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Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography–mass spectrometry

J. van der Heijden[a,*], Y. de Beer[a], K. Hoogtanders[a], M. Christiaans[b], G.J. de Jong[c], C. Neef[a], L. Stolk[a]

[a] Department of Clinical Pharmacy and Toxicology, Maastricht, University Hospital, The Netherlands
[b] Department of Internal Medicine, Maastricht, University Hospital, The Netherlands
[c] Department of Pharmaceutical Sciences, Utrecht, University, The Netherlands

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ABSTRACT

An assay of everolimus based on finger prick sampling and consecutive application as a blood spot on sampling paper has been developed. We explored several methods [K. Hoogtanders, J. van der Heijden, M. Christiaans, P. Edelbroek, J. van Hooff, L. Stolk, J. Pharm. Biomed. Anal. 44 (2006) 658–664; A. Allanson, M. Cotton, J. Tettey, et al., J. Pharm. Biomed. Anal. 44 (2007) 963–969] and developed a new method, namely the impregnation of sampling paper with a solution of plasma–protein, formic acid and ammonium acetate, in combination with the extraction of the blood spot by filter filtration. This kind of sample preparation provides new possibilities for blood spot sampling especially if analytes are adsorbed to the paper.

The dried blood spot was analysed using the HPLC–electrospray-tandem mass spectrometry method, with 32-desmethoxyrapamycin as the internal standard. The working range of our study was 2–30 μg/l. Within this range, intra-and inter-assay variability for precision and accuracy was <15%. Everolimus blood spot samples proved stable for 3 days at 60 °C and for 32 days at 4 °C. Everolimus concentrations of one stable out-patient were compared after both blood spot sampling and conventional venous sampling on various occasions. Results indicate that this new method is promising for therapeutic drug monitoring in stable renal transplant patients.

1. Introduction

Everolimus (Certican®) is a proliferation signal inhibitor used for the prophylaxis of acute and chronic rejection in transplantation. It has a macrolide lactone structure similar to sirolimus, but with an additional 2-hydroxyethyl moiety at position 40 on the sirolimus molecule [3]. See Fig. 1 (molecular weight = 958.2 Da).

In kidney transplant patients, a relationship between pre-dose everolimus blood concentration and various efficacy and safety parameters has been reported [3]. These data suggest that therapeutic drug monitoring (TDM) of everolimus would be beneficial for optimizing dosage regimens and improving clinical outcomes.

As we have previously demonstrated with tacrolimus [1,5], dried blood spots (DBS) sampling could be a promising alternative to venous sampling. In DBS sampling, the patient's capillary blood is first obtained from a finger prick with an automatic lancet. A drop of blood is then applied to a designated circle on special sampling paper. After it has dried, the paper with the blood spot sample is sent by mail to the laboratory. The laboratory punches out a disk from the blood spot. The filter paper disk must provide a volumetric measurement that is similar to liquid measurement devices [6]. The drug is extracted from this disk and ready for further analysis.

DBS sampling has become common practice for use with newborns. Over the past decade, many applications of DBS have been reported for both qualitative and quantitative screening of metabolic disorders [7]. TDM with the DBS method is rather new and is, thus, full of challenges, such as those we encountered when we started with liquid/liquid or liquid/solid extractions. DBS has been proposed for use with various classes of drugs, including antiretrovirals [8,9], antimalarials [10,11] and antidiabetics [12]. Still there is no relevant research done in doing TDM by DBS sampling. Important problems are sensitivity because of the limited sample volume, especially at lower therapeutic concentration levels, sampling by patients, and reproducible sample preparation.

Sensitivity could be solved by using more sensitive techniques like LC–MS/MS. Sampling could be done by two methods, like mentioned above with blood as a spot on sampling paper or with accurately pipetting capillary blood on sampling paper [6].

