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RESEARCH ARTICLE

Plasma, blood and liver tissue sample preparation methods for the separate quantification of liposomal-encapsulated prednisolone phosphate and non-encapsulated prednisolone

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Abstract

Besides the development of sample preparation methods for the determination of separate liposomal-encapsulated prednisolone phosphate and non-encapsulated prednisolone concentrations in murine plasma and blood, this article also presents the first description of an accurate sample preparation method for the determination of such separate concentrations in the murine liver. The quantitative differentiation is based on the immediate hydrolysis of prednisolone phosphate (PP) into prednisolone (P) after its release from the liposomes *in vivo*: PP represents the encapsulated drug, while P represents the non-encapsulated drug. The use of 10 ml methanol/g tissue during homogenization of liver tissue ensures complete liposome rupture, prevention of the dephosphorylation of PP released during homogenization, sufficient clean supernatants, excellent extraction of P and sufficient extraction of PP and excellent accuracies and precision complying with the internal guidelines for pre-clinical studies (80–120% and maximal 20%, respectively). Similarly, the matching sample preparation methods for plasma and blood involve protein precipitation with four equivalents of methanol also ensuring accuracies and precision complying with the internal guidelines for pre-clinical studies. Application of these sample preparation methods is going to generate the first pharmacokinetic (PK) profile of a liposomal preparation, in which the encapsulated and non-encapsulated drug concentrations in a tissue are measured separately. Such separated concentration profiles can gain important insights into the PKs of liposomal PP and probably also with regard to liposomal formulations in general, like the quantification of the *in vivo* drug release from the liposomes.

Keywords

Bioanalysis, liposomes, phosphatase, prodrug

History

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Introduction

Without doubt, the use of stealth liposomal drug delivery systems has been proven to be very valuable in the treatment of cancer, infections and inflammations. Due to the encapsulation of drugs in stealth liposomes, its pharmacokinetics (PKs) and corresponding biodistribution are changed. Consequently, an increased efficacy and/or a significant reduction of severe side-effects, like myelosuppression, mucositis and alopecia in the case of cytosatics, were observed compared to free drug formulations (Qian et al., 2012). However, numerous biodistribution studies showed not only the accumulation of liposomes in target tissue, but also in heavily perfused organs like the liver and the spleen (Gabizon et al., 2003; Metselaar et al., 2003; Newman et al., 1999; Schiffelers et al., 2005). Since liposomes are often used for the formulation of very toxic compounds like cytostatics, this accumulation of liposomes in healthy organs yielded new, dose-limiting, side-effects like hand-foot syndrome (Gabizon et al., 2003) and the significant reduction of the phagocytic activity of the liver macrophages resulting in a significantly reduced bacterial blood clearance (Daemen et al., 1997; Storm et al., 1998).

The explanation for the above-described increased efficacy and new side-effects rests in the PK profile of these liposomal formulations. Though, the complete PKs of liposomal drugs is still not elucidated. Although hundreds of biodistribution studies are performed (708 publications in PubMed, search terms ''liposome'' AND ''biodistribution''), only liposome concentrations or total drug concentrations in the tissues of interest were determined (Gabizon et al., 2003; Kwon et al., 2012; Metselaar et al., 2003). However, efficacy as well as toxicity is only related to the level of non-encapsulated drug (further referred to as free drug). In order to have a full PK profile, the free drug concentration profiles in plasma, whole blood, tumor and healthy tissues should be known separately from the encapsulated drug

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profiles. Such separate drug concentrations yield significant fundamental insights with regard to the functioning of liposomal formulations, like values for the drug release from the liposomes in different tissues *in vivo*. Once the complete PK profile is understood it will be possible to optimize liposomal formulations with regard to efficacy and side-effects.

While the separate quantification of encapsulated and free drug in plasma *in vivo* is becoming less rare (Druckmann et al., 1989; Griese et al., 2002; Krishna et al., 2001; Mayer & St.-Onge, 1995; Srigritsanapol & Chan, 1994; Zamboni et al., 2007), it is still absent in tumor and healthy tissues. Techniques, which were successful for the separate quantification of encapsulated and free drug in plasma, use the different physicochemical properties of the liposome and the free drug like charge, size and hydrophobicity. Unfortunately, these techniques are not suitable for the separate determination in tissues, since homogenization is required prior to their application. Homogenization induces liposome rupture followed by the release of encapsulated drug and, consequently, too high free drug concentrations would be obtained. Until now, the separate quantitative assessment of encapsulated and free drug in tissues was only approximated by techniques like dual-labeling and microdialysis or by using the ''sink'' characteristics of the nucleus in the case of doxorubicin. As for dual-labeling, the drug/lipid ratio in various tissues is measured by labeling the lipid as well as the drug, e.g. by using radiolabels (Zolnik et al., 2008). However, the drug/lipid ratio is not able to distinct between a lipid organized in a liposome and a free lipid. Moreover, the drug/lipid ratio resulting from encapsulated drug is equal to the drug/lipid ratio of free drug, which is already released from the liposome, but is still present in the tissue. Essentially, it is necessary to measure the true encapsulated and free drug concentrations instead of the ''liposome'' and free drug concentrations. During microdialysis (Zamboni et al., 2007), which is based on the passive diffusion of non-protein associated drug across the semi-permeable membrane of the microdialysis catheter, the non-protein bound part of the free drug is determined. Quantification of the non-protein bound free drug can yield information about the efficacy and toxicity of the liposome formulation. However, it does not yield information about the underlying PKs, since only an indication about the drug release from the liposomes is obtained instead of an accurate quantification. Further, the necessity to estimate the so-called probe recovery gives less accurate results of the non-protein bound drug concentration. Laginha et al. (2005) used a different, creative approach to approximate the separate concentrations in tumor tissue after intravenous administration of Doxil: once doxorubicin is released from the liposomes into the interstitial tissue in solid tumors it rapidly diffuses into cells. Then, a large proportion of the free doxorubicin accumulates in the cell nucleus and strongly binds to the nuclear DNA. Since the nucleus acts like a sink for doxorubicin, Laginha et al. used the nuclear doxorubicin concentration as a measure for the free doxorubicin concentration. As already stated by the authors themselves, this method yielded only a reasonable first approximation.

Therefore, there is still need for the development of an accurate, quantitative bioanalytical method, which is able to distinguish between the encapsulated and free drug in tumors and healthy tissues *in vivo*. The key behind the success of such a method lays in the careful development of the corresponding sample preparation method: the free drug has to be distinguished from the encapsulated drug in an accurate manner.

This study presents the development of a tissue sample preparation method, which enables the relatively simple determination of encapsulated and free drug concentrations in murine liver for a liposomal preparation of prednisolone phosphate (PP). In addition, the development of a matching sample preparation method suitable for plasma and whole blood samples was shown. Phosphate prodrugs like PP are known for their rapid *in vivo* dephosphorylation by phosphatases (Garg & Jusko, 1994; Möllmann et al., 1995) and, even more specific, the immediate dephosphorylation of PP that is released from the liposomes is demonstrated for murine tissues, i.e. liver and kidneys (Smits et al., 2013b), and whole blood [internal study similar to Smits et al. (2013b)]. Hence, the encapsulated concentration will then simply be represented by PP, whereas P represents the free drug concentration (Metselaar et al., 2003). In order to avoid overestimations of the free drug concentration, any conversion of PP released from the liposomes during storage and during sample preparation was prevented.

