias of 20%

Based on analyzed serum concentrations, the (*Z*)-endoxifen level was below the predefined threshold concentration of 5.9 ng/mL for eight patients. Based on calculated serum concentrations, ten patients had a low (*Z*)-endoxifen serum concentration; eight of these patients also had a low analyzed (*Z*)-endoxifen serum concentration, the other two patients had an analyzed serum concentration just above the threshold level - 6.3 and 6.2 ng/mL.

Discussion

With this study, we demonstrated that calculated serum concentrations of tamoxifen and (*Z*)-endoxifen based on DBS concentrations are in good agreement with analyzed serum concentrations. This is the first study to evaluate the correlation between tamoxifen and active metabolite concentrations in DBS and serum.

We showed that the difference between concentrations in DBS and serum samples can very well be explained by the analyte-specific blood cell-to-serum partitioning and haematocrit using a formula adapted from the literature [18,20]. For (*Z*)-endoxifen, this formula resulted in a very small bias between the calculated serum concentrations and the analyzed serum concentrations, only -0.042 ng/mL. For tamoxifen, this bias was substantially larger; 8.72 ng/mL. However, since tamoxifen is present in much higher concentrations in patients than (*Z*)-endoxifen and 84% of the calculated serum concentrations in patient samples were within 20% of the analyzed serum concentration, we believe this bias is acceptable.

The blood cell-to-serum partitioning was found to be independent of the concentration for both analytes and could therefore be imputed as a fixed value in the formula. Our target population consists of women treated for early breast cancer and receiving adjuvant tamoxifen treatment. This results in a relatively homogenous group of patients, since they are all adult females and physiological conditions having a major effect on Hct values are not expected. Therefore, the target population was expected to have Hct values within the normal range of Hct for adult females, between 0.36 and 0.46 L/L [22]. For eight of the included patients, the Hct value was obtained for purposes other than this study and shown to be between 0.34 and 0.42 L/L. Additionally, the validation of the bioanalytical method showed that analysis results of DBS samples with haematocrit values between 0.29 and 0.48 L/L fell within acceptable limits for assay performance [13]. This indicates that for this application, it is not necessary to impute patient-specific haematocrit values in the conversion formula and the average haematocrit for female

adults, 0.41L/L [22], could be imputed in the conversion formula as a fixed value. This finding is of importance, since Hct determination requires a venapuncture and would therefore diminish the large advantage of DBS sampling - the option for patients to self-sample at home, without the need for a laboratory visit and a venapuncture.
When using fixed values for both blood cell-to-serum partitioning and haematocrit, two simple formulas for the conversion of DBS concentrations to serum concentrations were obtained: $[Tamoxifen]_{serum,\ calculated} = [Tamoxifen]_{DBS}/0.779$ and $[(Z)\text{-}Endoxifen]_{serum,\ calculated} = [(Z)\text{-}Endoxifen]_{DBS}/0.663$. When this assay is to be used in patients with a Hct value known to deviate from validated values (0.29 – 0.48 L/L), an adequate correction can be made by using equation 6 and imputing the patient-specific haematocrit.
To the best of our knowledge, this is the first study comparing serum and DBS concentrations obtained from patient samples using a formula that includes both haematocrit and the analyte-specific blood cell-to-serum ratio. Several papers have reported the correlation between DBS and serum or plasma samples for other analytes; most of the authors showed that DBS and serum or plasma concentrations are well correlated but not identical, similar to our results. The majority of these studies showed higher concentrations in plasma or serum samples than in DBS samples. However, for voriconazole, fluconazole and posaconazole comparable concentrations in plasma and DBS samples have been described [23], and for venlafaxine [24] and carbamazapine [25] higher analyte concentrations in DBS samples than in plasma samples have been described. Eyles et al. [26] introduced a formula for the conversion of DBS to serum or plasma concentrations by correcting for haematocrit, which can be used for analytes that mainly partition in serum and therefore blood cells only function to dilute the serum sample: $[Analyte]_{plasma} = [Analyte]_{DBS}/(1\text{-}Hct)$. This formula was used for the comparison of plasma and DBS concentrations of propranolol [27], cocaine [28], Δ9-tetrahydrocannabinol [29], imatinib, nilotinib and dasatinib [30] and mycophenolic acid [31]. A formula introduced by Li and Tse [10] was based on the formula of Eyles et al., but used plasma protein binding as an additional factor. This formula was used to describe the correlation between plasma and DBS concentrations for nevirapine and efavirenz [32]. Taylor et al. [33] investigated the *ex vivo* blood-to-plasma distribution ratio of acetaminophen and explained the differences between DBS and plasma samples using this ratio, but haematocrit was not taken into account.
For 44 patients (88%) in our study at least one usable DBS was obtained. The DBS samples of the other 6 patients were too small to take out a subpunch of 6 mm. Although sample collection is not complicated, a small part of the obtained samples were not usable for the analysis. This emphasizes the need of proper training of

personnel and patients obtaining the spots, in order to keep the number of unusable spots to a minimum.

The minimum threshold (*Z*)-endoxifen serum concentration of 5.9 ng/mL suggested in the literature [8] was used as guidance for dose individualization. This value should be interpreted with care, since this is the result of a single study. However, since there is only one report available suggesting a certain threshold, this is the value used in this report. Indirect evidence for a dose-effect relation originates from the Oxford Overview data, where a significant test for trend was found for improved recurrence-free survival with increasing doses of adjuvant tamoxifen [36].

Using the conversion formula obtained in our study, the minimum concentration threshold of 5.9 ng/mL (*Z*)-endoxifen in serum is equal to 3.9 ng/mL in DBS. For two patients (4.5%), the calculated serum concentration was below the threshold level, but their analyzed serum concentration was above the threshold level. However, the analyzed (*Z*)-endoxifen serum level was at the boundary of acceptance for both patients; in practice the clinician would discuss the option to increase the tamoxifen dose with these patients as well. Therefore, it was not expected that clinical decisions would have been different when DBS concentrations were used.

Conclusions

The results of this study enable DBS sampling for the determination of tamoxifen and (*Z*)-endoxifen concentrations for clinical studies and therapeutic drug monitoring purposes. DBS concentrations were equal to serum concentrations after correction for haematocrit and blood cell-to-serum ratio. DBS concentrations can be easily converted to serum concentrations using the following formulas $[Tamoxifen]_{serum,\ calculated} = [Tamoxifen]_{DBS}/0.779$ and $[(Z)\text{-}Endoxifen]_{serum,\ calculated} = [(Z)\text{-}Endoxifen]_{DBS}/0.663$. The minimum therapeutic threshold of (*Z*)-endoxifen in dried blood spots is 3.9 ng/mL.

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Dried blood spot self-sampling at home for the individualization of tamoxifen treatment: a feasibility study

2.3

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Submitted for publication

Abstract

Background. The anti-estrogenic effect of tamoxifen is suggested to be mainly attributable to its metabolite (*Z*)-endoxifen and a minimum therapeutic threshold for (*Z*)-endoxifen in serum has been proposed. Dried blood spot (DBS) sampling may offer a less invasive and simple alternative to venous sampling. However, knowledge of patient capability of DBS self-sampling at home and acceptance of this procedure is limited. Therefore, the aim of this study is to assess the feasibility of DBS self-sampling at home for individualization of tamoxifen therapy based on (*Z*)-endoxifen concentrations.

Methods. Patients using tamoxifen after being cured from ER positive breast cancer were recruited from the outpatient clinic and instructed to self obtain a dried blood spot sample at home. Additionally, the patients were asked to fill out a short questionnaire about their preferred sampling method and sent the DBS samples and the questionnaire to the laboratory by regular mail service. The samples were analyzed using a previously validated HPLC-MS/MS method. Patients with (*Z*)-endoxifen levels below the predefined threshold were identified and the clinician was advised to discuss with their patients the option to increase the tamoxifen dose.

Results. In total, 38 patients were included in this study and 36 of these patients returned the DBS sample and questionnaire to the laboratory. Of these patients, 31 (86%) provided at least one DBS suitable for analysis. Most patients (92%) reported a positive experience with DBS self-sampling and 61% of the patients preferred DBS sampling over venous sampling.

Conclusion. This study supports the feasibility of dried blood spot sampling at home for tamoxifen treatment individualization.

Introduction

Tamoxifen is widely administered in the treatment of estrogen receptor (ER) positive breast cancer and has substantially reduced breast cancer recurrence and mortality. However, not all patients benefit from tamoxifen and there is great interest in identifying predictive factors associated with tamoxifen efficacy [1]. In recent years, it has become clear that some of the tamoxifen metabolites have a higher affinity for the ER than tamoxifen itself, where (*Z*)-*N*-desmethyl-4-hydroxytamoxifen ((*Z*)-endoxifen) is suggested to be the most therapeutically active [2–4]. A recent retrospective clinical trial with 1370 ER positive breast cancer patients showed a significant correlation between endoxifen serum levels and breast cancer outcome; patients with an endoxifen serum concentration above 5.9 ng/mL had a 26% lower recurrence rate than patients with a lower endoxifen serum concentration. [5] About 20% of the patients using 20 mg tamoxifen per day, according to standard of care, have (*Z*)-endoxifen serum levels below this threshold. Furthermore, it has been shown that a dose increase to 30 or 40 mg per day leads to higher serum concentrations of tamoxifen and its metabolites and 96% of the patients were able to reach the (*Z*)-endoxifen threshold concentration after the dose increase. [6] These findings encourage optimization of tamoxifen treatment by individual dosing based on (*Z*)-endoxifen concentrations. Current practice for (*Z*)-endoxifen quantification is by analysis of patient serum samples. In recent years, patient friendly, simple and cost-effective alternatives have been explored, of which dried blood spot (DBS) sampling appears to be a good option. DBS sampling consists of the collection of a whole blood sample obtained by a finger prick, on a paper DBS card. With DBS sampling, patients can self-sample at home and the samples can be sent to the laboratory by regular mail service, since no special conditions for transport and storage of DBS samples are required [7,8]. Our previous research revealed that tamoxifen and (*Z*)-endoxifen DBS concentrations were equal to serum concentrations after correction for haematocrit and blood cell-to-serum ratio. Consequently, the minimum therapeutic threshold of (*Z*)-endoxifen in serum, 5.9 ng/mL, could be converted to a minimum therapeutic threshold of 3.9 ng/mL in dried blood spots. [9]

However, before implementing DBS sampling at home in clinical practice, knowledge of patient capability and acceptability of this new method is essential. Therefore, the aim of this study was to assess the feasibility of DBS self-sampling at home for individualization of tamoxifen therapy based on (*Z*)-endoxifen concentrations.

Patients and methods

Patients

For this study, female patients receiving adjuvant tamoxifen after being cured from ER positive breast cancer were recruited from the outpatient clinic of the Netherlands Cancer Institute, Amsterdam, the Netherlands. Patients were recruited between February and June 2014. Each patient received a short training for sample collection and a package containing a DBS collection card (Whatman FTA™ DMPK-A DBS cards, GE Healthcare, Buckinghamshire, UK), a 1.8 mm contact-activating lancet (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), a zip-closure aluminum bag containing a desiccant, a written sampling instruction and a pre-paid envelope. Patients were instructed to obtain the DBS at a time point of their convenience. Given the long half-life of tamoxifen and its metabolites [10–12], wide variability in concentrations of tamoxifen and (Z)-endoxifen at steady-state as a result of differences in time between tamoxifen intake and blood sampling was not expected. Patients were instructed to allow the DBS card to dry for at least three hours at room temperature before placing the DBS card in the aluminum bag and send to sample to the laboratory by regular mail service.
Also, each patient received a short questionnaire regarding their daily dose of tamoxifen, the concomitant use of other prescribed and/or homeopathic medication and three questions regarding their experience with DBS sampling. These questions were if patients i) managed to obtain the DBS samples, ii) experienced the training adequate and iii) preferred DBS sampling, venous sampling or had no preference.
The report of Madlensky et al. 5, describing a minimum therapeutic threshold of (*Z*)-endoxifen in serum of 5.9 ng/mL, was used as a guidance for tamoxifen treatment individualization. Our previously published bridging study concerning serum concentrations versus DBS concentrations, showed that a (*Z*)-endoxifen serum concentration of 5.9 ng/mL is equal to a DBS concentration of 3.9 ng/mL 9. If the (*Z*)-endoxifen DBS concentration was below 3.9 ng/mL, the clinician was advised to discuss with the patient the option to increase the tamoxifen dose.
This study was approved by the ethics committee of the Netherlands Cancer Institute and written informed consent was provided by each participant after full explanation of the purpose and nature of the procedure.