Reproducible sample preparations is the topic of this paper. The degree to which sample preparations can be reproduced is

* Corresponding author at: Department of Clinical Pharmacy and Toxicology, Maastricht University Hospital, PO Box 5800, 6202 AZ Maastricht, The Netherlands. Tel.: +31 43 3874715; fax: +31 43 3874731.
E-mail address: j.vander.heijden@mumc.nl (J. van der Heijden).

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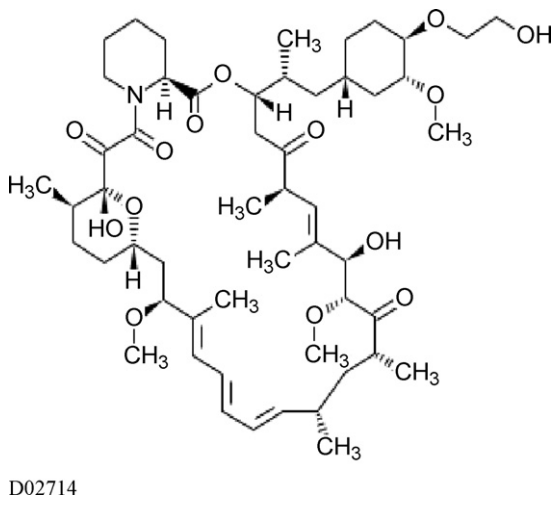

Fig. 1. Structure of everolimus.

dependent on the component to be determined, the sampling paper used and sample preparation procedure.

From the viewpoint of the patient or clinician, there are no disadvantages of DBS. Possible advantages of TDM with DBS are as follows: (1) patients are not obliged to leave their homes and no skilled personnel are required; (2) monitoring results are available prior to patients' visit to the clinician for routine control; (3) monitoring at any desired sampling time can be conducted conveniently; (4) there is no biohazard risk of sending samples by post; and (5) samples are often more stable in blood spots than in venous blood [8].

This paper describes the everolimus blood spot assay in combination with liquid chromatography–tandem mass spectrometry. Special attention has been paid to the pre-treatment of the filter paper and the extraction method.

2. Materials and methods

2.1. Routine assay for everolimus in venous blood

The routine everolimus assay in venous blood in our laboratory is based on LC–MS. The method for measuring everolimus in venous blood samples and our newly developed method using DBS samples are essentially the same, with the exception of the sample preparation procedure. The sample preparation of the venous blood samples involves the precipitation of 200 μl EDTA blood with a 200-μl precipitation reagent (internal standard solution mixed with zinc sulphate heptahydrate 89 mg/l in water in a 4:1 ratio). The supernatant is then transferred to another Eppendorf cup and 50 μl is injected into the liquid chromatographic system.

2.2. Chemicals

The everolimus used in our study was provided by Novartis Pharma AG (Basel, Switzerland), and the 32-desmethoxy-rapamycin used as an internal standard was provided by Wyeth–Ayerst Research (Princeton, NY, USA). Methanol LC/MS grade was obtained from Biosolve (Valkenswaard, The Netherlands), ammonium acetate, formic acid and ethanol absolute PA grade were obtained from Merck (Darmstadt, Germany). Lastly, the pasteurised plasma–protein solution 40 g/l was obtained from Sanguin (Amsterdam, The Netherlands).

The clinical check-controls for immunosuppressants used in this study were no. 8833, lot 547 (Recipe Chemicals and Instruments, Munich, Germany). The sampling paper used was Protein saver 903, ref no. 10531018, and was obtained from Whatman Gmbh (Dassel, Germany). Costar spin-X HPLC 0.2 μm nylon filter, catalogue no. 8169, was used as the micro-centrifuge filter. The peelable heat-sealing foil sheets used were obtained from Westburg (Epson, The UK). Everolimus-free blood was gathered from volunteers in the laboratory.

