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A validated method for the quantification of fosfomycin on dried plasma spots by HPLC-MS/MS: application to a pilot pharmacokinetic study in humans.

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Keywords

Fosfomycin; dried plasma spots; LC-MS/MS; pharmacokinetic; antibiotic

Highlights

- A method for the quantification of fosfomycin using dried plasma spot sampling.
- Validated to FDA guidelines and current scientific standards on microsampling.
- Includes a comparison to gold-standard liquid plasma sampling.
- Includes data from a critically ill patient in a pilot pharmacokinetic study.

Abstract

Quantification of fosfomycin in the plasma samples of patients is the basis of clinical pharmacokinetic studies from which evidence based dosing regimens can be devised to maximise antibiotic effectiveness against a pathogen. We have developed and validated a LC-MS/MS method to quantify fosfomycin using dried plasma spot sampling. Following HILIC chromatography, fosfomycin and ethylphosphonic acid, used as internal standard, were measured using negative-ion multiple reaction monitoring.

The method was linear over the calibration range of 5 to 2000 mg/L of fosfomycin. Intra-day assay results for dried plasma spot quality control samples at 15.6, 79.9 and 1581 mg/L of fosfomycin had precision of ± 4.2, 8.2, and 2.0%, respectively, and accuracy of +3.9, -0.1, and -1.2%, respectively. Recovery of fosfomycin from dried plasma spots was calculated as 83.6% and the dried plasma spot samples were found to be stable stored at room temperature for three months and when stored for four hours at 50˚C. A Bland-Altman plot comparing DPS to plasma sampling found a negative bias of 16.6%, with all but one sample within the mean limits of agreement (-2.6 to 30.6%). Dried plasma spot sampling provides a useful tool for pharmacokinetic research of fosfomycin.

1. Background

Fosfomycin is a broad-spectrum bactericidal antibiotic with a unique mechanism of action, inhibition of phosphoenolpyruvate transferase, the enzyme involved in the synthesis of peptidoglycan which is found in the cell wall of Gram-negative and Gram-positive bacteria [1, 2].

Fosfomycin is generating substantial interest as an intravenous or enteral therapy for multi-drug resistant (MDR) pathogens [3, 4]. With an increasing prevalence of MDR bacteria, combined with a steadily decreasing number of usable antibiotics available, fosfomycin offers a potential infection treatment option for critically-ill patients.

Critical illness is associated with a significant distortion of pharmacokinetics for many antibiotics [5]. Changes in fosfomycin pharmacokinetics can significantly impact concentrations at the site of infection, and as such, dose alterations may be required to ensure that optimal exposures are achieved [6]. Pharmacokinetic studies can provide evidence based dosing regimens and maximise antibiotic effectiveness against a pathogen.

Dry micro-sampling techniques offer several advantages over the gold-standard of traditional liquid plasma sampling for use in clinical pharmacokinetic studies including: low sample volumes, simplified collection, as well as a reduction in shipment and storage costs (traditional liquid plasma samples require frozen storage and transport).

However, dry whole-blood micro-sampling techniques, such as dried blood spots, are known to exhibit a bias due to hematocrit in quantitative analysis for sub-punched dried blood spots due to a non-homogeneous distribution of blood across the spot, viscosity-related diffusion properties of blood on dried blood spots, and may influence recovery and matrix effects in LC-

MS applications [7, 8]. Dried plasma spots may therefore be better suited for clinical pharmacokinetic studies in critically-ill patients, as these patients can commonly experience anaemia and consequently exhibit low levels of hematocrit [9].

Quantification of drugs using the dried plasma spot sampling technique has been described for daptomycin [10], valproic acid and gabapentin [11, 12], linezolid [13], paroxetine[14], acetaminophen [15], guanfacine [16], iothalamate [17], vigabatrin [18], triazoles [19], and anti-epileptic drugs [20], as well as anti-HIV drugs [21].

There are several analytical techniques available for the determination of fosfomycin in human plasma: using gas chromatography [22, 23], liquid chromatography (LC) - spectrophotometric detection [24], LC - photometric detection , capillary zone electrophoresis [25, 26], and, more recently, with derivatization and LC - atmospheric pressure chemical ionization mass spectrometry [27] and LC – tandem mass spectrometry (MS/MS) [28, 29]. However, no methods have been published describing the quantitation of fosfomycin in dried plasma spots.