Materials and methods

Materials

Unless mentioned otherwise, all materials were used as received. Dipalmitoyl phosphatidyl choline (DPPC) and poly(ethylene glycol) 2000-distearoyl phosphatidyl ethanolamine (PEG2000-DSPE) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Prednisolone disodium phosphate was from Bufa (IJsselstein, the Netherlands). Alkaline phosphatase from rabbit intestine, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), cholesterol, dexamethasone, dexamethasone disodium phosphate, prednisolone (P), Rolipram and anhydrous sodium hydroxide pellets ($\geq$98%) were from Sigma (St. Louis, MO). 0.01 M phosphate buffered saline (PBS) pH 7.4 was prepared using PBS powder in foil pouches from Sigma as described by the supplier. The commercial available phosphatase and protease inhibitor cocktails were from Sigma as well as from Roche Diagnostics (Mannheim, Germany). Methyl arachidonyl fluorophosphonate (MAFP) was from Tocris bioscience (Bristol, UK). Acetonitrile (ACN) LiChrosolv, ethylacetate and tetrahydrofuran Uvasol® were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) p.a., absolute ethanol and methanol HPLC gradient grade were from Mallinckrodt Baker BV (Deventer, The Netherlands). Hydrochloric acid for analysis, ~37% solution in water, was from Acros Organics (Geel, Belgium). Trifluoroacetic acid was from Fisher Scientific (Loughborough, UK). All water used was purified water prepared using a Milli-Q system from Millipore Corporation (Billerica, MA). Liver tissue, EDTA stabilized plasma and EDTA stabilized whole blood from male C57Bl/6J mice were from Janvier

(Le Genest-Saint-Isle, France). Nuclepore™ Track-Etch polycarbonate membrane filters used for liposome sizing were from Whatman GmbH (Dassel, Germany) and Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10 000 were from Pierce (Rockford, IL). 10 ml TC16 borosilicate glass tubes with matching polypropylene screw caps, both resistant to high-intensity focused ultrasound (HIFU), were from KBioscience (Hoddesdon, UK).

Liposome preparation and characterization

A batch of PEGylated liposomes encapsulating PP as described by Metselaar et al. (2003) was prepared. In short, a mixture of DPPC, cholesterol and PEG2000-DSPE in a molar ratio of 1.85:1.0:0.15, respectively, was dissolved in absolute ethanol. Hereafter, the ethanol was evaporated under reduced pressure using a rotary evaporator yielding a thin lipid film, which was additionally dried under a stream of nitrogen. Subsequently, the lipid film was hydrated with a 100 mg/ml prednisolone disodium phosphate aqueous solution inducing the self-assembly of the lipids and, consequently, the encapsulation of PP. The resulting liposome dispersion was sized by repeated extrusion through a variety of polycarbonate membrane filters of decreasing pore size from 600 to 50 nm. Finally, non-encapsulated PP was removed by dialysis using dialysis cassettes of 0.5–3 ml capacity against PBS pH 7.4 at 2–8 °C.

The total, encapsulated and free PP content of the liposome preparation were determined using a new method, in which the free PP is distinguished from the encapsulated PP by dephosphorylation into P using alkaline phosphatase (Smits et al., 2013a). In short, for the determination of the encapsulated and free PP content, 100 µl of an appropriate dilution of the liposome preparation were incubated with 100 µl of alkaline phosphatase solution (≥4.6 mg/ml). After 60 min, 400 µl of tetrahydrofuran were used for enzyme deactivation, liposome rupture and protein precipitation. After centrifugation, PP and P concentrations were determined using HPLC. To determine the total drug concentration, 200 µl of a hundred times diluted liposome preparation were used and the incubation step using alkaline phosphatase was skipped.

The mean liposome size was determined by dynamic light scattering as also described previously (Smits et al., 2013a).

HPLC

HPLC analysis was performed on an Agilent 1100 system equipped with a G1322A degasser, a G1312A high-pressure binary pump, a G1329A autosampler with a 100 µl injection loop, a G1316A column compartment and a G1314A variable wavelength UV detector with a 10-mm flowcel (Agilent Technologies, Palo Alto, CA). Empower Pro Software (Waters, Milford, MA) controlled all modules and was used for peak integration. PP, dexamethasone phosphate (DP), P and dexamethasone (D) could be measured in one single run using an Zorbax SB-C18 column (2.1 mm × 150 mm, 3.5 µm particle size, Agilent Technologies), which was maintained at 40 °C during analysis. The injection volume was set at 5 µl. When a higher sensitivity was required, the injection volume was increased to 10 µl. The mobile phase consisted of (A) 0.1 v/v % trifluoroacetic acid in water and (B) acetonitrile. A gradient was performed from 95% to 70% A over 2 min, followed by a gradient from 70% to 40% A over 8 min. Prior to the next run, the system was rinsed using 100% B for 4 min, followed by an 8-min equilibration step at 95% A. The flow rate was kept at 0.25 ml/min at all times and the eluate was monitored at 245 nm.

Tissue samples

During the development of the tissue sample preparation method, external calibration solutions of PP and P were prepared in methanol and in methanol–water (70:30, v/v). Additionally, the methanol and methanol–water, which was used during validation, contained the internal standards DP and D.

Plasma/whole blood samples

PP calibration standards were prepared by mixing one equivalent of a series of PP solutions in PBS pH 7.4 and a specific number of equivalents of methanol, followed by vortexing (30 s). During method validation, methanol containing the internal standards was used. Calibration curves for P were prepared in a similar way.

The range of the calibration curve was always chosen in such way to cover the expected sample concentrations. All calibration standards had to be within 15% of their nominal values after back-calculation.

High-intensity focused ultrasound

Drugs can be located within the cells of the tissues. In order to rupture these cells and to extract drug from these cells, HIFU was applied to the samples using a Covaris E210x (Covaris Inc., Woburn, MA) controlled by Covaris Sonolab software version Ev4.3.3 (Covaris Inc.). The Covaris was equipped with a metal rack holding a 12-well plate (Covaris Inc.).

The tubes containing the samples were placed in the metal rack held in a water bath with a maximal temperature limit of 15 °C. Then, the samples were exposed to HIFU by running the following process configuration, which is an adapted version of the treatment settings used by Melarange et al. (2007) for the sample preparation of rat liver. The progress configuration entails: 100 cycles/burst for 60 s, 1000 cycles/burst for 60 s, 100 cycles/burst for 30 s, 1000 cycles/burst for 30 s. The Power tracking mode was chosen from the Frequency tuning menu and the duty cycle and intensity were always kept at 50% and 10, respectively.