2.3. Stock and working solutions

Stock solutions: stock solutions of everolimus (1000 mg/l) and internal standard 32-desmethoxy-rapamycin (1000 mg/l) were prepared in ethanol. These solutions were further diluted until a concentration of 10 mg/l in ethanol was attained. This concentration was used as the working solution. The everolimus solutions were stored at −20 °C. The internal standard solution was stored at +4 °C. Under these conditions, all solutions are expected to be stable for at least 12 months.

2.4. Eluent A

The eluent A (2 mmol of ammonium acetate in water +0.1% (v/v) formic acid) was prepared by dissolving 0.31 g of ammonium acetate in 2000 ml water and then adding 2 ml of formic acid.

2.5. Eluent B

The eluent B (2 mmol of ammonium acetate in methanol +0.1% (v/v) formic acid) was prepared by dissolving 0.31 g of ammonium acetate in 2000 ml methanol and then adding 2 ml of formic acid.

2.6. Extraction solution blood spot with internal standard

The extraction liquid for the blood spot with internal standard was prepared by diluting 75 μl of the internal working solution (10 mg/l) into methanol up to 25 ml. This solution is then diluted 10 times with eluent B resulting in a concentration of 3 μg/l.

2.7. Impregnation of filter paper

50 mg ammonium acetate and 50 μl of formic acid were added to 20.0 ml of pasteurised plasma–protein solution to create the impregnation solution.

30 microliters of the impregnation solution were then applied to the predefined spot on the sampling paper. The paper was then allowed to dry at room temperature for a minimum of 4 h.

2.8. Instrumentation and chromatographic conditions

2.8.1. On line extraction and chromatographic conditions

Liquid chromatography was performed using a Waters 2795 Alliance HPLC system (Waters Ltd., Watford, UK). The sample was injected into a solid-phase extraction column (Waters Oasis HLB cartridge column, 2.1 mm × 20 mm, 25 μm). After flushing for half a minute with a mixture of eluent A and eluent B (40:60) at a flow rate of 3 ml/min, the analytes were foreflushed into the analytical column (Waters Atlantis dC18 3.0 mm × 100 mm, 5 μm) with another mixture of eluent A: eluent B (3:97) at a flow rate of 0.75 ml/min. Four minutes after the start of the cycle, the flow rate was set to 1.5 ml/min. After 6 min, the column-switching device was set to the starting position in order to equilibrate for the next injection that would take place 30 s later. The analytical column was heated to 60 °C and the pre-column remained at room temperature. The total cycle time was six and a half minutes for each injection.

2.8.2. Mass spectrometry

In this study, a Quatro Micro-tandem mass spectrometer fitted with a Z spray ion source was used (Waters, Manchester, The UK). The instrument was operated in the electrospray-positive ionization mode, and was directly coupled to the LC system. System control and data acquisition were performed with MassLynx 4.0 software.

Tuning of the instrument was done by infusing a solution of everolimus or 32-desmethoxy-rapamycin (1 mg/l) in mobile phase into the ion source, and the cone voltage was optimized to maximize the intensity of the $(M+NH_4)^+$ precursor ions of everolimus and 32-desmethoxy-rapamycin (m/z 975.8 and 901.5, respectively). The collision energy was then adjusted to optimize the signal for the most intense product ions (m/z 908.8 and 834.5, respectively). Typical tuning conditions were as follows: the electrospray capillary voltage was 1.0 kV, the sample cone voltage was 24 V, and the collision energy was 19 eV. Additionally, the source temperature was 120 °C while the desolvation temperature was 350 °C. The desolvation gas (nitrogen) flow was set to 800 l/h, and the collision gas used was argon. Sample analysis was performed in the multiple reaction monitoring mode of the mass spectrometer, with a dwell time of 0.2 s per transition. Calibration curves were constructed using linear least square regression with $1/x$ weighting.

2.9. Hole puncher

Paper disks with a diameter of 7.5 mm were punched with an electromagnetic driven hole puncher, which was specially developed for DBS analysis of drugs, and designed by P. Edelbroek (SEIN, Hoofddorp, The Netherlands), in cooperation with the Department of Mechatronics Engineering, Leiden University Medical Centre, Leiden, The Netherlands.