The aim of this work was to develop a reliable, quick and sensitive method for the quantitation of fosfomycin (structure in Figure 1) using a dried plasma spot (DPS) sampling technique, compare it to the gold standard of whole plasma sampling, and assess its suitability for a clinical pharmacokinetic study.

2. Experimental

2.1. Materials and reagents

Fosfomycin, ethylphosphonic acid (internal standard), methanol, and acetonitrile (HPLC gradient-grade solvent) were purchased from Sigma-Aldrich and ammonium acetate was obtained from Ajax Univar. Ultra-pure water was obtained using a four-module Hi-Pure

Permutit system manufactured by Permutit, Bayswater, Australia. Drug-free human plasma was obtained from the Australian Red Cross Blood Service. Whatman (GE Healthcare, Maidstone, U.K.) supplied the 903 Sample Collection Paper used for dried plasma spots.

2.2. Instruments and conditions

The LC-MS/MS used is a Shimadzu Nexera UHPLC equipped with a Shimadzu 8030+ triple quadrupole mass spectrometer (MS) detector. An electro-spray ionization (ESI) source interface operating in negative-ion mode was used for the multiple reaction monitoring (MRM) LC-MS/MS analysis with the compounds were separated on a Merck SeQuant zic-HILIC, 2.1 x 50 mm, 5.0 µm analytical column. LC and MS conditions for fosfomycin and the internal standard have been previously published [29]. The injection volume used was 1.0 µL. The retention time for both fosfomycin and ethylphosphonic acid was 2.4 min.

2.2.1. Standards for dried plasma spot analysis

Aqueous stock solutions for plasma standard preparation (at 10 000, 20 000 and 50 000 mg/L of fosfomycin) were stored at -80°C. These were diluted with drug free plasma to yield ten calibration standards from 5 to 2000 mg/L of fosfomcyin, ensuring the aqueous dilution volume in each standard was below 5% v/v. The calibration standards were then dispensed (20 µL) onto sample collection paper and dried for two hours at room temperature in a Class 2 Biosafety Cabinet with filtered, circulating air. The calibration standards were then stored in sealed plastic bags and processed alongside the clinical samples.

2.2.2. Internal standard solution

Ethylphosphonic acid in methanol was used as internal standard for the assay (at 10 mg/L) and stored at 4°C.

2.2.3. Quality control sample preparation

Quality control samples were prepared by spiking drug free plasma with fosfomycin stock solutions, prepared independently of standard solutions, to concentrations of 15, 80 and 1600 mg/L (ensuring the aqueous dilution volume in each standard was below 5% v/v), dispensed (20 µL) onto sample collection paper and dried for two hours at room temperature in a Class 2 Biosafety Cabinet with filtered, circulating air. The quality control samples were then stored at room temperature in sealed plastic bags.

2.3. Extraction of dried plasma spot samples for LC-MS/MS analysis

Dried plasma spot samples were extracted by manually punching a 3 mm disc from the centre of the DPS and inserting it into a 96-deep well plate containing 200 µL of internal standard solution (10 mg/L ethylphosphonic acid in methanol). The 96-deep well plate was capped and mixed for 30 minutes at 1200 r.p.m. on a lateral shaker. The dried plasma spot disc was removed, the 96-deep well plate re-sealed, vortex mixed for 3 seconds, followed by LC-MS/MS analysis.

2.4. Extraction of liquid plasma samples for LC-MS/MS analysis

The analysis of plasma samples for LC-MS/MS analysis was performed by protein precipitation with acetonitrile and has been published elsewhere [29] and is used here for comparative purposes with dried plasma spot clinical samples only.

2.5. Data analysis

The concentration of each clinical sample was back-calculated using least squares regression analysis based on the peak-area ratio (drug/internal standard area responses) against concentration (x) from the calibration curve prepared within the batch.

2.6. Method of validation

The validation was performed in accordance with the guidelines provided by the US FDA with pre-established acceptance criteria required to demonstrate the method is suitable for the intended purpose [30] and current scientific standards on microsampling [31-33]. The validation for extraction of fosfomycin from dried plasma spots was assessed for lower limit of quantification (LLOQ), linearity, inter-day precision and accuracy, sample spot volume, matrix effects, recovery, storage and transport stability.