Selection of tissue homogenization solvent

Since accumulation of PEGylated liposomes has been observed in the tumors, kidneys, livers and spleens of tumor-bearing mice after i.v. administration (Schiffelers et al., 2005), these tissues are the tissues of interest. The presence of phospholipids in the final sample is considered to be the major cause of matrix interference by endogenous compounds (Bennett & Van Horne, 2003). Based on the

phospholipid volume tissue fraction (Poulin & Theil, 2002), the liver contains the largest amount of phospholipids as compared to the kidneys and spleen (no data were available for tumor tissue). Moreover, the liver contains numerous amounts of phosphatases (Smits et al., 2013b). Therefore, the development of a sample preparation method suitable for liver tissue was expected to be the most challenging and, initially, the method development was performed using murine liver tissue.

To find a suitable manner to prevent the enzymatic hydrolysis of free PP, which is released after sampling, during and after tissue homogenization, phosphatase deactivation by various inhibitors and solvents (acetonitrile, 17.99 mM AEBSF, commercial available phosphatase and protease inhibitor cocktails, 7.80 mM EDTA, ethanol, ethanol–water (70:30, v/v), ethylacetate, 0.01 M HCl, 16.2 M MAFP, methanol, methanol–water (7:2, v/v), 2 M NaOH, 200.0 µM Rolipram, saturated ammonium sulfate solution, tetrahydrofuran, water of 100 °C) was evaluated as follows. Several murine livers were thawed at room temperature and homogenized using a General Laboratory Homogenizer (Omni, Kennesaw, GA). Aliquots of homogenate were transferred to 10 ml glass tubes. 2.5 ml of one of the inhibitors or solvents was added and the sample was vortexed immediately. To one of the aliquots of homogenate only 2.5 ml of water was added as control. After at least 30 min of incubation of the homogenate with the inhibitors/solvents, 150–200 µl of a PP solution (70.5 µM) were added and the samples were vortexed shortly. After incubation (overnight or for 30 min) acetonitrile was added, the samples were vortexed and proteins were precipitated by centrifugation. The resulting supernatants were transferred to HPLC vials and analyzed using HPLC as described previously. The percentage of P present in the different samples was estimated by calculating the percentage of the area of P compared to the sum total area (area PP + area P).

Liposome rupture

Tissue samples

To verify the complete liposome rupture by methanol during tissue sample preparation, different amounts of methanol were mixed with liposomes yielding final concentrations of 0.0030–0.45 mmol PP/l. These concentrations simulate the addition of 1, 2, 5, 10 and 35 ml methanol/g tissue to various tissues, among which the liver, containing their expected maximal *in vivo* liposome concentrations (Metselaar, 2003). A similar dilution series of liposomes was prepared in methanol–water (70:30, v/v). About 1 ml of each dilution was transferred to HPLC vials. Commonly, tissue samples are immersed in liquid nitrogen prior to homogenization. To determine the influence of this liquid nitrogen step on the recovery of PP, another 1 ml of each dilution was transferred to centrifuge tubes and cooled for ~10 s using liquid nitrogen. These samples were allowed to come to room temperature for at least 30 min and were also transferred to HPLC vials. PP concentrations were determined using HPLC as described previously. Recoveries were calculated using the nominal concentrations, which were calculated based on the total drug content of the liposome preparation.

Plasma samples

The amount of methanol[1] required to induce the complete liposome rupture in plasma samples was determined as follows. In duplicate, to 100 µl of a liposome dilution containing a PP concentration of 0.44 mM, which is similar to the expected maximal liposomal PP plasma concentration (Metselaar, 2003), volumes of 100 up to 500 µl of methanol were added. The resulting samples were vortexed for 30 s. As described previously, PP concentrations were determined using HPLC yielding the recovery of PP after liposome rupture.

Whole blood samples

The complete liposome rupture of 0.52 mM liposomal PP, which is similar to the expected maximal liposomal PP blood concentration (based on the dose and mouse blood volume (Brandenburg, 2000), by four equivalents of methanol[2] was verified in a similar way as described above for the plasma samples.

Immediate deactivation of phosphatases

Tissue samples

The volume of methanol or methanol–water (70:30, v/v) required to immediately stop the complete phosphatase activity during tissue sample preparation was determined as follows. Liver tissue was immersed for ~5 s in liquid nitrogen and homogenized using a General Laboratory Homogenizer. 11 aliquots of 250 mg homogenate were transferred to separate glass tubes. In duplicate, 1, 2, 5, 10 and 35 ml methanol/g homogenate containing 98.1 µM PP was added to 10 aliquots. To the 11th aliquot, an amount of 2 ml/g of plain methanol was added as control. The samples were vortexed and only one of the two samples per added amount of methanol was subjected to HIFU as described above. Subsequently, all samples were centrifuged at 2890 *g* for 15 min. The resulting supernatants were used for HPLC analysis. This procedure was repeated similarly for methanol–water. The percentage of P present in the samples treated with methanol was estimated using the calculated total area, which was based on the observed response of the above PP solution in methanol. The percentage of P present in the samples treated with methanol–water was estimated using the corresponding sum total response of the sample (area PP + area P).

Plasma samples

To determine the volume of methanol (refer footnote 1) necessary to immediately deactivate the plasma phosphatase activity, to 100 µl of plasma 100 up to 500 µl of a solution of

[1]After finishing the method development for the preparation of tissues, methanol appeared to be the solvent of choice (see section ''Selection of the homogenization and precipitation solvent''). Therefore, method development with regard to plasma sample preparation continued using methanol only.

[2]After development of the plasma sample preparation method it was evaluated whether the method could also be freely applied to whole blood samples.

PP in methanol (488 µM) was added in duplicate. Samples were vortexed for ~30 s and, after 30 min, the samples were centrifuged for 15 min at $8.8 \times 10^2 g$. The supernatant was used for HPLC analysis and analyzed using HPLC as described previously. The percentage of P after the addition of different volumes of methanol was estimated using the sum total response measured for the sample (area PP + area P).

Whole blood samples

The immediate phosphatase deactivation of whole blood samples by four equivalents of methanol (refer footnote 2) was verified in a similar way as described above for plasma samples. However, an additional step was introduced after vortexing, at which the samples were subjected to HIFU as described above.

Recovery, accuracy and precision

Tissue samples

The recovery and accuracy of PP as well as P after tissue sample preparation using different volumes of methanol or methanol–water (70:30, v/v) were determined as follows. Six murine livers were transferred to large centrifuge tubes of 50 ml. 25 µl of a P solution in DMSO (4.86 mM) was added to all six liver tissues. After 10 min, 25 µl of a liposome dilution in PBS pH 7.4 (4.6 mM PP) were also added. The tissue samples were cooled in liquid nitrogen for 10 s. Immediately, 5, 10 or 35 ml methanol/g tissue containing the internal standards D and DP were added and the tissues were homogenized using a General Laboratory Homogenizer. A representative aliquot of the resulting homogenates was transferred to 10 ml glass tubes and HIFU was applied as described above. Subsequently, the samples were centrifuged at 2890 *g* for 15 min and the resulting supernatants were transferred to HPLC vials. PP and P concentrations were determined using HPLC as described above.