2.10. Seal apparatus

The Eppendorf cups were sealed with peelable heat-sealing foil developed by a Westburg sealing apparatus (Leusden, The Netherlands).

2.11. Sampling

Venous sampling was done by venapuncture and the samples were collected in vacutainer tubes (Becton and Dickinson, Franklin Lakes, NY, USA) containing EDTA and stored at 4 °C until analysis. Finger-prick blood samples were collected using BD Microtainer Contact-Activated, lancet no. 366593 (Becton and Dickinson, Franklin Lakes, NY, USA). Patient samples were collected from the fingertip. The blood drop was collected to fill an 8 mm diameter predetermined circle on the above-mentioned sampling paper. The blood spots were allowed to dry at room temperature overnight.

2.12. Calibration standards and quality control samples.

2.12.1. DBS calibration standards

The everolimus working solution (10 mg/l) was diluted with distilled water to a concentration of 500 μg/l. This solution was added to fresh everolimus-free EDTA blood in order to obtain a 50-μg/l solution of everolimus in the blood. This solution was diluted with fresh EDTA blood to obtain the calibration standards 2, 3, 6, 9, 15 and 30 μg/l. A blank was also included.

2.12.2. DBS quality standards

DBS quality controls were prepared in our laboratory. Three concentrations of quality control blood pools were prepared with a concentration of 3, 8, and 24 μg/l, similar to the calibration standards used with fresh everolimus-free EDTA–blood. These were stored at 4 °C. The quality control standards and the calibration standards were compounded separately as different batches.

2.12.3. Calibration quality controls on sampling paper.

Thirty microliters of each calibration standard or quality control sample were applied with a positive displacement pipette onto impregnated sampling paper and kept overnight at room temperature to dry.

2.13. Method development sample preparation of the DBS.

Three DBS sample preparation methods were compared in a preliminary study, both with and without impregnation of the sampling paper.

The first sample preparation method we used was identical to the one we published earlier for tacrolimus [1,5]. Sample preparation consisted of punching out the DBS and placing them into glass tubes. Two hundred and fifty microliters of extraction solution were added. The tubes were shaken on a mechanical shaker for 60 min. The liquid phase was pipetted to an Eppendorf cup and 50 μl was injected on the HPLC system.

The second method we tried was sample preparation according to Allanson et al. [2] with vortex shaking in combination with ultrasonic vibration. The sample preparation consists of punching out the DBS and placing them into glass tubes. Two hundred and fifty microliters of extraction solution were added. The tubes were vortexed for 5 min and then placed into an ultrasonic bath for 5 min and then vortexed again for 5 min. The liquid phase was pipetted into an Eppendorf cup and 50 μl was injected onto the HPLC system.

The third method is the one finally chosen for the everolimus assay and validated. Paper disks were punched out of the DBS and placed on a micro-centrifuge filter. The punch is pressed onto the filter. Two hundred and fifty microliters of extraction solution were added. The tubes were placed in a ultrasonic bath with the liquid level of the bath just above the level of the blood spot. After 30 min of ultrasonic vibration the tubes were centrifuged 5 min at 4000 rpm. After centrifugation the filter was removed and the Eppendorf cup was mixed by vortexing for 10 s.To avoid evaporation the cups were sealed with peelable heat-sealing foil sheets. An aliquot of 50 μl of the samples was injected into the HPLC system. Fig. 2 shows a scheme of this sample preparation procedure.

All three blood spot sample preparation methods were compared in the preliminary study with and without impregnation of the sampling paper.

2.14. Analytical method validation

Analytical method validation was conducted according to the guidance of the industry [15].