Bioanalysis

On arrival in the laboratory, the blood spots were visually inspected to determine whether they fulfilled all criteria for analysis; complete and symmetrical filling of the preprinted circle of at least 6 mm and dark red color on both sides of the paper (Figure

1). The DBS were stored at room temperature, in a zip-closure aluminum bag containing a desiccant package, until the time of analysis. Acceptable DBS patient samples, DBS calibration standards and quality control samples were extracted and analyzed according to a previously published HPLC-MS/MS method for the quantification of tamoxifen (2.5-250 ng/mL) and (*Z*)-endoxifen (0.5-50 ng/mL) [13].

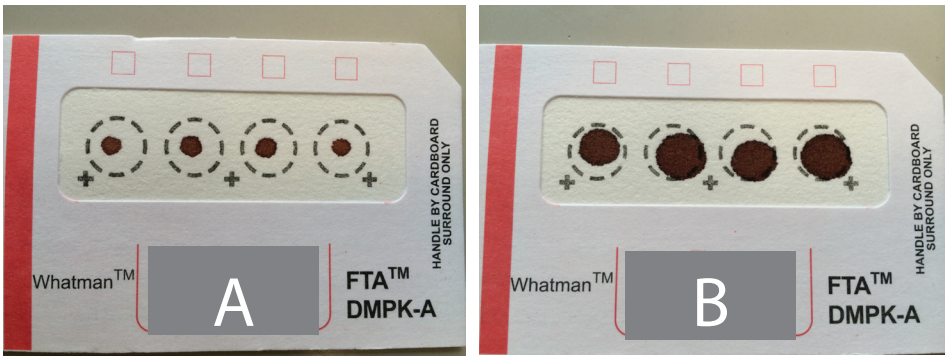

Figure 1 Dried blood spot samples obtained by self-sampling at home (A) fulfilling all criteria and (B) not fulfilling all criteria; not enough material to fill a circle of at least 6 mm

Results

Patients

In total, 38 patients, all female and with a mean age of 55 years (range 38-72), were included in this study. One patient used a dose of 10 mg tamoxifen per day, the other patients received a dose of 20 mg tamoxifen per day according to standard of care. In total, 36 of 38 patients (95%) returned the DBS samples and questionnaire by mail to the laboratory. Nine patients were contacted by telephone since no DBS sample was received within 3 weeks after recruitment and instruction. For seven of these patients, the DBS sample was received soon after the telephone call. In total, 31 patients (86%) provided at least one DBS suitable for analysis. The spots that were not usable for the analysis were mainly too small; i.e. the area of the 6 mm subpunch was not completely filled with blood on both sides of the card.

The questionnaire showed that 35 out of 36 patients (97%) patients found the training adequate, where one patient would have liked to receive more information during the training. The majority of the patients, 33 out of 36 (92%), stated that the self-sampling went well. Of these patients, 88% actually obtained at least one usable DBS.

One patient who stated she experienced difficulties with the sampling was not able to obtain a usable DBS, the other two patients obtained at least one usable DBS. In total, 61% (n=22) of the participants preferred DBS sampling over venous sampling, 11% (n=4) preferred venous sampling and 28% (n=10) had no preferred method.

Concentrations of tamoxifen and (*Z*)-endoxifen in DBS

Analyzed concentrations (n=31) of tamoxifen ranged from 40.8 to 190 ng/mL and for (*Z*)-endoxifen from 1.06 to 23.2 ng/mL. For 13 patients, the (*Z*)-endoxifen concentration was below the predefined DBS therapeutic threshold level of 3.9 ng/mL. One of these patients stated the use of a complementary alternative medicine, containing Salvia officinalis, that is reported to inhibit CYP metabolism [14] and the clinician was advised to discuss with the patient to stop the use of this drug. For the other 12 patients with low (*Z*)-endoxifen DBS concentrations, clinicians were advised to discuss with their patients the option to increase the tamoxifen dose.

Discussion

The results of this study show that the majority of the included patients is capable of performing DBS self-sampling at home and preferred this method over venous sampling. Determination of drugs in DBS is gaining popularity due to the simple and patient friendly sampling at home and easier transport and storage conditions. It could simplify logistics for clinical trials, since there is no need for a phlebotomist and samples can be transported and stored at room temperature. Also, it could be of interest for therapeutic drug monitoring purposes, since patients can obtain the sample at home by themselves and sent it to the laboratory by regular mail service. As a result, the clinician can have the analysis results of the patient sample before the patients' visit to the outpatient clinic. However, DBS sampling can only be implemented in clinical practice when the target population is capable and willing to perform DBS self-sampling at home. A few other studies evaluated home-based DBS sampling [15–18]. These studies all showed that the larger part of the included patients (83 – 95%) were able to provide at least one DBS suitable for analysis. Also, assessment of patient preferences showed a preference for DBS sampling over venous sampling (51 – 83%), where only a small part of the included patients preferred venous sampling (9 – 11%). These findings are in line with the results of our study in breast cancer patients, where 86% of the participants were able to provide at least one DBS suitable for analysis and 61% of the patients preferred DBS self-

sampling at home, where 11% of the patients preferred venous sampling.
For individualization of tamoxifen treatment, the minimum threshold (*Z*)-endoxifen serum concentration of 5.9 ng/mL suggested in the literature [5] was used as guidance. Of note, this value should be interpreted with caution, since it is the result of a single retrospective study. Future prospective studies are warranted to confirm this threshold level. However, since this is the only available report suggesting a certain threshold, this is the value used for treatment individualization described in this report.
One patient reported the use of a complementary alternative medicine (CAM) known to influence CYP enzymes. In this patient, the (*Z*)-endoxifen level was below the predefined threshold. Effect of comedication on active metabolite concentrations, via inhibition of CYP metabolism, is also reported in the literature [19–21]. Therefore, the use of medicines that could possibly affect CYP metabolism should be avoided during tamoxifen treatment, when this is possible. For this patient, the clinician was advised to discuss with this patient the option to discontinue the CAM. The other twelve patients with a DBS (*Z*)-endoxifen concentration below 3.9 ng/mL [9], were identified as eligible for a dose increase and the clinician was advised to discuss with their patients the option to increase the tamoxifen dose.
The main limitation of this study is possible selection bias for the recruited patients. Therefore, patient willingness and capability to self-sample at home might be overestimated.

Conclusion

Dried blood spot self-sampling at home is an acceptable and easy method for optimizing and personalizing tamoxifen treatment of breast cancer patients in the outpatient clinic.

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Clinical pharmacology of tamoxifen and its metabolites

3

Hot flashes are not predictive for serum concentrations of tamoxifen and its metabolites

3.1

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Abstract

Background. Tamoxifen has dramatically reduced the recurrence and mortality rate of estrogen receptor positive breast cancer. However, the efficacy of tamoxifen varies between individuals and 40% of patients will have a recurrence despite adjuvant tamoxifen treatment. Factors that predict tamoxifen efficacy would be helpful for optimizing treatment. Serum concentrations of the active metabolite, endoxifen, may be positively related to treatment outcome. In addition, hot flashes are suggested to be positively associated with tamoxifen treatment outcome.

Methods. We investigated in a series of 109 patients whether the frequency and severity of hot flashes were related to concentrations of tamoxifen and its metabolites. A serum sample of all patients was analyzed for the concentration of tamoxifen, *N*-desmethyltamoxifen, endoxifen and 4-hydroxytamoxifen, as well as for estradiol concentrations and several single nucleotide polymorphisms in CYP2D6. Additionally, these patients completed a questionnaire concerning biometric data and treatment side effects.

Results. We found no evidence supporting an association between concentrations of tamoxifen or metabolites and either the frequency or severity of hot flashes in the covariate unadjusted analyses. However, including interactions with menopausal status and pre-treatment hot flash (PTHF) history indicated that post-menopausal women with PTHF experienced an increasing frequency of hot flashes with increasing serum concentrations of tamoxifen and its metabolites. This finding was not altered when adjusting for potential confounding factors (duration of tamoxifen treatment, CYP2D6 phenotype, estradiol serum concentration, age and body mass index). In addition we observed a positive association between body mass index and both hot flash frequency ($p = 0.04$) and severity ($p < 0.0001$). We also observed that patients with lower estradiol levels reported more severe hot flashes ($p = 0.02$).

Conclusions. No univariate associations were observed between concentrations of active tamoxifen metabolites and either the frequency or severity of hot flashes during treatment. However, the frequency of hot flashes may be exacerbated by higher serum concentrations of tamoxifen and its metabolites in post-menopausal women with a history of hot flashes prior to tamoxifen treatment.

Introduction

For over 30 years tamoxifen, a selective estrogen receptor (ER) modulator, has been the standard treatment for estrogen receptor positive breast cancer patients, in both the adjuvant and metastatic setting. Tamoxifen has dramatically reduced the recurrence and mortality rate for patients with ER+ breast cancer [1]. However, as many as 40% of patients receiving adjuvant tamoxifen and almost all patients with metastatic disease eventually relapse and die from the disease [2]. Due to this high percentage of patients with an apparent lack of benefit, identification of early predictors of outcome of tamoxifen treatment may be helpful in the optimization of the treatment [3].
Tamoxifen itself is considered to be a prodrug that is converted into many metabolites. The metabolites with the highest therapeutic activity are 4-hydroxytamoxifen and *N*-desmethyl-4-hydroxytamoxifen (endoxifen), binding 100-fold more potent to the ER than tamoxifen itself [4]. The antiestrogenic activities of endoxifen and 4-hydroxytamoxifen are similar, although endoxifen, unlike 4-hydroxytamoxifen, also inhibits aromatase and is present at higher steady state concentrations in patients than 4-hydroxytamoxifen [4-7]. Recently, Madlensky et al. reported that low endoxifen levels are associated with worse outcome after tamoxifen treatment, suggesting that there is a minimum threshold serum level of endoxifen that when exceeded lowers the recurrence rate [8]. However, assays for routine measurement of concentrations of tamoxifen and its metabolites are not generally available in daily practice. Therefore, the quest for other biomarkers for treatment efficacy is still ongoing.
Tamoxifen is metabolized by cytochrome P450 (CYP) enzymes, in which the formation of endoxifen predominantly depends on CYP2D6. Inactivating genetic polymorphisms in CYP2D6 have been associated with lower endoxifen levels [9-11] and consequently CYP2D6 genotype has been suggested as a potentially useful marker for the prediction of treatment outcome. Recently, the ATAC and the BIG1-98 studies concluded that genetic variants of CYP2D6 are not predictive for outcome in tamoxifen-treated patients [12,13], although the validity of these findings has been questioned [14].
The occurrence of side effects, such as hot flashes, is a potential biomarker for treatment outcome, analogous to what has been described with EGFR inhibitors and skin-toxicity [15]. It is known that breast cancer patients treated with tamoxifen suffer more frequently from hot flashes, compared to placebo-treated breast cancer patients [16]. The severity of hot flashes is suggested to increase during the first three months of tamoxifen treatment, followed by a plateau or even a decrease for the duration of treatment [17,18].
Mortimer et al. showed that the occurrence of hot flashes is positively related to

outcome after tamoxifen treatment [19]. Cuzick et al. investigated whether the occurrence of treatment-related symptoms (vasomotor symptoms or joint symptoms) is associated with breast cancer recurrence. They found a trend that patients using tamoxifen who experienced newly emergent vasomotor symptoms (e.g. hot flushes, night sweats and cold sweats) had a lower recurrence rate, although these results were not statistically significant [20].

Recently, Lorizio et al. reported that the serum concentration of endoxifen is positively associated with the probability of reporting any side effect from tamoxifen (hot flashes, vaginal dryness, sleep problems, weight gain, and depression, irritability or mood swings combining all side effects and grades). When focusing on hot flashes only, this association was not statistically significant. Irvin et al. performed a genotyped tamoxifen dose-escalation study and found no correlation between endoxifen concentrations and the extent to which patients were bothered by hot flashes, neither at baseline nor at four months after dose escalation [10].

In order to clarify whether there is an association between concentrations of tamoxifen and its main metabolites and either frequency or severity of hot flashes, we investigated a series of 109 patients treated with tamoxifen, taking into account potentially influencing factors such as menopausal status, pre-treatment hot flashes, duration of tamoxifen treatment, CYP2D6 phenotype, estradiol serum concentrations, age and body mass index (BMI).