2.6.1. Limit of quantification and detection limits

The LLOQ for fosfomycin was evaluated by analysis of replicate standards (n=5) prepared as dried plasma spot samples and subsequently extracted in a single batch. The acceptance criteria was established as that the back-calculated concentration results should have precision that does not exceed 20% of the CV and accuracy within 20% of the nominal concentration. The limit of detection (LOD) for fosfomycin was calculated based on its definition as being the lowest peak reliably distinguished from the background noise and calculated as ≥ three-times the noise of the blank dried plasma spot sample.

2.6.2. Linearity

To investigate linearity, three calibration curves consisting of ten non-zero standards, using the concentration range of 5 to 2000 mg/L of fosfomycin in plasma and prepared in different runs (n=3) as dried plasma spots. The linearity selected was the simplest calibration model and weighting that satisfied the criteria of results being within 20% deviation from the LLOQ and within 15% deviation of standards other than the LLOQ, from nominal concentrations. Linear regression models were evaluated using a linear and quadratic regression analysis, with

unweighted and weighted parameters evaluated, including weighting factors of $1/x$, $1/x^2$, $1/y$, $1/y^2$.

2.6.3. Precision and accuracy

Intra-assay (n=5) precision and accuracy for fosfomycin throughout the calibration range for dried plasma spot sampling was evaluated by the analysis of quality control samples at three different concentrations, determined against a standard calibration curve in a single batch. Inter-assay precision and accuracy was evaluated similarly, for the duplicate analysis of quality control samples in two separate batches (n=4). The acceptance criteria was established as that the mean value at each concentration should have precision that does not exceed 15% of the CV and accuracy within 15% of the nominal concentration.

2.6.4. Sample spot volume

The effect of sample spot volume was evaluated for a range of dried plasma spot sampling volumes by identifying a trend in fosfomycin concentration across a range of sample spot volumes (from 5 to 30 µL), and determining if there was unacceptable variability (>5%) in peak area obtained from samples prepared from low volumes (5 µL) to high volumes (30 µL).

2.6.5. Matrix effects

Matrix effects were evaluated to identify any suppression or enhancement of signal from an interfering substance around the retention time of fosfomycin by using the matrix factor test. Dried plasma spot samples were prepared with five different blank matrices and extracted in duplicate. The blank matrix extracts and non-matrix (water) samples were spiked with internal standard and low and high concentration levels of fosfomycin and the peak areas measured. The precision of the matrix factor (normalized against internal standard, where applicable) was

used to determine if any concentration level demonstrated unacceptable variability from the expected result.

2.6.6. Recovery

The recovery of fosfomycin was evaluated by comparing the peak area for triplicate samples spiked with fosfomycin in matrix prior to sample preparation, with triplicate samples spiked after sample preparation. Care was taken to ensure the injection matrix was identical in comparable samples.

2.6.7. Storage and transport stability

Stability of fosfomycin stored as dried plasma spot samples at room temperature was assessed by comparing the peak area ratio of the dried plasma spot samples stored at room temperature for three months to the peak area ratio of freshly prepared dried plasma spot samples, with each sample prepared at one concentration and analysis in triplicate. Stability of fosfomycin during transport was assessed by comparing the peak area of dried plasma spot samples, at two concentrations, stored at 50 °C for four hours to the peak area of dried plasma spot samples stored at room temperature for four hours, with each sample prepared in triplicate. Results for stability were considered acceptable when the % difference of the stored sample from the original result was within 15%.

2.7. Pharmacokinetic application

The method was developed and validated to determine the suitability of the analysis of fosfomycin samples stored as dried plasma spots from a pharmacokinetic clinical trial with critically ill patients receiving intravenous fosfomycin for the treatment of an infection in an Intensive Care Unit.

One critically ill patient was administered an intravenous dose of 6 g fosfomycin disodium, every eight hours. Blood samples (3 mL) were taken prior to dosing (0 h) and 0.5, 0.75, 1, 1.5, 2, 4, and 6 h post administration using heparinized vacuum tubes (Greiner Bio-One, Vacuette® LiHep) on the second day of fosfomycin administration and on the fifth day of fosfomycin dosing. Blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma samples. Plasma samples were transferred into 2 mL polypropylene tubes, capped and stored at -80°C. On receipt in to the laboratory, and immediately prior to plasma analysis, samples were thawed and 20 µL dispensed onto dried plasma spot paper. The dried plasma spot clinical samples were then dried for 2 hours at room temperature in a Class 2 Biosafety Cabinet with filtered, circulating air. The dried plasma spot clinical samples were then stored in sealed plastic bags at room temperature until analysis.