Again this procedure was repeated using methanol–water containing the internal standards D and DP, at which 10 or 35 ml/g tissue was added. To improve the purity of the supernatants of samples treated with methanol–water, also a second aliquot of homogenate was transferred to 10 ml glass tubes for all methanol–water samples. These samples were treated as described above with the exception that the samples were cooled for 15 min at 2–8 °C prior to centrifugation. If necessary, the ultracentrifuge was applied additionally.

Plasma samples

The amount of methanol (refer footnote 1), yielding a good recovery and accuracy after plasma sample preparation, was determined as follows: in sextuple, 10 µl of a P solution in DMSO (260.0 µM) were added to 180 µl of plasma and the samples were mixed gently using the pipette. After 10 min, 10 µl of a liposomal PP dilution in PBS pH 7.4 (3.8 mM PP) were added to the samples and again the samples were mixed gently using the pipette. Hereafter, either 600, 800 or 1000 µl of methanol containing the internal standards was added. The samples were vortexed for 30 s, cooled for 15 min at 2–8 °C and centrifuged for 15 min at $8.8 \times 10^2 g$. The supernatants were used for HPLC analysis. PP and P concentrations were determined using HPLC as described above.

Whole blood samples

The recovery and accuracy after processing blood samples containing either a high (PP: 0.45 mM; P: 0.4748 mM) or a low (PP: 45 µM; P: 47.5 µM) drug concentration using four equivalents of methanol (refer footnote 2) was determined similarly as compared to the plasma samples. After vortexing, these samples were subjected to HIFU as described above. The samples were centrifuged at 20 817 *g* for 15 min. Samples were analyzed using HPLC as described above.

Recoveries were determined by comparing the nominal concentrations and the measured PP and P concentrations obtained without the use of the internal standards. The nominal concentrations were corrected for the small spiking volumes and for concentrating due to precipitation of solid tissue matter during centrifugation. To do so, the water contents of the liver tissue, plasma or whole blood was used and the density of biological matter was assumed to be equal to 1 ml/g. The liver (70.81%) and plasma (93.33%) water contents were determined in-house by freeze-drying and loss-on-drying. The used water content of whole blood, as determined by Sahin et al. (2006), was 81.1%. To determine the accuracies, the nominal concentrations and the PP and P concentrations obtained when using the internal standards were compared. In this case, the internal standards correct for the volume contribution of the biological matter and the small spiking volume. The precision is expressed by the relative standard deviation (RSD) of the calculated PP and P accuracies. The accuracy and precision were assessed according to internal guidelines for pre-clinical studies aiming for an accuracy of 80–120% and an RSD of maximal 20%, respectively.

Freeze-thaw stability of whole blood samples

The freeze-thaw stability of whole blood samples was assessed as follows: 15 µl liposome dispersion were added to 235 µl of whole blood yielding samples containing a high concentration of 0.54 mM PP and samples containing a low concentration of 0.10 mM PP. After gently mixing of the samples none, one or three freeze-thaw cycles were applied. One freeze-thaw cycle included storage at −20 °C for at least 24 h, followed by storage at room temperature for ~7.5 h with exception of the last thawing step, which was always shorter in order to process the samples. In addition, four samples were subjected to three freeze-thaw cycles containing a freezing step at −20 °C for at least 24 h and a thawing step of only 30 min. After the required number of freeze-thaw cycles, the samples were gently homogenized and divided into two aliquots of 100 µl. To determine the ''true'' encapsulated and free drug ratio, the first aliquot was processed by an adapted version of the method described by Smits et al. (2013a). In short, 100 µl of an alkaline phosphatase solution in PBS pH 7.4 (4.64 mg/ml) was added and the sample was gently mixed. After 60 min, the enzymatic activity was stopped by the addition of 800 µl

methanol containing the internal standards. After mixing using the vortexer for 30 s, the samples were subjected to HIFU as described above and centrifuged for 15 min at 20 817 *g*. The supernatants were injected into the HPLC. To evaluate the validity of the here developed sample preparation method after storage, 400 µl of methanol containing the internal standards were added to the second aliquot. After vortexing for 30 s, HIFU and centrifugation (15 min at 20 817 *g*), also these supernatants were injected into the HPLC.

Statistical analysis

To reinforce the observed results, the following statistical tests were performed using SigmaPlot version 8.02 from Systat Software Inc. (San Jose, CA). To evaluate whether one can expect the recovery corresponding to a sample treatment to be different from the nominal value of 100%, *p* values were calculated by performing a one-sample *t*-test using 100 as the test mean. To verify significant differences between various groups a two-sample *t*-test or one-way ANOVA was performed. For pairwise comparisons after ANOVA, the Holm–Sidak test was applied. To evaluate whether one can expect the accuracy corresponding to a sample treatment to be between 80% and 120%, (1) the observed recoveries should be between 80 and 120 and (2) *p* values were calculated by performing two one-sample *t*-tests using 80 and 120 as the test mean. To evaluate whether one can expect the precision corresponding to a sample treatment to be <20%, a two-sample *t*-test was performed using the individual results of the two injections of each sample within one treatment group, at which the results of the injections corresponding to the sample exhibiting the lowest results were multiplied by 1.2. *p* Values of <0.05 were considered statistically significant. Only results, at which the null hypothesis was rejected, were taken into consideration.

When the data were too limited, the estimated standard deviations (SD) and RSDs between the various treatments were compared to the (R)SDs due to sample and HPLC variability as observed during this study.

Results and discussion

To avoid overestimations of the free drug concentration and to develop accurate methods, any conversion of PP released from the liposomes during storage and during sample preparation had to be prevented. To safeguard this, it was chosen to freeze the tissue samples directly after sampling and to not thaw them prior to homogenization. At such low temperatures, phosphatases are not active. Since the sample temperature will increase and major drug release from the liposomes is expected during homogenization, the sample preparation method must be so that during homogenization phosphatases are deactivated immediately. In addition, prior to HPLC analysis, liposome rupture should be complete. Thus, in this case the tissue sample preparation method should not only involve: (1) homogenization, (2) analyte extraction and (3) sample clean-up, but also (4) immediate deactivation of the phosphatases during homogenization and (5) complete liposome rupture. To avoid too many steps during sample preparation, the homogenization solvent is not only anticipated to perform analyte extraction and sample clean-up, but also to immediately deactivate the present phosphatases and ensure the complete liposome rupture.