Methods

Patients, both pre- and postmenopausal, who used tamoxifen for at least two months at the moment serum concentrations of tamoxifen and metabolites were determined as part of routine clinical care were eligible for this study. Retrospectively, these patients were asked whether they would be willing to complete a single, short questionnaire concerning biometric data and the side effects they had experienced. The questionnaire was sent to the patients along with an informative letter, stating the goal of this study and explicitly giving the patients the option to opt-out, by returning the questionnaire without filling it out. By this questionnaire, patients were asked if they had been experiencing hot flashes prior to beginning tamoxifen treatment, and also if they experienced hot flashes during tamoxifen treatment (around the time the blood sample was drawn). In both cases the patients were asked to record the frequency of the flashes per week and the average severity of the experienced hot flashes (severity categories:

mild, <5 minute duration; moderate, 5 to 15 minute duration; severe, 15 to 20 minute duration; very severe, >20 minute duration). These definitions were based on the methodology and instruments for conducting hot flash studies [21,22].
We performed this observational study with a simple, single questionnaire according to the national act on Ethics Committees (Dutch Act on medical research involving humans, February 26, 1998) and in compliance with Good Clinical Practice guidelines [23]. As a further interpretation of these GCP guidelines there is the "code of conduct of Human Tissue and Medical Research: Code of conduct for responsible use (2011)" by the Federa (http://www.federa.org/codes-conduct). In this code of conduct is stated that anonymous left-over body material may be used in observational clinical trials without explicit consent of the individual patients.

Serum sample handling and determination of tamoxifen and metabolites
The serum samples were collected in serum gel tubes and stored at −70°C for some weeks, in order to analyze more patient samples during one HPLC-MS analysis. Patient samples, calibration standards and quality control samples were handled according to the method described by Teunissen et al. [24]. The liquid chromatography – tandem mass spectrometry (LC-MS/MS) method developed by Teunissen et al. [24] was slightly modified and used for the determination of tamoxifen (5 to 500 ng/mL), *N*-desmethyltamoxifen (10 to 1000 ng/mL), (*E*)-endoxifen (1 to 100 ng/mL), (*Z*)-endoxifen (1 to 100 ng/mL), *N*-desmethyl-4'-hydroxytamoxifen (1 to 100 ng/mL), 4-hydroxytamoxifen (0.4 to 40 ng/mL) and 4'-hydroxytamoxifen (0.4 to 40 ng/mL). Detection was performed on a triple-quadrupole MS/MS detector with an electrospray ionization source (API4000, AB Sciex, Foster City, USA) operating in the positive ion mode. A partial validation was executed and all requirements for acceptance, as defined in the FDA and EMA guidelines on bioanalytical method validation [25,26] were fulfilled.

Genotyping and predicted phenotype
DNA was isolated from 200 µL serum that was left over from the tamoxifen and metabolite analysis, using the MagNA Pure LC Total Nucleic Acid Isolation Kit I and the automated MagNA Pure™ LC system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's manual.
Genotyping was performed according to Standard Operating Procedures, using assays that were validated by direct sequencing. In each run, positive and negative controls were included. All patients were genotyped for *CYP2D6*3, *4, *6* and **41* variant alleles, which will identify 95% of CYP2D6 poor metabolizers (PMs) using Taqman allelic discrimination assays with primers and probes designed by Applied Biosystems

(Carlsbad, California, USA), as described earlier [27]. Polymerase chain reactions (PCR) were carried out in a reaction volume of 10 µl, containing 1 ng genomic DNA. The thermal profile consisted of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and 1 minute at 60°C for annealing and extension. Genotypes were scored through measuring allele-specific fluorescence using the SDS 2.2.2 software for allelic discrimination (Applied Biosystems).
On the basis of CYP2D6 genotype patients were classified into three predicted phenotype groups. Patients without nonfunctional alleles (*CYP2D6*3, *4* or **6*) were defined as extensive metabolizers (EMs). Intermediate metabolizers (IMs) consisted of patients that (i) carry *CYP2D6*41* alleles either homozygous or in combination with a nonfunctional allele or (ii) were heterozygous for the *CYP2D6*3, *4, *6* allele (**3/wt, *4/wt* or **6/wt*). Patients were classified as PM in case of two nonfunctional alleles (*CYP2D6*3/*3, *3/*4* or **4/*4*).

Estradiol concentration

The estradiol concentration was measured in the left over serum sample on a Modular Analytics E170 immunoassay analyzer, using the electrochemiluminescence technique (Roche Diagnostics), routinely used in the Netherlands Cancer Institute.

Statistical methods

The relation between hot flashes and several factors was investigated, where the serum concentrations of tamoxifen and three of its main metabolites (*N*-desmethyltamoxifen, endoxifen and 4-hydroxytamoxifen) were considered of primary interest. In addition there were seven secondary factors that may have a potential confounding role: menopausal status, a history of hot flashes prior to tamoxifen treatment, duration of tamoxifen treatment, estradiol serum concentration, age, BMI and CYP2D6 predicted phenotype. The association between all factors and menopausal status was assessed using Mann–Whitney-Wilcox, Fisher exact and linear-by-linear tests as appropriate. Spearman's rho was used to assess pairwise covariate associations between the four primary factors (tamoxifen and metabolite serum concentrations), age, BMI and estradiol concentration. Linear by linear trend tests were used to assess the association between CYP2D6 phenotype and the four primary factors. Kruskal-Wallis tests was used to determine if the four factors differed due to menopausal status and pre-treatment hot flash history. The association between reported hot flash frequency and both primary and secondary factors was assessed using over-dispersed Poisson models, both unadjusted (univariable) and multivariable regressions. Similarly, the association between all factors and the severity of hot flashes was assessed using proportional-

odds ordinal regressions. It was assumed that these associations may be influenced by menopausal status and the occurrence of pre-tamoxifen treatment hot flashes (PTHF). Due to the small number of pre-menopausal women reporting PTHF the influence of menopausal status and PTHF was assessed via pair-wise interactions with a three level menopausal and pre-treatment hot flash status variable (pre-menopausal versus post-menopausal & PTHF versus post-menopausal & no PTHF). In the multivariable analyses, estradiol concentrations were log transformed and missing estradiol and CYP2D6 values due to insufficient material were imputed with population medians. Due to the large number of individuals missing for the CYP2D6 assessments, sensitivity analyses were performed; once with these individuals imputed as poor-intermediate metabolizers and once excluding these individuals. For samples with an estradiol concentration level below the lower limit of quantitation (43 pmol/L), half of the lower limit of detection (21.5 pmol/L) was imputed. The level of significance for all tests was set at 0.05. The analysis was performed using the R (v3.0.1) using package MASS for ordinal regression and coin for linear by linear tests (http://cran.r-project.org/).

Results

Cohort

Between July 2008 and December 2011, serum samples from 165 patients treated with tamoxifen at the Netherlands Cancer Institute, Amsterdam, the Netherlands were obtained and analyzed for tamoxifen and metabolite concentrations. These 165 patients received the questionnaire. 33 patients did not respond to the questionnaire that was sent and 13 patients returned the reply form empty, thereby choosing the option to opt-out and not participate in this study. In total, 119 patients returned a filled out questionnaire, of which 115 forms were correctly completed. Six patients were excluded for the following reasons: one patient had an uncertain menopausal status at the moment of blood sampling; one patient was taking medication to relieve menopausal complaints; it turned out that two patients used tamoxifen less than two months at the moment of blood sampling and two patients used tamoxifen for distant metastases for an exceptionally long time (over 6 years). In total, 109 patients (all female, mean age 51 years (range 22–76)) were enrolled in the study. The patients were divided into two groups, based on menopausal status. Table 1 presents an overview of patient characteristics.

Table 1 Patient characteristics

Characteristic	Total cohort n=109 n (%)	Pre-menopausal n=56 n (%)	Post-menopausal n=53 n (%)	p-value
Median age at assessment (years)	51	45	58	<0.0001
Range	22 - 76	22 - 54	40 - 76	
Median Body Mass Index	24	24	24	0.44
Range	17 - 43	17 - 34	18 - 29	
T-status (TNM)				0.73
T1	48 (44%)	23 (41%)	25 (47%)	
T2	27 (25%)	16 (19%)	11 (21%)	
T3	2 (1.8%)	1 (1.8%)	1 (1.9%)	
Unknown	32 (29%)	16 (29%)	16 (30%)	
N-status (TNM				1.00
N0	45 (41%)	24 (43%)	21 (40%)	
N+	49 (45%)	26 (46%)	23 (43%)	
Unknown	15 (14%)	6 (11%)	9 (17%)	
AJCC stage (7th ed.)				0.87
Stage I	26 (24%)	14 (24%)	12 (23%)	
Stage IIa	32 (29%)	16 (29%)	16 (30%)	
Stage IIb	7 (6%)	5 (9%)	2 (4%)	
Stage IIIa	9 (8%)	4 (7%)	5 (9%)	
Stage IIIb	0	0	0	
Stage IIIc	6 (6%)	3 (5%)	3 (6%)	
Unknown	29 (27%)	14 (25%)	15 (28%)	
Estrogen receptor				NA
Positive	93 (85%)	50 (89%)	43 (81%)	
Unknown	16 (15%)	6 (11%)	10 (19%)	
Progesterone receptor				1.00
Positive	10 (9%)	5 (9%(	5 (9%)	
Negative	83 (76%)	45 (80%)	38 (72%)	
Unknown	16 (15%)	6 (11%)	10 (19%)	
HER2 status				1.00
Positive	38 (35%)	20 (36%)	18 (34%)	
Negative	55 (50%)	30 (54%)	25 (47%)	
Unknown	16 (15%)	6 (11%)	10 (19%)	
Median duration of treatment (months)	9	9	9	0.73
Range	2 - 70	2- 59	3 - 70	
Tamoxifen (daily dose)				0.17
10 mg	1 (1%)	1 (2%)	0 (0%)	
20 mg	102 (94%)	50 (89%)	53 (98%)	
40 mg	6 (6%)	5 (9%)	1 (2%)	

T-status: Tumor status, N-status: Lymph node status, HER2: Human Epidermal growth factor Receptor 2.

Table 2 shows that the serum concentrations of tamoxifen and its metabolites were not significantly different between pre- and postmenopausal patients. A total of 92 patients (84%) reported experiencing hot flashes during tamoxifen treatment, with considerable variation in reported hot flash severity. Of patients who reported experiencing no hot flashes before start of tamoxifen treatment, 65 (79%) reported developing hot flashes during treatment whereas all patients who reported experiencing hot flashes prior to starting tamoxifen treatment reported experiencing hot flashes during treatment. The frequency and severity of the reported hot flashes during tamoxifen treatment did not differ significantly between pre- and postmenopausal patients. For two patients, estradiol values were missing, due to an insufficient amount of input material. For 70 (64%) samples the analyzed estradiol concentration was below the lower limit of quantification (LLOQ, 43 pmol/L).

Genotyping

CYP2D6 genotype predicted phenotype was evaluable for 89 patients (81.7%). 5 (4.6%) patients were classified as poor metabolizers (PM), 30 (27.5%) as intermediate metabolizers (IM) and 54 (49.5%) as extensive metabolizers (EM) (see Table 2). For the other 20 patients (18.3%) the DNA quality was not sufficient to allow genotyping.

Covariate associations

Spearman's correlation coefficients indicated a positive association between tamoxifen and its three main metabolites and a negative association between age and estradiol levels. In addition, linear by linear tests indicated associations between CYP2D6 predicted phenotype and endoxifen ($p < 0.0001$), *N*-desmethyltamoxifen ($p = 0.009$) and 4-hydroxytamoxifen serum concentrations ($p = 0.05$), but not tamoxifen concentrations ($p = 0.65$). Kruskal-Wallis tests indicated no pairwise associations between the combined menopausal and PTHF status variable and tamoxifen nor its three metabolites.