This clinical procedure was conducted in accordance with the principles laid down by the ICH guidelines for Good Clinical Practice and approved by the University of Queensland Medical Research Review Committee (clearance # 2012000870) and the Epistimoniko Symvouleio (Scientific Committee) of Attikon University Hospital (approval ΜΕΘ-84/13-3-12).

The samples from the pharmacokinetic profile acquired are used to evaluate the correlation between the use of dried plasma spot sampling to the gold-standard sampling technique of frozen plasma. Correlation between the two methods was assessed with a Bland Altman plot (Figure 2b) using Microsoft® Excel® for Mac 2011, version 14.2.2, with 95% confidence intervals reported for the mean and limits of agreement.

3. Results and discussion

3.1. Use of dried plasma spot extraction

The use of dried plasma spot allows a very simple extraction of drug from the proteinaceous matrix. The extraction performed in this method was clean and did not require centrifugation prior to LC-MS/MS analysis. The LOD and repeatability of LLOQ demonstrate the ability of the method to use very small samples - with a sub-punch from a 20 µL sample being used in the extraction – and sample volume testing demonstrating it is not necessary to apply precise volumes of plasma in order to obtain accurate results.

While the venous sampling and centrifugation of dried plasma spots is more onerous for staff performing than the sampling than dried blood spots, the advantages of transportation, storage and simplicity of extraction are still realised relative to traditional liquid plasma samples. The use of membrane filtration devices to directly form dried plasma spot samples may offer a simplification to preparation [16, 34] and with this the dried plasma spot sampling techniques may offer more of the benefits currently available with dried blood spots – smaller sample volumes from thumb or needle-prick, no pre-treatment prior to shipment – without the inherent challenges of samples containing hematocrit.

3.2. Validation

The precision and accuracy (n=5) of the dried plasma spot sample extraction at the LLOQ are reported in Table 2, with all five individual accuracy results within 10%. The limit of detection (LOD) is defined as being reliably distinguished from the background noise and calculated as ≥ three-times the noise of the blank plasma sample. From the validation the LLOQ ratio of signal

to noise was calculated as 35, and therefore the LOD was estimated as being approximately 0.5 mg/L for the dried plasma spot sample extraction.

A linear regression provided the simplest calibration model across the concentration range, and the 1/concentration^2 weighting improved the correlation coefficient considerably. This model met the acceptance criteria with the back-calculated concentration results being within 20% deviation from the LLOQ and within 15% deviation of standards other than the LLOQ, from nominal concentrations, for eight out of ten of the non-zero standards. The calibration range, mean correlation coefficient (r^2) and the percentage of maximum deviation (inaccuracy) of the standards of the three calibration curves are presented in Table 1.

The precision and accuracy of the dried plasma spot sample assay at the prepared QC levels are presented in Table 2; the acceptance criteria was met in all cases.

Sample volume testing demonstrated no trends across a range of sample spot volumes and a +1.6% difference in peak area obtained between a sub-punch from a 5 µL sample spot and a 30 µL sample spot.

No signal suppression/enhancement was evident for fosfomycin or the internal standard (ethylphosphonic acid) for extraction using dried plasma spot samples, from the matrix studies performed as evidenced by significant bias or variability. The results of the matrix effect evaluations are described in Table 2.

Validation revealed adequate recoveries for both fosfomycin (83.6%) and ethylphosphonic acid (87.1%) from dried plasma spots. The performance of the method was not adversely affected by the less than complete recovery as demonstrated by there being sufficient signal at the LLOQ, and acceptable precision and accuracy validation.

Stability of fosfomycin in plasma when applied to sample collection paper and stored at room temperature for 3 months was acceptable with a difference in peak area ratio observed of -4.9% at 500 mg/L of fosfomycin. Stability of fosfomycin during transport at 50˚C for four hours was acceptable with a mean difference observed of 0.2%.