Similarly, it was also chosen to freeze plasma and blood samples immediately. However, plasma and blood samples have to be thawed prior to processing. Therefore, ethylenediaminetetraacetic acid (EDTA), which reduces phosphate prodrug dephosphorylation in plasma (Samtani et al., 2004), was chosen as anticoagulant and additional experiments were performed to verify the prevention of the hydrolysis of significant amounts of PP in EDTA stabilized plasma and blood samples due to freeze-thaw. Since EDTA does not prevent the dephosphorylation reaction completely (Samtani et al., 2004) causing the hydrolysis of large amounts of PP in the long-term, the precipitation solvent used during the matching sample preparation method for plasma and whole blood samples should meet similar criteria as described above for the tissue sample preparation method.

Since the methodology is intended for use during fundamental research, it is not restricted to extensive bioanalytical method validation as defined by the Food and Drug Administration authorities (U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) et al., 2001). Still, to guarantee suitable and reliable performance characteristics, the selectivity, linearity, freeze-thaw stability (of plasma and whole blood samples), recovery, accuracy and precision were evaluated in a comprehensive way.

Liposome characteristics

The total drug content, liposome size and polydispersity was similar as described by Metselaar (2003): the liposome batch contained 8.9 ± 0.3 mM PP, of which $9.1 \pm 0.2\%$ is present as free drug. The presence of a minor free drug amount in liposome preparations is common (Bellott et al., 2001; Crommelin & Storm, 2003; Krishna et al., 2001). After self-assembly of the liposomes during liposome preparation, the non-encapsulated drug is removed by dialysis. Dialysis is based on an equilibrium between the donor and acceptor phase leaving always a small amount of free drug in the donor phase. Although a smaller free drug amount can be desirable for certain liposome formulations during clinical applications, the observed free drug amount in this study is very acceptable with regard to the analytical scope. The peak diameter appeared to be $\sim 98 \pm 27$ nm. The polydispersity index was 0.055 indicating that the liposome preparation is monodisperse. The phospholipid content was 60 mM (Metselaar et al., 2003).

Selectivity

During the evaluation of blanks containing liver, plasma or whole blood matrix no evidence of significant interfering impurities was observed: either no impurities or only insignificant amounts of impurities eluted together with PP, DP, P and D. Peak areas of the co-eluting impurities were always $\leq 7\%$ compared to peaks corresponding to the smallest PP, DP,

P and D concentrations present in the samples. Such amounts of impurities are considered insignificant, because accuracies were still between 80% and 120% regardless whether the impurity peak area was included in the peak area of the analytes and internal standards.

PP, DP, P and D eluted at 8.7–9.1, 9.4–9.8, 10.1–10.5 and 11.2–11.6 min, respectively. The variation in retention time was caused by the use of multiple Zorbax columns and Agilent 1100 systems and it is not caused by the variation in matrices.

Linearity

During the development of the tissue sample preparation method, linearity was observed for all used calibration curves in methanol as well as methanol–water (70:30, v/v). Noteworthy are: 48.8–488 µM PP in methanol (injection volume: 5 µl); 0.976–97.6 µM PP in methanol (injection volume: 10 µl); 47.3–473 µM PP in methanol–water (injection volume: 5 µl); 1.18–118 µM PP in methanol–water (injection volume: 10 µl); 1.03–25.6 µM for P in methanol (injection volume: 5 µl) and 1.00–25.0 µM for P in methanol–water (injection volume: 5 µl). The coefficient of determination (R^2) was always $\geq$0.9998 and after back-calculation almost all calibration standards were within 4% of their nominal values. Exceptions were found for the calibration standards containing the lowest drug concentrations, which were within 10% of their nominal values.

With regard to the development of a plasma preparation method, linearity was observed for all calibration curves used (injection volume was always 5 µl): 184–584 µM for PP (PBS pH 7.4 – methanol ratio 1:1–1:5); 49.4–247 µM for PP (PBS pH 7.4 – methanol ratio 1:3–1:5; ratio 1:1 and 1:2 not tested) and 5.40–26.98 µM for P (PBS pH 7.4 – methanol ratio 1:3–1:5; ratio 1:1 and 1:2 not tested). R^2 was always $\geq$0.9997 for PP and $\geq$0.9986 for P. All calibration standards were within 2% and 3% of their nominal values for the PP and P calibration curves, respectively.

During the analysis of whole blood samples, linearity was observed for the PP calibration curves in the range 50.3–654 µM and for P over 51.0–664 µM, at which almost all calibration standards were within 6% of their nominal values. This is with exception of the lowest calibration standards at which the determined concentrations were within 15% and 12% of their nominal values for PP and P, respectively. The R^2 for calibration curves of PP was always $\geq$0.9945, for P $\geq$0.9976. The linearity of calibration curves using internal standards was superior to calibration curves without the use of internal standards.

For all three matrices, the analyte responses of the calibration standards containing the lowest analyte concentrations were larger than five times the response of the liver, plasma or blood containing blank. Therefore, the analyte concentrations in these calibration standards were considered to be the lower limit of quantification.

Selection of the homogenization and precipitation solvent

PP and P exhibit very different physical characteristics yielding different partitioning during sample clean-up methods like liquid/liquid extraction (unpublished data) and solid phase extraction. This is in disservice of the recovery for at least one of the compounds. Therefore, sample clean-up by protein precipitation is preferred.

A large variety of solvents and inhibitors, including acetonitrile and methanol, was tested for their capability as homogenization solvent to deactivate the liver phosphatase activity. Methanol and methanol–water (7:2, v/v) offered the best perspective. In contrast to most solvents and inhibitors tested, they were able to prevent any conversion of PP in P completely. After 30 min of incubation of PP with liver homogenate, which was treated overnight with these solvents, no measurable P amounts were observed. It should be noted that this does not mean that these solvents deactivate phosphatases ''immediately''. This is evaluated in the section titled ''Immediate deactivation of phosphatases''. Furthermore, the largest UV response was observed in favor of methanol and methanol–water.

Although acetonitrile is widely used in bioanalysis to initiate protein precipitation in plasma samples and it is assumed to denaturate enzymes, the solvent was not able to deactivate liver phosphatase activity completely and a P amount of $\sim$47 area% was observed. In comparison, in the control sample a P amount of $\sim$81 area% was observed. It appeared that the phosphatase activity in EDTA plasma at room temperature is only significant for small PP concentrations (data not shown), which clarifies the accurate results obtained worldwide when using acetonitrile. Further, also the use of commercial available inhibitor cocktails did not yield complete phosphatase inhibition.

Plasma and whole blood, both anti-coagulated with EDTA, contain less impurities and active phosphatases compared to liver tissue. Therefore, because of the above promising results with regard to tissue sample preparation, methanol was also selected as the solvent of choice for the sample processing of plasma and whole blood samples.