Associations with hot flashes

In the univariable Poisson and ordinal regressions no associations were found between the levels of tamoxifen, endoxifen or the two other metabolites and either the frequency or severity of hot flashes (see Table 3). When including a pairwise interaction with menopausal and PTHF status it was observed that the associations between tamoxifen and metabolite serum concentrations and the frequency of hot flashes were increasing for post-menopausal women with a pre-treatment history of hot flashes (see Table 3). Adjusting for potential confounding factors did not alter these results. Figure 1 presents the associations between serum concentrations of tamoxifen and its metabolites and

Table 2 Hot flash frequency and severity and pharmacological and biochemical characteristics of study participants during treatment with tamoxifen

Characteristic	Total cohort	Pre-menopausal	Post-menopausal	p-value
	n=109 n (%)	n=56 n (%)	n=53 n (%)	
Pre-treatment history of hot flashes				0.04
No	82 (75%)	47 (84%)	35 (66%)	
Yes	27 (25%)	9 (16%)	18 (34%)	
Median frequency of hot flashes per week	21	21	21	0.77
Range	0 - 168	0 - 168	0 - 168	
Average severity of hot flashes				0.56
None	17 (16%)	9 (16%)	8 (15%)	
Mild	22 (20%)	13 (23%)	9 (17%)	
Moderate	55 (50%)	26 (46%)	29 (55%)	
Severe	11 (10%)	7 (12%)	4 (8%)	
Very severe	4 (4%)	1 (2%)	3 (6%)	
Median tamoxifen (ng/mL)	95.4	93.8	97.9	0.61
Range	39.7 - 237	50.0 - 220	39.7 - 237	
Median *N*-desmethyltamoxifen (ng/mL)	181	177	187	0.82
Range	82.3 - 532	94.3 - 532	82.3 - 439	
Median endoxifen (ng/mL)	9.12	8.59	9.16	0.75
Range	1.73 - 22.6	1.73 - 20.3	2.14 - 22.6	
Median 4-hydroxytamoxifen (ng/mL)	1.69	1.77	1.43	0.91
Range	0.74 - 4.23	0.74 - 4.23	0.78 - 3.51	
Median estradiol (pmol/L)				0.06
<LLOQ*	70	33	37	
43.0 - 67.0	12	6	6	
67.0 - 361	14	8	6	
> 361	11	9	2	
Missing	2	0	2	
CYP2D6 phenotype				0.66
Extensive metabolizer	54 (50%)	28 (50%)	26 (49%)	
Intermediate metabolizer	30 (28%)	19 (34%)	11 (21%)	
Poor metabolizer	5 (5%)	2 (4%)	3 (6%)	
Missing	20 (18%)	7 (12%)	13 (24%)	

* < LLOQ is below the minimal quantification limit.

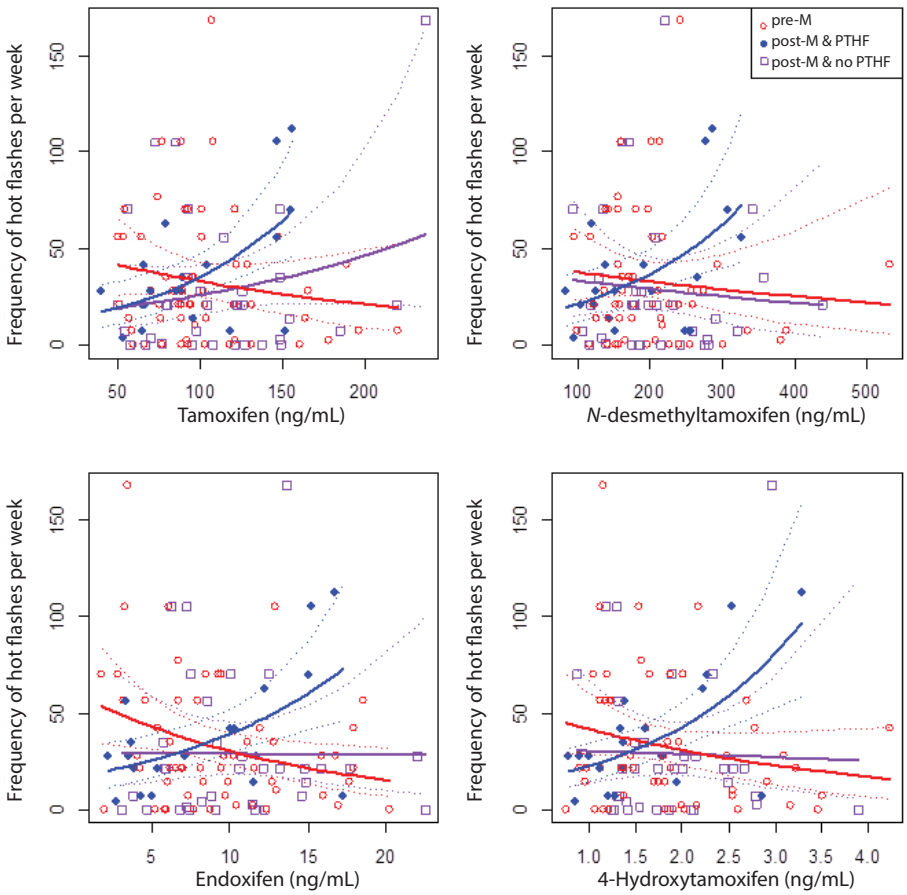

Figure 1 Hot-flash frequency plotted against tamoxifen and its metabolites, for pre- and post-menopausal women separately
Pre-M: pre-menopausal patients, post-M: post-menopausal patients. PTHF: pretreatment hot flash history.

patient-reported hot flash frequency in the menopausal and PTHF subgroups. Positive associations were found between BMI and both hot flash frequency ($p = 0.04$) and severity ($p < 0.0001$) (Table 3A). We also observed that pre-menopausal patients with lower estradiol levels reported more severe hot flashes ($p = 0.02$) (Table 3B). Both of these results remained significant in the multivariable analyses. The sensitivity analyses indicated that the estimated coefficients were unaffected by the imputation of the missing CYP2D6 levels. While the tests for interaction remained significant when the missing data were imputed (both as poor-intermediate and as extensive metabolizers), these tests were non-significant in the analysis excluding missing values, possibly due to the 18% reduction in sample size. CYP2D6 predicted phenotype was not associated with hot flash frequency ($p = 0.61$) nor hot flash severity ($p = 0.99$) (Table 3).

Table 3 Univariable Poisson regression associations with hot flash frequency (3A) and ordinal regression associations with hot flash severity (3B)

3A	Univariable (n=109)		Inter.	Pre-M (n=56)	Post-M & PTHF (n=18)	Post-M & no PTHF (n=35)							
	Coef	SE	p-value	p-value	Coef	SE	p-value	Coef	SE	p-value	Coef	SE	p-value
Tamoxifen	0.002	0.0024	0.41	0.03	-0.0045	0.004	0.27	0.012	0.0038	0.01	0.0058	0.0044	0.19
N-desmethyltamoxifen	–0.00002	0.0013	0.99	0.13	-0.0013	0.002	0.50	0.0053	0.002	0.02	-0.0014	0.0031	0.66
Endoxifen	-0.015	0.022	0.50	0.01	-0.069	0.03	0.03	0.085	0.028	0.01	-0.0021	0.05	0.97
4-Hydroxytamoxifen	-0.05	0.14	0.73	0.03	-0.3	0.19	0.13	0.63	0.17	0.002	-0.056	0.37	0.88
post-M & PTHF v pre-M	0.13	0.27	0.67										
post-M & no PTHF v pre-M	-0.14	0.24											
Age	-0.0059	0.0098	0.55										
Estradiol concentration	-0.12	0.095	0.21										
BMI	0.048	0.023	0.04										
Tamoxifen duration	0.084	0.087	0.34										
CYP2D6: EM versus I/PM	-0.11	0.21	0.61										
3B	**Univariable (n=109)**		**Inter.**	**Pre-M (n=56)**	**Post-M & PTHF (n=18)**	**Post-M & no PTHF (n=35)**							
	Coef	SE	p-value	p-value	Coef	SE	p-value	Coef	SE	p-value	Coef	SE	p-value
Tamoxifen	0.0026	0.0045	0.57	0.60	0.0026	0.0065	0.69	0.018	0.014	0.16	0.0019	0.0075	0.80
N-desmethyltamoxifen	-0.00043	0.0022	0.85	0.30	-0.00048	0.003	0.88	0.01	0.0067	0.11	-0.0024	0.0039	0.53
Endoxifen	-0.013	0.039	0.73	0.72	-0.027	0.055	0.62	0.044	0.092	0.63	0.025	0.072	0.73
4-Hydroxytamoxifen	-0.20	0.25	0.43	0.64	-0.11	0.31	0.74	0.39	0.070	0.58	-0.36	0.52	0.48
post-M & PTHF v pre-M	0.94	0.51	0.11										
post-M & no PTHF v pre-M	-0.17	0.42											
Age	-0.018	0.017	0.31										
Estradiol concentration	-0.34	0.14	0.02										
BMI	0.19	0.048	<0.0001										
Tamoxifen duration	0.25	0.18	0.15										
CYP2D6: EM versus I/PM	0.0058	0.41	0.99										

Inter: Interaction,.pre-M: pre-menopausal patients, post-M: post-menopausal patients, PTHF: pre-treatment hot flashes, v: versus, Coef: coefficient, SE: standard error, BMI: body mass index, EM: extensive metabolizers, I/PM: intermediate to poor metabolizers.
For tamoxifen and its metabolites the test of interaction with menopausal and PTHF status, and the within-group associations are also reported

Discussion

In this study we were unable to find evidence supporting the hypothesis that either frequency or severity of hot flashes are associated with higher levels of tamoxifen or any of its main metabolites during treatment in our entire cohort, consisting of both pre- and postmenopausal patients. No differences were detected in the frequency of reported hot flashes between pre- and post-menopausal women, however the association between concentrations of tamoxifen and its metabolites and patient-reported hot flash frequency appeared to be influenced by menopausal status and pre-treatment hot flash history.

Previously, Lorizio et al. have suggested that the endoxifen serum concentration was associated with increased risk of hot flashes, although this finding was not statistically significant [28]. Irvin et al. found no association between the extent to which patients were bothered by hot flashes and endoxifen concentration, neither at baseline, nor at four months after dose escalation [10]. We initiated this study to investigate the association of concentrations of tamoxifen and its main metabolites and both severity and frequency of hot flashes, taking potential confounding factors, such as menopausal status, pre-treatment hot flash history, duration of tamoxifen treatment, CYP2D6 phenotype, estradiol levels, age and BMI, into account. We could, however, find no evidence to support this hypothesis in the whole cohort. In the earlier mentioned BIG1-98 study, the authors also investigated hot flash incidence and the aggravation of hot flashes in the first two years of tamoxifen therapy. They found an association between CYP2D6 phenotype and tamoxifen-induced hot flashes ($p = 0.02$): both PM and IM phenotypes had an increased risk of tamoxifen-induced hot flashes compared with EM phenotype [13], contradictory to what was expected. Additionally, Sestak et al. [29] and Goetz et al. [30] reported that they were unable to detect an association between CYP2D6 phenotype and the occurrence of hot flashes. In this study we also found no evidence supporting the hypothesis that either hot flash frequency or severity is associated with CYP2D6 predicted phenotype, however genotyping data was missing in 18% of the cases. The large percentage of genotyping failures can be explained by the fact that DNA was isolated from serum, since this matrix was left over from the tamoxifen and metabolite analysis, which is a reproducible and validated method for genotyping in our lab, however the yield is low. Although the physiology of hot flashes, in both healthy women and women with breast cancer, remains unclear, it has been observed that healthy postmenopausal women who experience hot flashes have lower estradiol levels than women who do not experience hot flashes [31-34]. In our series, we correspondingly observed that patients, especially pre-menopausal patients, with lower

estradiol levels reported more severe hot flashes.

Another physiological factor that may influence the occurrence of hot flashes in healthy women is body mass index (BMI), although this relationship is still a matter of debate. Some studies found a positive association [35], others a negative association [36,37] or no association [38]. In our series patients with higher BMIs reported suffering from more frequent and severe hot flashes.

Tamoxifen is metabolized into many different metabolites by cytochrome P450, the formation of endoxifen is mainly dependent on CYP2D6 activity. As with other studies [9-11], we were able to demonstrate a positive association between CYP2D6 activity and serum concentrations of active tamoxifen metabolites.

Our study has the following limitations. The hot flash data was collected retrospectively. Consequently, we are unable to completely exclude recall-bias concerning the grade and frequency of the hot flashes. Also, the modest sample size of this retrospective study requires that these results should be interpreted with care. Furthermore, only a single questionnaire was completed per patient, and as such we are unable to identify fluctuations in frequency and severity of hot flashes over the course of the tamoxifen treatment period. To adjust for any potential confounding, the duration of tamoxifen treatment was included as a covariate in the analyses. Finally, we have insufficient data concerning co-medication, other than medication to relieve hot flashes, to include this factor in our analyses, however, in the ATAC analyses medication use was not found to be an independent predictor [12].

This is the first study reporting a difference within post-menopausal patients based on their pre-treatment hot flash history in the association between tamoxifen and its main metabolite serum concentrations and hot flash frequency. This possible effect should be investigated further and requires validation in other series.