Stock solution stability for fosfomycin has been previously reported as being stable for aqueous solutions stored for over 16 months at -80°C and for over 11 months at -20°C; freeze-thaw stability in liquid plasma samples has also been previously reported as being stable across three freeze-thaw cycles [29]. In-house data on storage of frozen plasma samples containing fosfomycin was acceptable with a difference in concentration of 11.6% at -20°C for 20 months.

3.3. Application

This method has been successfully applied to samples of a clinical pharmacokinetic study. The plasma concentration-time patient profile for one patient is presented in Figure 2. A 58 year old male provided samples after receiving his fourth and thirteenth dose of 6 g intravenous fosfomycin disodium, every 8 hours. The results of the Bland-Altman plot (Figure 2b) demonstrate that the DPS concentrations were subject to a consistant negative bias (16.6%) compared to the plasma samples, with the precision from the mean result relatively limited (7%). The 95% confidence interval for the mean is -12.9 to -20.4%. All but one sample were within the mean limits of agreement (-2.6 to 30.6%). The 95% confidence interval for the upper limit of agreement was -9.1 to 3.8%, and for the lower limit of agreement was -37.1 to -24.2%. Of the 16 samples analysed the differences between the DPS and plasma concentrations 75% were below 20% of the mean result, thereby fulfilling the acceptance criteria of a incurred sample reanalysis, criteria which is intended for replicate analysis were the results here are from different matrix preparations, different extraction procedures, analysed in small and

single analytical runs, and separate analytical runs (with whole plasma performed separately to the DPS analysis), and prepared from different standard solution preparations.

4. Conclusion

The method presented here offers a validated quantitative analysis of fosfomycin using a dried plasma spot sampling technique. The assay performance is accurate and precise, with sufficiently sensitivity and range of calibration for a clinical pharmacokinetic study. The dried plasma spot sampling technique has advantages over traditional plasma sampling in terms of storage temperature, transport and simplicity of sample preparation. The results of the dried plasma spot samples from a clinical pharmacokinetic study were subject to a negative bias of 16.6%. Of the 16 samples analysed the differences between the DPS and plasma concentrations 75% were below 20%, thereby fulfilling the acceptance criteria of a incurred sample reanalysis. This method has been demonstrated to be suitable for clinical pharmacokinetic study applications for fosfomycin and may provide greater opportunities for collaborative research in critically ill populations, including, burns or obese patients.

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Table 1: Linearity Analysis

Sample	Calibration range	Correlation coefficient *	Maximum deviation (%)**
dried plasma spot	5 to 2000 mg/L	0.9983	7.6

*Mean (n = 3)

** Reported maximum deviation from nominal (%) across all standard curves and concentration levels

Table 2: Intra-assay and inter-assay precision and accuracy, matrix, recovery and stability studies

Study	Concentration (mg /L)	Sample #	Mean	Accuracy (%)	Precision (%)
LLOQ	5	5	4.96	-0.8	±6.3
Intra-assay Precision & Accuracy	15	5	15.6	+3.9	±4.2
	80	5	79.9	-0.1	±8.2
	1600	5	1581	-1.2	±2.0
Inter-assay Precision & Accuracy	15	4	15.7	+4.3	±9.0
	80	4	77.8	-2.8	±5.2
	1600	4	1632	+2.0	±2.3
Matrix	50	5	1.00[a]		±0.8
	500	5	1.02[a]		±2.7
Recovery	500	3	83.6%		±4.7
Stability RT[b]	500	3		-4.9 loss	
Stability Trans[c]	500	3		0.2 increase	

[a] matrix factor: calculated as a ratio of peak area of analyte in the presence of matrix to the peak area in the absence of matrix (normalized using the internal standard).

[b] stability at room temperature: calculated as a ratio of peak area analyte to internal standard compared over three months of storage as a dried plasma spot sample.

[c] stability during transport at 50°C: calculated as a ratio of peak area analyte to internal standard compared to 4 hours of storage as a dried plasma spot sample

Figure 1: Structure of fosfomycin (FOM, left) and the internal standard, ethylphosphonic acid (EPA, right),[35,36].