Liposome rupture

The PP recovery of samples representing the expected *in vivo* tissue concentrations after liposome rupture by different volumes of methanol and methanol–water (70:30, v/v) was determined. In addition, the influence of the immersion in liquid nitrogen on the recovery of PP was evaluated. Full recoveries (99–107%) were observed for the samples representing the liposome rupture by the smallest volumes of solvent (0.045–0.45 mmol PP/l). Considering the random sample variability, variability of the HPLC analysis and the SD of the total drug content of the liposome preparation, which is used for the calculation of the nominal values, it is not worthwhile to compare different treatments. However, for these smallest volumes of methanol and methanol–water and regardless whether liquid nitrogen was used, the recoveries were well within 80–120% also when considering the SD of the total drug content of the liposome concentration. This indicates the liposome rupture is sufficient to prevent inaccurate results. The samples representing the liposome rupture by the largest volumes of solvent (3.0–18 µmol PP/l) were below the linear range. However, since the smaller volumes of solvent are already able to yield

sufficient liposome rupture, this most likely also applies for these larger volumes. Further, significant influence of the liquid nitrogen step on the recovery of PP is unlikely: the difference in the response (RSD $\leq 2\%$) is rather caused by the variability of the HPLC analysis than due to the liquid nitrogen step. Besides, considering the aimed accuracy of 80–120% such deviations observed due to the use of liquid nitrogen are not interesting. Hence, all tested treatments are suitable and the development of an accurate method was not restricted by insufficient liposome rupture. In spite of the observed full recoveries, for methanol–water (slightly) cloudy samples were obtained for the smaller methanol–water volumes. The development of the sample preparation method was therefore continued with 1, 2, 5, 10 and 35 ml/g for methanol and 5, 10 and 35 ml/g for methanol–water only.

The liposome rupture of one equivalent containing the maximal expected *in vivo* liposomal PP plasma concentration was induced using different equivalents of methanol (refer footnote 1). The mean of the observed recoveries per group, which were treated with different amounts of methanol, varied from $106 \pm 1\%$ to $108 \pm 0.1\%$. All recoveries were well within the aimed accuracy of 80–120%, even when considering the SD of the total drug content of the liposomal preparation. This indicates the liposome rupture by all volumes is sufficient to prevent inaccurate results. Thus, also for plasma the development of an accurate method was not restricted by insufficient liposome rupture.

After using four equivalents of methanol (refer footnote 2) in combination with the maximal expected *in vivo* whole blood concentration, the mean of the observed recoveries yielded $96\% \pm 0.4$. Even when considering the SD of the total drug content, the recoveries were well within 80–120%. No inaccurate results are expected due to insufficient liposome rupture.

Immediate deactivation of phosphatases

The ability of different volumes of methanol or methanol–water (70:30, v/v) to immediately stop all phosphatase activity during tissue homogenization was evaluated. Logically, the higher the amount of PP converted into P, the less the immediate phosphatase activation. All observed P areas, with exception of these observed in the samples treated with 1 ml methanol/g liver, were below the linear range. This indicates the P amounts in these samples are maximally 1.3–1.8 area%. The estimated area% of P in these samples as well as the calculated area% for the samples treated with 1 ml/g of methanol is shown in Figure 1(a) and (b). Since only one of the duplos was subjected to HIFU, the estimated SD of the percentage of P in Figure 1 represents the variation due to HIFU as well as the random sample variability and variability of the HPLC analysis. Either the corresponding SDs of the absolute values of the P response were rather caused due to random sample and/or HPLC variability or no significant amounts of P were observed at all. Hence, no significant differences were observed for samples subjected and samples not subjected to HIFU. Neither chemical instability of PP (or P) during HIFU nor the release of intracellular phosphatases during HIFU in the presence of methanol or methanol–water does yield significant unwanted hydrolysis of PP into P. When volumes of 5, 10 and 35 ml/g methanol and 10 and 35 ml/g of methanol–water were used only insignificant amounts of P (<1 area%) were observed, which probably originated from the raw PP product and/or small amounts of co-eluting impurities. Volumes of 1 or 2 ml/g of methanol yielded larger percentages of P. Roughly, it seems that a larger conversion of PP is observed when smaller volumes of homogenization solvent are used. However, larger volumes of solvent did not change the partition of the impurities and the analytes between the tissue material and solvent. Therefore, the use of small volumes of homogenization solvent, which still prevent significant hydrolysis of P, are most favorable because of the resulting smaller dilution and the most advantageous lower limit of quantification.

In addition, the ability of different equivalents of methanol (refer footnote 1) to immediately deactivate the minor amount of active phosphatases in EDTA plasma was evaluated. The use of one or two equivalents of methanol resulted in cloudy supernatants and irreversible damage of the column. In order

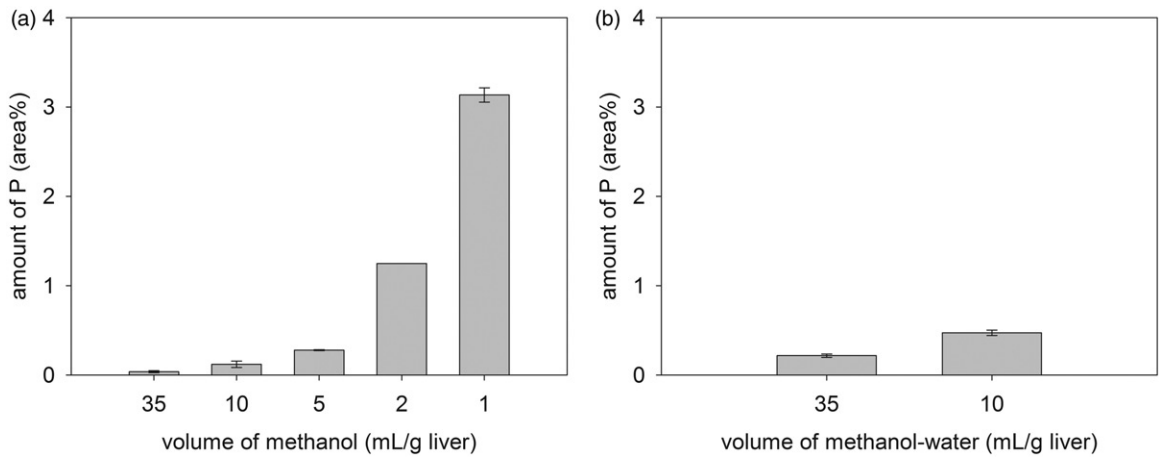

Figure 1. The ability of various volumes of methanol (a) or methanol–water (70:30, v/v) (b) to stop the phosphatase activity immediately during homogenization ($n = 2$). This was expressed as the relative amount of PP which was converted into P by liver phosphatases in the presence of the solvents.

Table 1. The recoveries and accuracies observed for the encapsulated PP and free P after applying the tissue sample preparation method, at which the volume of the homogenization solvents was varied.

Homogenization solvent	Volume of homogenization solvent (ml/g tissue)	Recovery PP (%) ± SD	Accuracy PP (%) ± SD	Recovery P (%) ± SD	Accuracy P (%) ± SD
Methanol ($n = 2$)	5	65 ± 1	102 ± 3	97.4 ± 0.9	99 ± 2
	10	67 ± 0.4	97 ± 0.3	102 ± 2	101 ± 0.3
	35	79 ± 0.3	92 ± 2	99 ± 4	95 ± 4
Methanol–water ($n = 2$)	10	90 ± 4	110 ± 8	104 ± 1	107 ± 3
	35	97 ± 3	101 ± 1	103 ± 0.2	101 ± 1

The theoretical concentrations (in nmol/g ± SD) were: 86 ± 9 for PP and 91 ± 9 for P.