As we are unable to show that hot flash assessments are unambiguously indicative for therapeutic serum concentrations of endoxifen, and given that the value of pharmacogenomics is currently under debate, we think that future research could focus on measurement of active metabolite concentrations as a potential surrogate biomarker for tamoxifen efficacy.

Conclusions

We are unable to confirm positive associations between active tamoxifen metabolite concentrations and either the frequency or severity of hot flashes during tamoxifen treatment, when ignoring menopausal status and pre-treatment hot flash history. However, within the post-menopausal women experiencing hot flashes prior to treatment, there is evidence for positive associations between serum concentrations of tamoxifen and its metabolites with hot flash frequency.

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Tailored tamoxifen treatment for breast cancer patients

3.2

NGL Jager, SC Linn, JHM Schellens, JH Beijnen

A position paper

Abstract

Tamoxifen, an endocrine agent, is widely used in the treatment of estrogen receptor-positive breast cancer. It has greatly reduced recurrence and mortality rates of breast cancer patients, however, not all patients benefit from tamoxifen treatment as in about 25-30% of the patients the disease recurs. Many researchers have sought to find factors associated with endocrine treatment outcome in the past years, however this quest has not been finished yet. It seems clear that there are several potential mechanisms by which resistance to endocrine therapy might evolve. In this paper, we focus on one factor that might influence outcome of tamoxifen treatment; inter-patient variability in tamoxifen pharmacokinetics. In recent years it has become clear that tamoxifen undergoes extensive metabolism and that some of the formed metabolites are much more pharmacologically active than tamoxifen itself. Despite the wide inter-patient variability in tamoxifen pharmacokinetics and pharmacodynamics, all patients receive a standard dose of 20 mg tamoxifen per day. The issue of the suitability of individual dosing for tamoxifen treatment continues to fuel controversies, therefore, we make a plea for further clinical pharmacological research to investigate the value of individual dosing for tamoxifen treatment optimization.

Introduction

Each year, around 1.4 million cases of breast cancer are diagnosed across the world [1]. About 70% of the breast cancers are estrogen receptor (ER) positive; their growth is thought to be dependent on the binding of estrogen to the ER on tumor cells. Endocrine therapy forms the cornerstone of systemic treatment for women with ER positive breast cancer at every stage of management and is directed against the growth-stimulating effects of estrogen on breast tissue. Endocrine agents abrogate estrogenic signaling through distinct mechanisms; either by impeding the transcriptional activity of the ER or by diminishing estrogen synthesis. The most widely used endocrine agent is tamoxifen, which is used by either pre- and postmenopausal women. Tamoxifen binds to the ER, leading to an altered receptor conformation and thereby preventing the binding of co-activators and inhibiting transcription [2]. The development of aromatase inhibitors (AIs) has provided an alternative form of endocrine therapy. Recently, these agents have become the standard treatment for most postmenopausal women with ER positive breast cancer. AIs suppress estrogen levels in postmenopausal women by inhibiting aromatase, the enzyme responsible for the synthesis of estrogens from androgenic substrates [3]. Before menopause, the major source of estrogen is production in the ovaries, which is not catalyzed by aromatase, and therefore AIs are not indicated for premenopausal women. After menopause, aromatization of androgens in adipose tissue and muscle is the major source of circulating estrogens. For premenopausal women, guidelines dictate the use of adjuvant tamoxifen for 5 years and the recently published ATLAS and aTTom trials even suggest to use tamoxifen for 10 years [4,5]. The recommended adjuvant endocrine therapy in postmenopausal women is either an aromatase inhibitor for at least 5 years, or sequential treatment with tamoxifen followed by an aromatase inhibitor or vice versa. Despite improvements in recurrence rates and breast cancer mortality using these adjuvant therapies, around 25-30% of breast cancer patients relapse within 10 years and will eventually die from the disease [6]. This relatively high number of patients are at risk for side effects, but will not gain benefit of the endocrine treatment. Understanding the mechanisms underlying resistance is of importance to determine whether a patient is likely to benefit from the intended treatment. Many researchers have sought to find factors associated with endocrine treatment outcome in the past years, however this quest has not been finished yet [7]. It seems clear that there are several potential mechanisms by which resistance to endocrine therapy might evolve. These include variation in expression of the ER, modifications of the ER, increased levels or activity of ER coactivators, ER independent growth by additional activated growth factor signaling pathways or stabilization of the

ER despite the presence of tamoxifen [7,8]. Apart from these tumor-related mechanisms, also patient-related factors may influence the response to endocrine therapy. In this paper, we focus on a patient-related factor that might influence outcome of tamoxifen treatment; inter-patient variability in tamoxifen pharmacokinetics. In recent years it has become clear that tamoxifen undergoes extensive metabolism and that some of the formed metabolites are much more pharmacologically active than tamoxifen itself. Despite the wide inter-patient variability in tamoxifen pharmacokinetics and pharmacodynamics, all patients receive a standard dose of 20 mg tamoxifen per day. The issue of the suitability of individual dosing for tamoxifen treatment continues to fuel controversies, therefore, we make a plea for further clinical research to investigate the value of individual dosing for tamoxifen treatment optimization.

Pharmacokinetics of tamoxifen

The metabolism of tamoxifen leads to the formation of at least 22 phase I metabolites in humans [9,10]. The main metabolic pathway involves demethylation, particularly by CYP3A4/5, to form *N*-desmethyltamoxifen, which is next hydroxylated by CYP2D6 to N-desmethyl-4-hydroxytamoxifen (endoxifen). To a smaller extent tamoxifen is

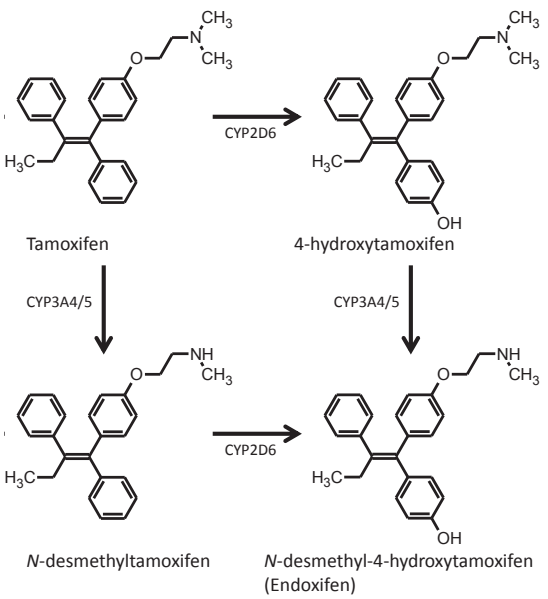

Figure 1 Part of the biotransformation of tamoxifen

hydroxylated to form 4-hydroxytamoxifen, which is subsequently demethylated to endoxifen. This part of the biotransformation of tamoxifen is depicted in Figure 1. Tamoxifen is administered as a pure *Z* (zusammen) isomer [11] and its metabolites are also generated primarily in the *Z*-form. There are large differences in the pharmacological activity of the tamoxifen metabolites, where (*Z*)-endoxifen and (*Z*)-4-hydroxytamoxifen are reported to have the highest anti-estrogenic activity, being 30 to 100-fold more potent towards the ER than *N*-desmethyltamoxifen and tamoxifen itself [12]. (*Z*)-endoxifen is suggested to be the most important metabolite, considering it is present at a steady-state serum concentration about five times higher than (*Z*)-4-hydroxytamoxifen in patients using tamoxifen [13]. Two *in vitro* studies have fueled the controversies around the added value of the metabolites to tamoxifen efficacy, by showing that the ER is already saturated by more than 99% in the presence of tamoxifen alone [14,15]. However, these highly simplified in vitro models have several limitations, for example, the high proportion of tamoxifen bound to proteins and the binding of tamoxifen to sites other than the ER are not taken into account [15]. Moreover, another *in vitro* study was conducted to determine whether effects of endoxifen on breast cancer cells were maintained in the presence of tamoxifen and its primary metabolites. In this study, ER positive breast cancer cells were simultaneously exposed to tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen, in a fixed concentration similar to what is found in patients using 20 mg tamoxifen per day, and increasing concentrations of endoxifen. The results of this experiment showed that the addition of endoxifen affected cell transcription and proliferation, and that this effect was concentration dependent [16], contradicting the theory that the role of endoxifen in tamoxifen treatment efficacy would be irrelevant.

The combination of wide inter-patient variability in tamoxifen pharmacokinetics and large differences in biological activity of tamoxifen metabolites form the rationale for individual dosing of tamoxifen. Different approaches can be pursued to individualize tamoxifen treatment; genotyping, phenotyping and therapeutic drug monitoring. These three strategies will be discussed concisely below.

Approaches to personalize tamoxifen treatment

Genotyping

Based on the finding that the formation of (*Z*)-endoxifen predominantly depends on CYP2D6, genotyping patients for CYP2D6 polymorphisms has been suggested as a tool

to individualize tamoxifen therapy. Inactivating genetic polymorphisms in CYP2D6 are reported to be associated with lower (*Z*)-endoxifen levels [17–19] and several studies showed an association between CYP2D6 genotype and recurrence free survival [20–23]. Other studies failed to show this association [24–26] and recently, the large ATAC and BIG1-98 studies concluded that genetic variants of CYP2D6 are not predictive for outcome in tamoxifen-treated patients [24,27]. However, the validity of these findings has been questioned. The main remark on the pharmacogenomic part of the BIG1-98 trial was the large deviation from the Hardy-Weinberg equilibrium, which is suggested to be caused by the source of the genotype; this was obtained from the tumor genome and not from the host genome, or by deviation from the standard genotyping assay protocol [28]. The results of the ATAC study have been criticized because of the insufficient statistical power in the pharmacogenomic study population [28]. As a result, the value of genotyping to optimize tamoxifen treatment and dosing remains unclear, hitherto. Genotyping provides time invariant information on the individual patient's metabolizing capacity, however, other factors such as nutrition, environmental factors and comedication are also known to affect tamoxifen pharmacokinetics. Of importance is the concomitant use of selective serotonin reuptake inhibitors, that are commonly prescribed to patients using endocrine therapy in order to treat depression and to alleviate hot flash symptoms. This group of drugs, paroxetine in particular, is reported to possibly affect endoxifen levels by inhibition of CYP2D6 [29–31]. Another drawback of genotyping is the dependency on the SNPs that can be identified by the specific genotyping assay that is used. Moreover, the finding of new SNPs affecting CYP activity could possibly change the formerly used classification and affect study results.

Phenotyping

A phenotyping probe, a drug metabolized in a similar way as the target drug, can be used to predict the individual pharmacokinetic profile of the target drug. By this approach, a combination of genotype and other factors influencing the metabolism such as nutrition and comedication are taken into account. Dextromethorphan was suggested as a possible phenotyping probe, and its serum levels were shown to be suitable to predict endoxifen exposure [32]. Also, a ^{13}C-dextromethorphan breath test could adequately predict endoxifen serum levels [33]. However, both procedures are rather time-consuming and a somewhat cumbersome approach to predict the endoxifen exposure. Moreover, although the metabolism of the probe and target drug is similar, it is never identical. Also, when comedication or other influencing factors change, the tamoxifen biotransformation can be affected and the phenotyping results from the initial test are not usable anymore.

Therapeutic drug monitoring

Therapeutic drug monitoring (TDM), using actual measured drug concentrations instead of predicting them, seems the most direct way to individualize tamoxifen treatment. With TDM, the combined result of all factors affecting tamoxifen pharmacokinetics, such as genotype, comedication, nutrition and other (unknown) factors is measured. Also, TDM can be used to establish compliance, which is reported to be a point of concern in long term tamoxifen treatment [34]. The wide interindividual differences in systemic exposure of endoxifen, a positive dose–exposure relationship [13,17,18] and a positive exposure–efficacy relationship [35] form the rationale for application of TDM. The association between exposure and treatment outcome is the most important prerequisite for TDM. However, the essential role of tamoxifen metabolites was only suggested several years ago and clinical studies investigating treatment outcome take many years to be completed. As a result, at this moment only one report describing an association between metabolite concentrations and outcome has been published [35]. The WHEL study was initiated to investigate the effect of a healthy diet on reducing breast cancer events and early death in women with early-stage invasive breast cancer; the analysis of an association between active metabolite levels and outcome was secondary to the original hypotheses. A total of 1370 ER-positive breast cancer patients included in this study used tamoxifen and a significant correlation between endoxifen serum levels and breast cancer outcome was reported. The results of this study show that patients with an endoxifen serum concentration above 5.9 ng/mL, 80% of the included patients, had a 26% lower recurrence rate than patients with a lower endoxifen serum concentration (hazard ratio 0.74; 95% confidence interval [0.55 - 1.00]) [35]. Previous research has shown that most patients who receive a dose increase based on low (*Z*)-endoxifen levels when using a standard dose of 20 mg per day, are able to reach the threshold level when using 30 or 40 mg tamoxifen per day [13].