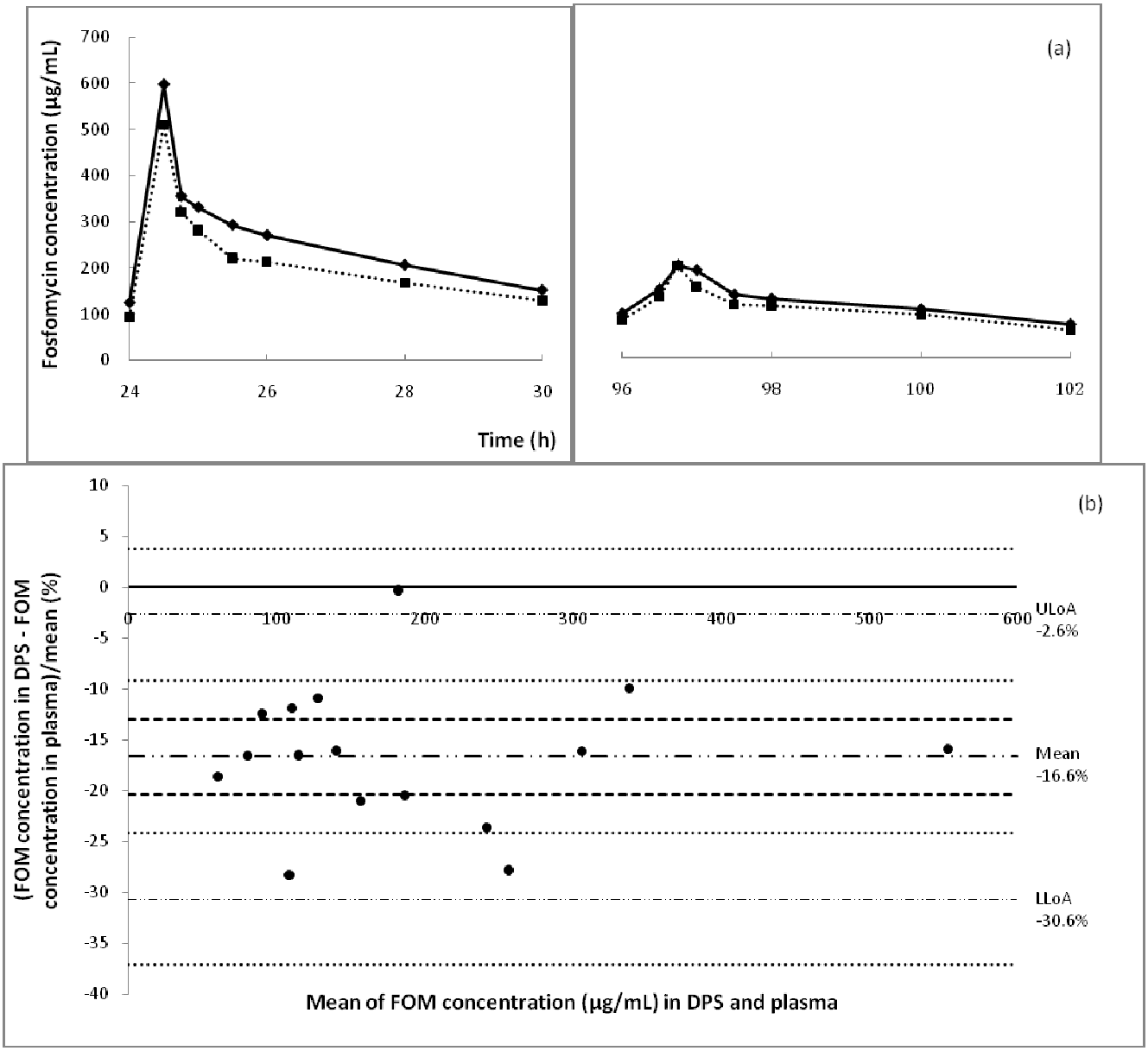

Figure 2(a): Plasma concentration – time profiles of fosfomycin in a critically ill patient receiving a 6 g fosfomycin IV dose every 6 hours, for the second and fifth doses, prepared using an extraction from plasma (diamond, full-line) and dried plasma spots (DPS, squares, dotted line); 2(b): Bland-Altman plot between plasma and DPS samples obtained for fosfomycin (FOM) concentrations from a critically-ill patient. The mean difference demonstrated a negative bias of 16.6%, with all but one sample within the mean limits of agreement (-2.6 to 30.6%).