Table 2. The recoveries and accuracies observed for the encapsulated PP and free P after applying the plasma sample preparation method, at which the sample/methanol ratio was varied.

Plasma/methanol ratio	Recovery PP (%) ± SD	Accuracy PP (%) ± SD	Recovery P (%) ± SD	Accuracy P (%) ± SD
1:3	97 ± 0.3	103 ± 0.3	93.1 ± 0.1	97.6 ± 0.4
1:4	96 ± 0.3	106 ± 1	95.1 ± 0.8	100 ± 1
1:5	95 ± 1	105 ± 1	93 ± 22	97 ± 22

The theoretical concentrations were: 0.19 mM for PP and 13.0 μM for P. For all ratios $n = 2$.

to obtain a robust method, the use of one or two equivalents was further avoided. Larger quantities yielded clean supernatants. These contained P amounts of approximating 0.5 area%, at which the differences between the use of three, four or five equivalents of methanol are probably caused due to random sample and HPLC variability. Most probably, such P amounts are originating from the raw PP product and/or small amounts of co-eluting impurities. Most relevant, in these samples substantial hydrolysis of PP which is released after sampling was prevented. In spite of the clean samples observed for a ratio of 1:3, the ratio 1:4 was selected. This guarantees the presence of sufficient methanol also when somewhat more impurities are present in the plasma assuring a robust method.

The processing of whole blood samples using four equivalents of methanol (refer footnote 2) yielded similar results compared to the plasma samples. The observed response for P was below the linear range and the amount of P was estimated at 0.12 ± 0.01 area%. Again, such small amounts of P are rather caused by P in the raw PP product and/or small amounts of co-eluting impurities than by dephosphorylation of PP. Thus, the use of four equivalents of methanol deactivates blood phosphatases immediately, avoiding the dephosphorylation of free PP which may be released after sampling.

Recovery, accuracy and precision

The recovery, intra-run accuracy and intra-run precision of PP and P after tissue sample preparation using different volumes of methanol (35, 10 and 5 ml/g) or methanol–water (70:30, v/v) (35 and 10 ml/g) were determined to confirm the validity of this sample preparation method. The observed recoveries and accuracies are shown in Table 1. Although nice recoveries and accuracies are observed after extraction of PP and P using different amounts of methanol–water, cloudy supernatants were observed regardless of the volume used, even when the samples were additionally cooled or centrifuged using an ultracentrifuge. As explained above such cloudy samples and thus the use of methanol–water should be avoided. Fortunately, clear supernatants were obtained for all livers homogenized in methanol. For all volumes of methanol, the extraction of P from the liver tissue is excellent: all observed recoveries are within a few percentages of 100%. The recoveries observed for PP are significantly <100% ($p \leq 0.010$) for all volumes of methanol used. This is probably due to partitioning of PP between the tissue and methanol, which is supported by the fact that the use of larger volumes of methanol yields significant larger recoveries ($p < 0.001$). Nonetheless, the observed PP recoveries are not that dramatic, since at least ~2/3 of the PP is still recovered, and the internal standard DP can correct for this, yielding excellent accuracies as shown in Table 1. For all volumes of methanol used, the observed accuracies of PP and P comply with the internal guidelines for pre-clinical studies. Also, the FDA requirements with regard to accuracies in human clinical trials were met (U.S. Department of Health and Human Services, FDA, CDER et al., 2001), even when the SD of the total drug content in the liposomal preparation was considered. The RSDs of the PP and P accuracies were 4% or smaller and the precision of the tissue sample preparation method complies with the internal guidelines for pre-clinical studies ($p \leq 0.025$). Hence, methanol yields clean supernatants, suitable recoveries and excellent accuracies and precision for all volumes tested. Since homogenization using 5 ml/g is very inconvenient from a practical point of view and the larger PP recovery observed when using 35 ml/g does not counterbalance the large sample dilution, 10 ml methanol/g tissue is favorable.

A similar validity investigation was done on the plasma sample preparation. Again, the plasma/methanol ratio was varied and the corresponding recoveries and accuracies are summarized in Table 2. Excellent recoveries approaching 100% were observed for all ratios for PP as well as P, indicating extraction of both drugs is optimal. Subsequently, the use of all plasma/methanol ratios tested results in accuracies, which comply with the internal guidelines for pre-clinical studies ($p \leq 0.042$) and nearly meet the FDA requirements for human clinical trials (U.S. Department of Health and Human Services, FDA, CDER et al., 2001) ($p \leq 0.071$). Even when considering the SD of

Table 3. The recoveries and accuracies observed for the encapsulated PP and free P after applying the blood sample preparation method, at which the concentrations of encapsulated PP and free P were varied.

Theoretical PP concentration (mM)	Recovery PP (%) ± SD	Accuracy PP (%) ± SD	Theoretical P concentration (μM)	Recovery P (%) ± SD	Accuracy P (%) ± SD
0.45	76 ± 17	120 ± 28	475	78.4 ± 0.4	96.9 ± 0.3
0.045	79 ± 3	106 ± 7	47.5	92 ± 5	102 ± 7

For both concentrations $n = 2$.

Table 4. Freeze-thaw stability of whole blood samples containing high drug concentrations (0.54 mM).

	''True'' encapsulated/free drug ratio		PP/P drug ratio according to new method	
Number of freeze-thaw cycles applied	[Liposomal drug] (%) ± SD	[Free drug] (%) ± SD	[PP] (%) ± SD	[P] (%) ± SD
0	93 ± 0.1	7.0 ± 0.1	>99.5 ± 0.002	<0.46 ± 0.002
1	31 ± 1	69 ± 1	98 ± 0.1	1.8 ± 0.1
3	<8.4 ± 0.03	>92 ± 0.03	43 ± 1	57 ± 1
3[a]	–	–	96 ± 0.02	4.1 ± 0.02

The PP and P concentrations according to the new method are compared to the ''true'' ratio of the encapsulated drug concentration and free drug concentration after none, one or three freeze-thaw cycles. SD, standard deviation.
[a]Thawing step was decreased from 7.5 h to 30 min.

the total drug content of the liposome preparation. This with exception for P when using five equivalents of methanol, for which it was not possible to statistically verify that the use of five equivalents yields the aimed accuracies. This is due to an exceptional large deviation (RSD of 22%) caused by a low recovery of one of the individual samples. However, the average and individual accuracies are still between 80% and 120%. The other RSDs of the PP and P accuracies in plasma samples were 1% or smaller and thus easily comply with the aimed precision of 20%.