Indirect evidence for a dose–effect relation originates from the Oxford Overview data, where a significant test for trend was found for improved recurrence rates with increasing doses of adjuvant tamoxifen (20, 30 and 40 mg per day) [6]. Also, several in vitro studies have shown that the anti-estrogenic effects of (*Z*)-endoxifen are concentration-dependent [16,36,37]. Several studies investigating tamoxifen at different dose levels (20 vs. 30 or 40 mg per day) showed no significant difference in the frequency and severity of the experienced side effects, but did not report data on long-term serious toxicity [38, 39]. However, these studies included a relatively limited number of patients and no data on long-term serious toxicity, such as endometrial cancer, were reported. One report suggests a slightly higher risk of endometrial cancer when using 40 mg tamoxifen per day, compared to 20 mg tamoxifen per day [40].

Given the long half-life of tamoxifen and its metabolites [41], wide variability in serum concentrations of tamoxifen and its metabolites at steady-state during the day is not expected, therefore it is not required to obtain patient samples at specific time points. Furthermore, intra-patient variability over several months is shown to be low [13], indicating that serum concentrations of tamoxifen and its metabolites are fairly constant during the steady-state phase of tamoxifen treatment. However, a patients' comedication and nutrition can change over time; obtaining TDM samples on a regular basis will be of value to monitor the effects of these factors on metabolite levels.

Future perspectives

At this moment, TDM seems the most direct and promising approach to tailor tamoxifen treatment. However, incontrovertible evidence for an exposure-outcome relationship is lacking at this point in time. Also, the complex metabolism of tamoxifen leads to the formation of multiple metabolites, with different pharmacological activities. In recent years, the focus has been on endoxifen alone, however, the contribution of other metabolites to treatment outcome cannot be ruled out. A possible strategy to investigate this could be to establish an Antiestrogenic Activity Score (AAS) based on *in vitro* activity of different metabolites, as suggested by Barginear et al. [17], and to correlate this score to treatment outcome.

In order to investigate the added value of TDM in treatment efficacy, a randomized prospective clinical trial is warranted. A possible study design could be to randomize patients who are about to start adjuvant tamoxifen therapy into two groups; group 1 starts with 20 mg tamoxifen per day, according to standard of practice, and a TDM sample is obtained every three months. Based on the analyzed metabolite levels, these patients are advised to increase their daily dose to 30 or 40 mg tamoxifen per day. At this time, the suggested serum concentration threshold of 5.9 ng/mL endoxifen could be used, until more studies suggesting a certain threshold or demonstrating the value of an AAS become available. The control group receives 20 mg tamoxifen per day, according to standard of practice and receives no dose adjustment.

Recently, a dried blood spot (DBS) method for the quantification of tamoxifen and (*Z*)-endoxifen was developed [42]. With DBS sampling, a sample is obtained by means of a finger prick and drops of blood are collected on a DBS card. The use of DBS sampling in a prospective clinical trial enables easier logistics; the patient can self-sample at home and there is no need for a phlebotomist. Furthermore, the samples can be transported using

regular mail service and stored at room temperature, since no special transport and storage conditions are required.

In conclusion, individualization of tamoxifen treatment based on active metabolite levels might be of value to reduce resistance to tamoxifen treatment. However, one should be aware that there are multiple mechanisms that can contribute to and induce resistance to tamoxifen. In order to fully personalize breast cancer treatment, it is of importance that also other markers possibly predicting resistance, and thus treatment outcome, are identified. Many *in vitro* studies have provided leads to understand the biology of endocrine therapy resistance, now is the time to validate these in clinical practice. The appreciation of the heterogeneity of possible resistance inducing pathways within the same tumor will help to choose the right drug, or maybe the right combinatory treatment, at the right dose, for the right patient.

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Conclusions and perspectives

Conclusions and perspectives

Tamoxifen is widely used in the treatment of estrogen receptor (ER) positive breast cancer. Although it significantly reduced breast cancer recurrence and mortality, not all patients benefit from tamoxifen as in about one-third of the patients the disease recurs despite adjuvant treatment with tamoxifen [1]. In order to optimize the treatment, it is of importance to identify mechanisms underlying this treatment failure. A possible predictive factor for tamoxifen treatment outcome is the patient specific level of active metabolites of tamoxifen. 4-Hydroxytamoxifen and endoxifen are considered to be the most biologically active, and a retrospective analysis suggests a correlation between endoxifen serum levels and recurrence free survival [2]. The aim of this thesis project was to develop sensitive and selective bioanalytical assays to quantify tamoxifen and its metabolites in patient samples, and to implement these assays in clinical practice.

Bioanalytical aspects

The availability of reliable and sensitive bioanalytical methods is a prerequisite in the determination of drug exposure in patients. Currently, the golden standard for drug quantification is the use of high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). We developed and successfully validated a sensitive and selective HPLC-MS/MS method to simultaneously analyze tamoxifen and five of its metabolites in patient serum samples.

The mean serum levels of tamoxifen and its metabolites obtained by different clinical studies reported in the literature, that all included patients using 20 mg tamoxifen per day and analyzed the samples with HPLC–MS/MS, differ largely, more than expected purely based on inter-patient variability. Our research showed that lack of selectivity of the analysis method causes a factor 2-3 overestimation of the concentration of endoxifen and 4-hydroxytamoxifen, by overlapping chromatographic peaks of other tamoxifen metabolites that show marked resemblance in molecular structure and have similar masses and fragmentation patterns. These results emphasize the use of highly selective HPLC-MS/MS assays for the accurate quantification of tamoxifen and its metabolites in patient samples.

Dried blood spots

Dried blood spot sampling, where samples are obtained by a finger- or heel prick instead of via a venapuncture, is an alternative to venous sampling. In this thesis, we present an overview of bioanalytical challenges related to DBS assays, followed by a set of concrete recommendations for the validation of a DBS-based assay. This report will help

researchers to execute a thorough validation of their DBS-based assay, in order to obtain robust and reliable assays for the support of (pre)clinical studies and therapeutic drug monitoring (TDM).

To ease the logistics of future clinical trials and TDM, an HPLC-MS/MS assay to determine tamoxifen and (*Z*)-endoxifen in DBS was developed. Regular pure cellulose DBS cards, Whatman 903 cards, were shown to be unsuitable for this application, due to an uncomprehended increasing extraction recovery over time for endoxifen. When using coated DBS cards, Whatman DMPK-A cards, the analytes were both fully recovered from the matrix and the extraction recovery remained constant over time. We demonstrated that the difference in extraction recovery between the two types of DBS cards is mainly attributable to the presence of SDS, one of the constituents of the DMPK-A coating. Also, the presence of a physical coating further optimized the extraction recovery of endoxifen. Although SDS caused matrix effects, the stable isotope labeled internal standards were most effective to compensate for these effects. The assay using DMPK-A cards was fully validated according to FDA and EMA guidelines and the recommendations described in this thesis, and showed good performance. Of importance is the finding that adequate concentration levels for both analytes could be measured in DBS with haematocrit (Hct) values between 29 and 48%, a wide enough range to include all adult females with Hct values within and just outside the normal range (36 to 46% [3]). The analytes were shown to be stable in DBS when stored at room temperature for at least four months, and at the temperature range expected during transport; 2-8˚C to 37˚C, for 24 hours. These results indicate that the DBS samples can be sent by regular mail service and can be stored for a period of time that is feasible for the support of clinical studies and TDM.

DBS concentrations are usually not similar to serum concentrations, due to differences in analyte-specific blood cell-to-serum partitioning ($K_{BC:serum}$) and patient-specific haematocrit (Hct). The bridging study described in this thesis showed that the difference between tamoxifen and endoxifen concentrations in DBS and serum samples can very well be explained with the formula $[Analyte]_{serum,\ calculated} = [Analyte]_{DBS}/([1-Hct]+ K_{BC:serum} * Hct)$. The blood cell-to-serum partitioning was found to be independent of the concentration for both analytes and could therefore be imputed as a fixed value in the formula. Also, Hct could be imputed as a fixed value in this formula, since our target population consists of a relatively homogenous group of patients; adult females treated for early breast cancer and receiving adjuvant tamoxifen treatment, where physiological conditions having a major effect on Hct values are not expected. When using fixed values for both blood cell-to-serum partitioning and Hct, two simple formulas for the conversion of DBS concentrations to serum concentrations were obtained:

$[Tamoxifen]_{serum,\ calculated} = [Tamoxifen]_{DBS}/0.779$ and
$[Endoxifen]_{serum,\ calculated} = [Endoxifen]_{DBS}/0.663$.
Additionally, it is shown that patients using tamoxifen are capable of collecting DBS samples by themselves at home and that they prefer this sampling method over venous sampling. These results enable DBS sampling for the determination of tamoxifen and endoxifen concentrations for clinical studies and TDM purposes. Implementation of this sampling method in clinical practice will result in easier logistics for both the patient, who can self-sample at home, and the for clinician and/or researcher, since there is no need for special transport or storage conditions.

Clinical pharmacological aspects

Wide inter-individual differences in systemic exposure of a drug in combination with a positive dose-exposure relationship are prerequisites for the use of individual dosing in order to reach target serum concentrations. In a large cohort of outpatients in the Netherlands Cancer Institute, wide inter-individual differences, but low intra-individual differences, in systemic exposure to tamoxifen and its metabolites were demonstrated. Additionally, it was shown that the prescribed tamoxifen dose was related to endoxifen exposure and that increasing the tamoxifen dose leads to a significantly higher serum concentration of tamoxifen and its metabolites.
Next to the levels of the active metabolites, also the occurrence and severity of side effects might be predictive for treatment outcome. In our study, however, the frequency and severity of hot flashes, a common side effect of tamoxifen treatment, was not related to serum concentrations of tamoxifen and its metabolites.

Future perspectives

Further clinical research to investigate the value of TDM for tamoxifen treatment optimization is highly encouraged. Two prerequisites for TDM; wide inter-individual variability and a positive dose-exposure relationship, are demonstrated in this thesis. However, incontrovertible evidence for an exposure-outcome relationship, the third prerequisite for TDM, is lacking at this point in time.
Of importance is the determination of an adequate target level of the active metabolites of tamoxifen. A previously published retrospective trial suggested a threshold level of 5.9 ng/mL endoxifen [2]. This value should be confirmed in more cohorts before it can be used in clinical practice.
Secondly, a prospective randomized clinical trial where one group receives a TDM-based dose of tamoxifen, and where the control group receives the standard dose of 20 mg tamoxifen per day, will be of great value. The DBS method described in this thesis will

ease the logistics of these clinical trials, since patients can self-sample at home and no special conditions for transport or storage of the samples are required. With DBS self-sampling at home, it is possible to obtain a DBS sample of the participants on a regular basis, for example, every three months. This type of research will provide information about the added value of TDM in the efficacy of tamoxifen treatment.

DBS sampling will also ease the logistics of treatment individualization in clinical practice, since the patient can self-sample at home and the clinician will have the analysis result before the patients' visit to the outpatient clinic. Also, compliance to treatment can be monitored when DBS samples are obtained on a regular basis.

In recent years, endoxifen has been the metabolite of interest, however, it is possible that other metabolites also play a role in tamoxifen treatment efficacy. Therefore, the combined effect of several metabolites, for example by means of the sum of the (active) metabolites, should be investigated in the future.

The development of aromatase inhibitors (AI) has provided an alternative form of endocrine therapy. Currently, AIs are standard treatment for most postmenopausal women with ER positive breast cancer. Generally, these agents are given either alone, or in sequence, before or after, tamoxifen. Recent studies indicate that AIs are more effective than tamoxifen to improve disease-free and overall survival [4]. However, also for a part of the group of breast cancer patients being treated with AIs, the disease recurs. Possible resistance mechanisms to AIs are, just as for tamoxifen, not fully understood. However, a wide variability in pharmacokinetics has been shown for letrozole, which is predominantly metabolized by CYP2A6 [5]. Also, a dose response relationship has been demonstrated for letrozole; 2.5 mg per day was shown to be more effective than 0.5 mg per day [6]. These data suggest that there also might be a role for individual dosing in letrozole treatment, though further research is warranted to investigate the added value of TDM. The research described in this thesis can serve as a guidebook for the preparations of these trials, in particular the development of a DBS-based assay, to ease trial logistics.

It has been reported that some women who experience failure of one type of endocrine therapy can subsequently derive benefit from the other type [7]. Switching from one endocrine agent to another offers the opportunity to increase the number of lines of hormone therapy before making the inevitable switch to more toxic chemotherapy, thus potentially improving the quality of life for the patient.

AIs have a different risk profile than tamoxifen; AI use is associated with an increase in bone fractures and cardiovascular disease, and tamoxifen use is associated with an increase in venous thrombosis and endometrial carcinoma [8]. Hot flashes are reported for tamoxifen and AIs, where some trials report similar percentages for both agents and

other studies state a slightly higher percentage of tamoxifen treated patients reporting these side effects [9].

For premenopausal women, tamoxifen remains the endocrine agent of choice since AIs have no effect on the preferred pathway of estrogen synthesis in premenopausal women, which is not catalyzed by aromatase.

Considering the above, and also the differences in costs between tamoxifen and AIs, tamoxifen will remain an important therapeutic agent in the treatment of breast cancer. Moreover, recent studies suggest to extend the use of tamoxifen from five to ten years [10,11], since this results in a further reduction in recurrence and mortality. However, this will also prolong the burden of possible side effects for patients using tamoxifen, emphasizing the need for optimal treatment during these ten years.

Individualization of breast cancer treatment based on active tamoxifen metabolite levels might be of value to improve treatment outcome. However, one should be aware that there are several other mechanisms that can also possibly induce resistance to tamoxifen, or AIs. Examples of these mechanisms are variation in expression of the ER on the tumor, modifications of the ER or ER independent growth by additional activated growth factor signaling pathways. In order to fully personalize breast cancer treatment, it is of importance to identify different markers possibly predicting resistance, and thus treatment outcome The appreciation of the heterogeneity of possible resistance inducing pathways within the same tumor will help to choose the right drug, or maybe the right combinatory treatment, at the right dose, for the right patient.

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Summary

Summary

The cornerstone of systemic treatment for patients with estrogen receptor (ER) positive breast cancer is endocrine therapy, with tamoxifen being the most widely used endocrine agent. Tamoxifen binds to the ER, thereby blocking the proliferative actions of estrogen on mammary tissue. It has been used for over 40 years and has significantly reduced recurrence and mortality rates of ER positive breast cancer patients. However, not all patients benefit from tamoxifen treatment as in about one third of the patients the disease recurs. Studies on the pharmacology of tamoxifen revealed extensive biotransformation and showed that some of its metabolites, especially 4-hydroxytamoxifen and *N*-desmethyl-4-hydroxytamoxifen (endoxifen), have a much higher affinity for the ER than tamoxifen itself. Endoxifen is suggested to be the most important metabolite, considering it is present at a much higher steady-state serum concentration in patients using tamoxifen than 4-hydroxytamoxifen. There is wide inter-patient variability in tamoxifen pharmacokinetics, however, in general all patients use a standard dose of 20 mg tamoxifen per day. These findings provide a new possibility for tamoxifen treatment optimization; individual dosing based on active metabolites levels. This approach is encouraged by the results of a recent large retrospective clinical trial, where a significant correlation between endoxifen serum levels and breast cancer recurrence rate was shown and a minimum therapeutic threshold endoxifen serum level was suggested.
In this thesis, the development of sensitive and selective bioanalytical assays to quantify tamoxifen and its metabolites in patient samples has been described. Furthermore, these assays were implemented in clinical practice and applied to clinical studies. In order to ease the logistics of patient sampling and as a patient-friendly alternative to venous sampling, a dried blood spot method has been developed, where samples are obtained by means of a finger prick.

In Chapter 1, two bioanalytical assays for the quantification of tamoxifen and its metabolites in patient samples have been described. The development and validation of a sensitive and selective HPLC-MS/MS method for the simultaneous quantification of tamoxifen and five of its phase I metabolites; *N*-desmethyltamoxifen, endoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen, in human serum samples is described in Chapter 1.1. This method was validated over clinically relevant ranges for all analytes. Chapter 1.2 describes the quest for the cause of large variations in mean serum levels of tamoxifen and its metabolites obtained for different clinical studies reported in the literature. Although the patients

included in these studies all used the standard dose of 20 mg tamoxifen per day and all samples were analyzed with HPLC–MS/MS, the reported mean serum levels of especially endoxifen and 4-hydroxytamoxifen differed more than expected purely based on inter-patient variability. It has been shown that lack of selectivity of some analytical methods resulted in a factor 2-3 overestimation of the concentration of endoxifen and 4-hydroxytamoxifen. This overestimation is caused by overlapping chromatographic peaks of tamoxifen metabolites that show marked resemblance in molecular structure and have similar masses and fragmentation patterns to endoxifen or 4-hydroxytamoxifen. This research shows that the use of highly selective HPLC-MS/MS assays is of major importance for the accurate quantification of tamoxifen and its metabolites in patient samples.

Chapter 1.3 and 1.4 focus on dried blood spot (DBS) sampling, a patient-friendly alternative to venous sampling. In Chapter 1.3, an overview of bioanalytical challenges related to DBS assays is presented, followed by a set of concrete recommendations for the validation of a DBS-based assay. The development and validation of a bioanalytical assay for the determination of tamoxifen and endoxifen in DBS, with the focus on the previously discussed challenges, is described in Chapter 1.4. Regular pure cellulose DBS cards, Whatman 903, were unsuitable for this application, due to an uncomprehended increasing extraction recovery over time for endoxifen. When using coated DBS cards, Whatman DMPK-A, the analytes were both fully recovered from the matrix and the extraction recovery remained constant over time. The difference in extraction recovery between the two types of DBS cards was demonstrated to be mainly attributable to the presence of SDS, one of the constituents of the DMPK-A coating. Also, the presence of a physical coating further optimized the extraction recovery of endoxifen. SDS caused matrix effects, however, these where compensated by the stable isotope labeled internal standards. Adequate concentration levels for both analytes could be measured in DBS with haematocrit (Hct) values between 29 and 48%, a wide enough range to include all adult females with Hct values within and just outside the normal range (36 – 46%). Tamoxifen and endoxifen were both stable in DBS when stored at room temperature for at least four months, and at the temperature range expected during transport; 2-8˚C to 37˚C, for 24 hours.

In Chapter 2, the clinical application of the bioanalysis of tamoxifen and its metabolites has been described. From a large cohort of outpatients in the Netherlands Cancer Institute serum concentrations of tamoxifen and its metabolites were determined using the previously described HPLC-MS/MS method. Wide inter-individual differences, but low intra-individual differences, in systemic exposure to tamoxifen and its metabolites were

demonstrated. The prescribed tamoxifen dose was shown to be related to endoxifen exposure and increasing the dose led to a higher serum concentration of tamoxifen and its metabolites in all patients (Chapter 2.1). In Chapter 2.2, a bridging study to establish the relationship between DBS and serum concentrations of tamoxifen and endoxifen has been described. Paired samples, serum and DBS, were obtained from 44 patients using tamoxifen. Although DBS and serum concentrations correlated well, serum concentrations were consequently higher than DBS concentrations for both analytes. This difference could very well be explained by patient-specific haematocrit (Hct) and analyte-specific blood cell-to-serum ratio (KBC:serum), using the formula $[Analyte]_{serum,\ calculated} = [Analyte]_{DBS}/([1-Hct]+ K_{BC:serum} * Hct)$. The $K_{BC:serum}$ was found to be independent of the concentration for both analytes and was therefore imputed as a fixed value in the formula. Hct was also imputed as a fixed value in this formula, since the target population consists of a relatively homogenous group of patients; adult females treated for early breast cancer and receiving adjuvant tamoxifen treatment, where physiological conditions having a major effect on Hct values are not expected. Also, the validation of the bioanalytical method has demonstrated a minimal difference in analysis results for Hct values between 29 and 48%. When using fixed values for both KBC:serum and Hct, two simple formulas for the conversion of DBS concentrations to serum concentrations were obtained: $[Tamoxifen]_{serum,\ calculated} = [Tamoxifen]_{DBS}/0.779$ and $[Endoxifen]_{serum,\ calculated} = [Endoxifen]_{DBS}/0.663$. Serum concentrations calculated from DBS concentrations were within 20% of analyzed serum concentrations in 84% of the patient samples for tamoxifen, and in 100% of the patient samples for endoxifen. This study enables the use of DBS samples in clinical practice, even when reference values are determined in serum. In Chapter 2.3, the feasibility of DBS self-sampling at home for individualization of tamoxifen therapy based on endoxifen concentrations was assessed. In total, 38 patients were asked to self-obtain a DBS sample at home and to fill out a short questionnaire regarding their experiences and preferences and sent these to the laboratory. Of this group, 36 patients (95%) returned the DBS sample and questionnaire to the laboratory. Of these patients, 31 (86%) provided at least one DBS suitable for analysis. Most patients (92%) reported a positive experience with DBS self-sampling and 61% of the patients preferred DBS sampling over venous sampling. This study confirms the feasibility of DBS sampling for the determination of tamoxifen and endoxifen concentrations for clinical studies and TDM purposes.

Clinical pharmacologic aspects of tamoxifen treatment have been described in Chapter 3. In Chapter 3.1, the investigation of a relationship between the frequency and severity of hot flashes occurring during tamoxifen treatment and concentrations

of tamoxifen and its metabolites in patient samples has been described. In a series of 109 patients, no univariate associations were observed between concentrations of tamoxifen and its metabolites and either the frequency or severity of hot flashes during treatment. However, the frequency of hot flashes may be exacerbated by higher serum concentrations of tamoxifen and its metabolites in post-menopausal women suffering from hot flashes prior to tamoxifen treatment. Finally, in Chapter 3.2, the position of active metabolite concentration-guided dosing in tamoxifen treatment has been discussed. Therapeutic drug monitoring seems a promising approach to tailor tamoxifen treatment, however, incontrovertible evidence for an exposure-outcome relationship is lacking. Therefore, a plea for further clinical research to investigate the value of individual dosing for tamoxifen treatment optimization was made.

Nederlandse samenvatting

Nederlandse samenvatting

Endocriene therapie speelt een belangrijke rol in de behandeling van oestrogeen-receptor positieve borstkanker. Tamoxifen, het meest voorgeschreven endocriene geneesmiddel, bindt aan de oestrogeen-receptor waardoor de proliferatieve effecten van oestrogeen op borstweefsel geremd worden. Het wordt al meer dan 40 jaar gebruikt en heeft een aanzienlijke daling van borstkankersterfte veroorzaakt. Echter, niet alle patiënten hebben baat bij tamoxifen behandeling; ongeveer een derde van de patiënten krijgt een recidief. Kennis over factoren die voorspellend zijn voor de behandeluitkomst is van grote waarde voor het verbeteren van de tamoxifen behandeling. Studies naar de farmacologie van tamoxifen hebben laten zien dat tamoxifen uitgebreid gemetaboliseerd wordt en dat sommige van haar metabolieten, met name 4-hydroxytamoxifen en *N*-desmethyl-4-hydroxytamoxifen (endoxifen), een grotere affiniteit hebben voor de oestrogeen-receptor dan tamoxifen zelf. Er wordt gesuggereerd dat endoxifen de belangrijkste metaboliet is, aangezien het in een hogere steady-state concentratie in patiënten aanwezig is dan 4-hydroxytamoxifen. Er is een grote inter-patiënt variabiliteit in de farmacokinetiek van tamoxifen, echter, in het algemeen gebruiken alle patiënten een standaard dosis van 20 mg tamoxifen per dag. Deze bevindingen hebben geleid tot een nieuwe mogelijkheid voor het optimaliseren van de tamoxifenbehandeling; individueel doseren op basis van de concentraties van de actieve metabolieten. Deze benadering wordt ondersteund door de resultaten van een recent gepubliceerde grote retrospectieve klinische studie, waar een significante correlatie tussen endoxifen serum concentraties en het terugkeren van borstkanker werd gevonden. Tevens werd gesuggereerd dat er een therapeutische ondergrens is voor de serum concentratie van endoxifen.
Dit proefschrift beschrijft de ontwikkeling van bioanalytische methoden om de concentraties van tamoxifen en haar metabolieten in patiëntensamples te analyseren. Tevens zijn deze methoden geïmplementeerd in de kliniek en gebruikt om klinische studies te ondersteunen. Om de logistiek van de bloedafname makkelijker te maken, is een dried blood spot methode ontwikkeld, waarbij samples worden afgenomen door middel van een simpele vingerprik, in plaats van afname uit de arm.