Recoveries and accuracies observed after preparation of whole blood samples containing either a high or a low-analyte concentration are summarized in Table 3. The observed recoveries were always sufficient at which the vast majority of PP and P was extracted from blood cells, proteins and liposomes. The observed accuracies comply with the guidelines for pre-clinical studies and nearly meet the FDA requirements for human clinical trials (U.S. Department of Health and Human Services, FDA, CDER et al., 2001). This also applies when considering the SD of the total drug content of the liposome preparation. The observed RSDs after application of the sample preparation method for blood is maximal 7%. This with exception of the precision observed for a high PP concentration in blood: a precision of 23% was determined, which was due to the large peak area observed for PP in one of the individual samples. Most probably, this large peak area is caused by carry-over from the previous injected sample. While carry-over can be prevented by injecting the samples from low to high PP concentration, in this case the sample order was unfavorable due to human error.

Freeze-thaw stability of whole blood samples

Unlike tissue samples, plasma and whole blood samples have to be thawed prior to processing in order to accurately transfer a known volume. Major release of PP from the liposomes is expected during freeze-thaw (Hays et al., 2001; Van Bommel & Crommelin, 1984) and during the thawing step the phosphatase activity will increase again. Consequently, PP that is released during freeze-thaw will be (partly) hydrolyzed into P yielding overestimations of the free drug concentration. To prevent such overestimations, it was chosen to use EDTA as anti-coagulant during sampling, since EDTA also shows phosphatase inhibiting properties (Samtani et al., 2004). However, the dephosphorylation reaction might not be completely inhibited by EDTA (Samtani et al., 2004). To exclude significant hydrolysis of the PP released after sampling, the freeze-thaw stability of the whole blood samples was investigated.

As can be seen in Table 4, PP leaks out of the liposomes significantly ($p < 0.001$) due to freeze-thaw: after one freeze-thaw cycle only one-third of the drug is still encapsulated and after three freeze-thaw cycles (almost) all encapsulated drug is released. And, this might even be an underestimation of the drug release caused by freeze-thaw, since the free PP is differentiated from the encapsulated PP by hydrolysis using alkaline phosphatase and the alkaline phosphatase activity is probably reduced due to the presence of EDTA. Such major drug release during storage would lead to inaccurate results when the conventional techniques based on the physical separation of the liposomes and free drug would be used. The method described in this current article does not lead to inaccurate results, as long as all PP, which was still encapsulated at the moment of sampling, is not converted into P. The amounts of PP and P after none, one or three freeze-thaw cycles followed by the new sample preparation procedure are also shown in Table 4. After one freeze-thaw cycle, the new method yields only little amounts of P, which are significantly smaller than the free drug amount before freeze-thaw ($p < 0.001$). Such small amounts of P are probably caused by the presence of P in the raw PP product or

by dephosphorylation of non-encapsulated PP in the liposome preparation during the small period in between spiking and freezing. Hence, one freeze thaw cycle causes no overestimations of the free drug concentration using the new method. After three freeze-thaw cycles, the amount of P is significantly ($p < 0.001$) larger than the free drug amount before freeze-thaw. Apparently, the long periods at room temperature were sufficient to ''reactivate'' the phosphatases and, indeed, EDTA does not prevent the dephosphorylation reaction in blood completely. Three freeze-thaw cycles should be prevented. However, if the thawing step is decreased from 7.5 h to 30 min only, which could be the case in a situation where the samples are repeatedly removed from the freezer to remove a few and store the remaining samples again, only $4.1 \pm 0.02\%$ of P is observed. This P amount is significantly lower than the free drug amount before freeze-thaw ($p < 0.001$) and the corresponding dephosphorylation of PP is too small to cause any underestimations of the encapsulated drug with regard to the aimed accuracy ($p < 0.001$). Note, after such a thawing step of 30 min, the protein precipitation method must be so that phosphatases are inhibited immediately. This was safeguarded as described in the section titled ''Immediate deactivation of phosphatases''. Of course, the relative amount of PP converted for smaller concentrations would be expected to be larger. However, for whole blood samples containing a low PP concentration of 1.0×10^2 µM the relative amount of PP converted after one freeze-thaw cycle was $<4.7\% \pm 0.04$ and after three freeze-thaw cycles containing thawing steps of 30 min amounted to be $4.9\% \pm 0.3$. Also here, the corresponding dephosphorylation of PP is too small to cause any underestimations of the encapsulated drug with regard to the aimed accuracy ($p = 0.001$ and $p = 0.008$, respectively). Since whole blood samples exhibit more phosphatase activity compared to plasma, the defined storage conditions for whole blood samples also prevent significant free drug overestimations in plasma samples.

Conclusions

The accurate determination of separate encapsulated and free drug concentrations in tissue, plasma and whole blood is desirable. Suitable methodology to measure such separate concentration profiles in tissues was not yet available.

A murine liver tissue sample preparation method for the accurate determination of such separate concentrations for liposomal PP was developed. Thorough method development and optimization guarantee that under- and overestimations of encapsulated and free drug concentrations are prevented: The use of 10 ml methanol/g tissue (containing the internal standards DP and D) during tissue homogenization verifies: (1) complete liposome rupture, (2) immediate phosphatase deactivation, (3) sufficient clean supernatants, (4) convenient homogenization, (5) excellent extraction of P and sufficient extraction of PP and (6) excellent accuracies and precision complying with the internal guidelines for pre-clinical studies. Similarly, a matching plasma sample preparation method was developed. Here, proteins were precipitated using four equivalents of methanol containing the internal standards. By adding one step, at which the samples were subjected to HIFU to extract possible intracellular drug, the plasma sample preparation method can be freely applied to whole blood samples still yielding accurate results complying with the internal guidelines for pre-clinical studies. One prerequisite: More than one freeze-thaw cycle of whole blood as well as plasma samples should preferably be prevented. The protocols corresponding to the developed sample preparation methods are summarized in Table 5.

Table 5. Schematic representation of the sample preparation methods for murine liver, plasma and whole blood samples to determine the liposomal encapsulated and free drug concentration profiles of liposomal PP.

Liver samples
Immerse in liquid nitrogen for 5–10 s
Immediately, add 10 ml/g methanol containing the internal standards
Immediately, homogenize using a General Laboratory Homogenizer (3×5 s, level 6)
Apply HIFU as described above
Centrifuge for 15 min
Transfer supernatant to vial
Plasma/whole blood samples
Add four equivalents of methanol containing the internal standards
Vortex 30 s
Apply HIFU as described above[a]
Centrifuge for 15 min
Transfer supernatant to vial

[a]Only required for whole blood samples.

Application of above sample preparation methods is going to generate the first PK profile of a liposomal preparation, in which also the encapsulated and free drug concentrations in a tissue are measured separately. Through combining these data with a physiologically based PK model, the *in vivo* drug release from the liposomes can be quantified, which will form an important component in assessing the true PKs.

Although these sample preparation methods are specifically suitable for liposomal encapsulated phosphate prodrugs and possibly, after additional experiments, also for other prodrugs and carrier systems, it is not able to distinguish between a liposomal anthracycline and the free anthracycline drug. However, it can gain important insights into the PK of liposomal formulations in general.

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Declaration of interest

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