In Hoofdstuk 1 worden twee methoden voor het kwantificeren van tamoxifen en haar metabolieten in patiëntensamples beschreven. De ontwikkeling en validatie van een sensitieve en selectieve LC-MS/MS methode voor de gelijktijdige bepaling van tamoxifen en vijf van haar fase I metabolieten; *N*-desmethyltamoxifen, endoxifen, *N*-desmethyl-4'-hydroxytamoxifen, 4-hydroxytamoxifen en 4'-hydroxytamoxifen is

beschreven in Hoofdstuk 1.1. Deze methode is gevalideerd over een klinisch relevante concentratiereeks voor alle analieten. In Hoofdstuk 1.2 is de zoektocht beschreven naar de oorzaak voor de grote verschillen in gemiddelde serum concentraties van tamoxifen en haar metabolieten in klinische studies gerapporteerd in de literatuur. Hoewel de patiënten in deze studies allen de standaarddosering van 20 mg tamoxifen per dag gebruikten en alle samples werden geanalyseerd met behulp van LC-MS/MS, liggen de gerapporteerde gemiddelde concentraties van met name endoxifen en 4-hydroxytamoxifen verder uit elkaar dan verwacht wordt puur op basis van inter-individuele variabiliteit. Een gebrek aan selectiviteit van sommige analytische methoden bleek de oorzaak te zijn van een factor 2-3 overschatting van de serum concentratie van endoxifen en 4-hydroxytamoxifen. Deze overschatting werd veroorzaakt door overlappende chromatografische pieken van tamoxifen metabolieten die grote overeenkomsten vertonen in moleculaire structuur, en waarvan de massa's en fragmentatiepatronen gelijk zijn aan endoxifen of 4-hydroxytamoxifen. Uit dit onderzoek blijkt dat het gebruik van zeer selectieve HPLC-MS methoden van groot belang is voor een accurate kwantificering van tamoxifen en haar metabolieten in patiëntensamples. Het gebruik van dried blood spots (DBS) maakt het mogelijk om samples af te nemen met een simpele vingerprik en wordt gezien als een patiëntvriendelijk alternatief voor de reguliere veneuze afname. In Hoofdstuk 1.3 wordt een overzicht gepresenteerd van bioanalytische uitdagingen gerelateerd aan DBS methoden, gevolgd door een aantal concrete aanbevelingen voor de validatie van een DBS methode. De ontwikkeling en validatie van een bioanalytische methode voor het meten van de concentratie van tamoxifen en endoxifen in DBS, waarbij de focus ligt op de uitdagingen beschreven in het voorgaande hoofdstuk, wordt beschreven in Hoofdstuk 1.4. Reguliere cellulose DBS kaartjes, Whatman 903, waren niet geschikt voor deze toepassing doordat de opbrengst van endoxifen uit de matrix toenam in de tijd. Met het gebruik van gecoate kaartjes, Whatman DMPK-A, werden beide analieten volledig teruggewonnen uit de matrix en de extractie opbrengst was constant in de tijd. De verschillen in extractie opbrengst tussen de twee typen DBS kaartjes was voornamelijk toe te schrijven aan SDS, een van de bestanddelen van de DMPK-A coating. Ook de aanwezigheid van een fysieke coating bleek bij te dragen aan de extractie opbrengst. De aanwezigheid van SDS tijdens de analyse veroorzaakte weliswaar een matixeffect, maar deze werd gecompenseerd door de stabiele isotopen die zijn gebruikt als interne standaard. Voor beide analieten werden adequate concentraties gemeten bij hematocriet (Hct) waarden tussen 29 en 48%, een klinisch relevante range voor volwassen vrouwen met Hct waarden binnen of net buiten de normaalrange (36 – 46%). Tamoxifen en endoxifen waren beiden minimaal vier maanden stabiel in DBS als deze werden bewaard bij kamertemperatuur, en gedurende

minimaal 24 uur bij temperaturen die worden verwacht tijdens transport van de samples; 2-8˚C tot 37˚C.

Hoofdstuk 2 is gericht op de klinische toepassing van de bioanalyse van tamoxifen en haar metabolieten. Van een grote groep niet-geselecteerde polipatiënten die behandeld worden met tamoxifen in het Nederlands Kanker Instituut werden de serumspiegels van tamoxifen en haar metabolieten gemeten met de eerder beschreven HPLC-MS/MS methode. Er werd een grote inter-individuele variatie, maar een kleine intra-individuele variatie gevonden in spiegels van tamoxifen en haar metabolieten. Er was een duidelijk verband tussen de voorgeschreven tamoxifen dosering en endoxifen spiegels en het verhogen van de tamoxifen dosis resulteerde voor alle patiënten in een hogere serum concentratie van tamoxifen en haar metabolieten (Hoofdstuk 2.1). In Hoofdstuk 2.2 wordt de relatie tussen DBS en serum concentraties van tamoxifen en endoxifen beschreven. Gepaarde samples, serum en DBS, werden afgenomen van 44 patiënten die tamoxifen gebruikten. Er werd een sterke relatie gevonden tussen beide concentraties, echter, de serum concentraties waren voor beide analieten consequent hoger dan de DBS concentraties. Dit verschil kon worden verklaard door het patiëntspecifieke hematocriet en de analietspecifieke bloedcell-serum ratio ($K_{BC:serum}$), met de formule $[Analiet]_{serum,\ berekend} = [Analiet]_{DBS}/([1-Hct]+ K_{BC:serum} * Hct)$. De $K_{BC:serum}$ bleek voor beide analieten onafhankelijk te zijn van de concentratie en werd daarom als vaste waarde in de formule ingevuld. Ook Hct kon als vaste waarde in de formule worden gezet, omdat de beoogde patiëntenpopulatie bestaat uit een relatief homogene groep; volwassen vrouwen behandeld voor vroege borstkanker, waarbij aandoeningen die een groot effect hebben op het Hct niet worden verwacht. Daar komt bij dat de eerder beschreven validatie van de bioanalytische methode heeft uitgewezen dat Hct waarden tussen de 29 en 48% geen noemenswaardig effect hebben op de analyseresultaten. Door het gebruiken van een vaste waarde voor zowel Hct en $K_{BC:serum}$, werden twee simpele formules voor het omrekenen van DBS naar serum concentraties verkregen: $[Tamoxifen]_{serum,\ berekend} = [Tamoxifen]_{DBS}/0.779$ en $[Endoxifen]_{serum,\ berekend} = [Endoxifen]_{DBS}/0.663$. Serum concentraties berekend op basis van DBS concentraties waren binnen 20% van de gemeten serum concentraties voor tamoxifen in 84% van de patiëntensamples, en voor endoxifen in 100% van de patiëntensamples. Deze studie maakt het gebruik van DBS samples mogelijk, ook als referentiewaarden in serum bepaald zijn. In Hoofdstuk 2.3 werd de haalbaarheid van thuis-afname van DBS samples voor individualisatie van tamoxifen behandeling op basis van endoxifen concentraties geëvalueerd. In totaal werden 38 patiënten gevraagd om thuis zelf een DBS af te nemen en een korte vragenlijst over hun ervaringen en voorkeuren in te vullen, en deze op te sturen naar

het laboratorium. Van deze groep stuurden 36 patiënten (95%) de DBS en de ingevulde vragenlijst terug. Van deze patiënten waren er 31 (86%) die een DBS opstuurden die geschikt was voor de analyse. Het merendeel van de patiënten (92%) gaf aan dat zij de afname goed vonden gaan en 61% van de patiënten prefereerde de DBS afname methode boven veneuze afname. Derhalve laat deze studie zien dat thuis-afname van DBS geschikt is voor toepassing in klinische studies en therapeutic drug monitoring.

Klinisch farmacologische aspecten van tamoxifen behandeling zijn beschreven in Hoofdstuk 3. In Hoofdstuk 3.1 wordt een studie naar de relatie tussen de frequentie en ernst van opvliegers tijdens tamoxifen behandeling en concentraties van tamoxifen en haar metabolieten in patiëntensamples beschreven. In een groep van 109 patiënten werden geen univariate associaties gevonden tussen concentraties van tamoxifen en haar metabolieten en de frequentie en/of ernst van de opvliegers. Er is wel een aanwijzing gevonden dat bij post-menopausale vrouwen die last hadden van opvliegers voorafgaand aan de behandeling, hogere concentraties van tamoxifen en haar metabolieten aanleiding geven tot frequentere opvliegers tijdens de behandeling. Tenslotte wordt in Hoofdstuk 3.2 de positie van tamoxifen dosering op geleide van actieve metabolietconcentraties beschreven. Therapeutic drug monitoring lijkt een geschikte manier om de tamoxifen behandeling te individualiseren, echter, onweerlegbaar bewijs voor een relatie tussen metabolietconcentraties en behandelresultaat is er op dit moment nog niet. Verder klinisch onderzoek naar de toegevoegde waarde van individueel doseren is van groot belang voor het optimaliseren van de behandeling met tamoxifen.

Dankwoord

Dankwoord

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Nynke
Amsterdam 2014

Curriculum vitae

Curriculum vitae

Nynke Jager werd op 7 oktober 1985 geboren in Dordrecht en groeide op in Tilburg. In 2003 behaalde zij haar VWO diploma aan het Theresialyceum te Tilburg en in datzelfde jaar is zij gestart met haar studie Farmacie aan de Rijksuniversiteit Groningen. In 2007 behaalde zij haar bachelordiploma. Als onderdeel van de masteropleiding volgde zij een wetenschappelijke stage aan de divisie Farmaceutische Wetenschappen van de Universiteit van Tasmanië, Australië. Zij heeft daar een bioanalytisch onderzoek uitgevoerd, met als doel de structuur van de flavonoiden van de *Carpobrotus rossii,* een typisch Australische plant met mogelijk therapeutische eigenschappen, te identificeren. In juli 2010 ontving zij haar masterdiploma Farmacie (*cum laude*). In september van dat jaar startte zij in de apotheek van het Slotervaartziekenhuis en het Nederlands Kanker Instituut te Amsterdam met het promotieonderzoek dat is beschreven in dit proefschrift. Dit onderzoek werd uitgevoerd onder begeleiding van prof.dr. J.H. Beijnen en prof.dr. J.H.M. Schellens en copromotor dr. H. Rosing. Naast haar promotieonderzoek heeft Nynke de opleiding tot klinisch farmacoloog gevolgd, waarvoor in juni 2014 de certificering werd toegekend door de Nederlandse Vereniging voor Klinische Farmacie & Biofarmacie (NVKFB). In oktober 2014 zal zij starten met de opleiding tot ziekenhuisapotheker in het AMC te Amsterdam en het MCA te Alkmaar.

Nynke Jager was born October 7th 1985 in Dordrecht and grew up in Tilburg, the Netherlands. In 2003 she finished secondary school at the Theresialyceum in Tilburg and she subsequently started to study Pharmacy at the University of Groningen. In 2007, she received her Bachelor's degree. As part of the Master's program, she completed a scientific research project at the Division of Pharmaceutical Sciences at the University of Tasmania, Australia. She investigated the chemical structure of the flavonoids of *Carpobrotus rossi*, a plant with therapeutical potential typically found in Tasmania. In July 2010 she obtained her Master's degree (*cum laude*). In September that same year she started the PhD project described in this thesis at the Department of Pharmacy & Pharmacology of the Slotervaart Hospital and the Netherlands Cancer Institute, Amsterdam, the Netherlands. This research project was supervised by Prof.dr. J.H. Beijnen and Prof.dr. J.H.M. Schellens and joint-supervisor Dr. H. Rosing. During her PhD-program, Nynke followed a training for clinical pharmacologist and certification was granted in June 2014 by the Dutch Society for Clinical Pharmacology & Biopharmacy. In October 2014, she will start her Hospital Pharmacist training at the University Medical Centre (AMC) in Amsterdam and the Medical Centre Alkmaar in Alkmaar, the Netherlands.

List of publications

List of publications

NGL Jager, H Rosing, SC Linn, JHM Schellens, JH Beijnen. Individualization of tamoxifen therapy by dried blood spot self-sampling at home: a feasibility study. Submitted for publication

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NGL Jager. Importance of highly selective LC-MS/MS analysis for the accurate quantification of tamoxifen and its metabolites. Oral presentation AB Sciex symposium, Eindhoven, 2012