2.14.1. Accuracy, limit of detection and lower limit of quantification

The accuracy and precision of the method were investigated by analyzing 30 μl of spiked fresh venous blood placed onto the sampling paper at three different concentrations (3, 8, and 24 μg/l). Analysis was done in duplicate on 6 separate days over a period of 3 months (inter-assay and trueness). On the first day, each concentration level was analysed six times with one calibration line (intra-assay). Each concentration of the calibration curve was also analysed five times on the first 3 days (precision profile).

Acceptance criteria were precision and trueness <15%. The limit of detection was based on a peak height three times greater than the largest baseline fluctuation in a 1-min window at the elution time of the analyte in an analytical blank. The lower limit of quantification

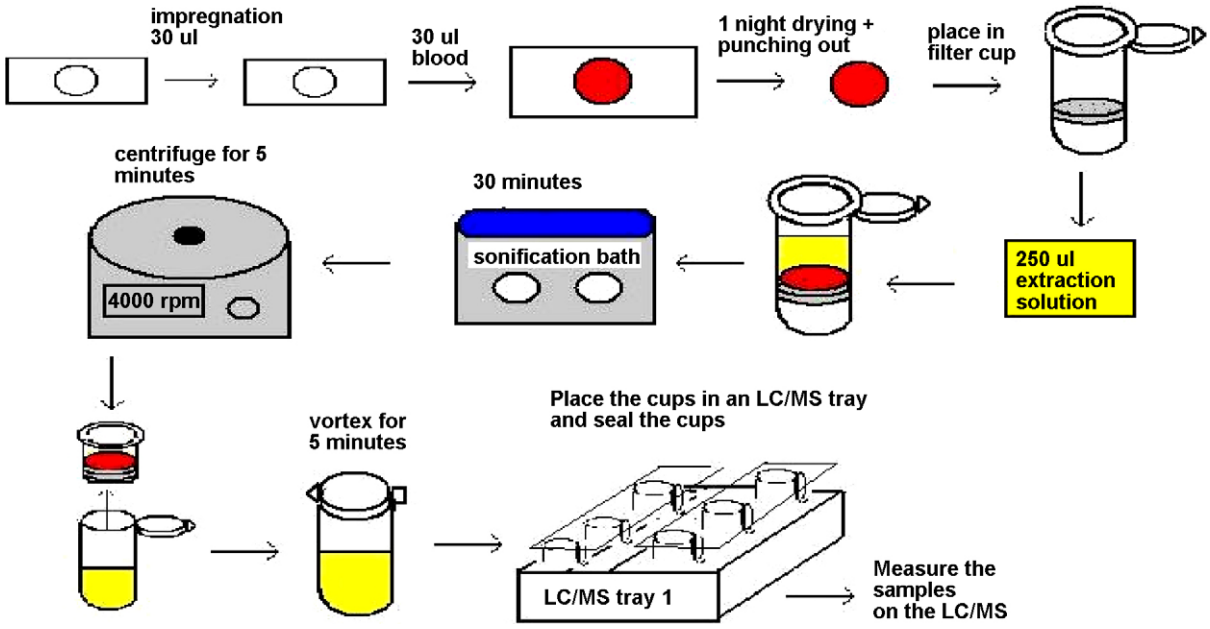

Fig. 2. Sample preparation method.

was defined as the concentration at which the analyte response of a spiked sample had a precision of <20% and trueness of <20%.

2.14.2. Linearity

Calibration graphs were constructed using seven concentration levels in the range of 2–30 μg/l and a blank, all in duplicate. The ratio of the everolimus peak area and the internal standard area at each concentration was plotted as a function of the everolimus concentration, and 1/*x*-weighted linear regression was performed.

2.14.3. Extraction recovery

For the extraction recovery, the everolimus peak areas from 15 μl blood spots of quality controls were compared with the areas after direct injection of the standards at the same nominal concentrations which followed only reconstitution. We used 15 μl instead of 30 μl in order to recover the complete blood spot with the hole puncher and to compare it with the same standard of 15 μl.

2.14.4. Matrix effects (ion-suppression)

For the study of MS ion suppression, extracts of six everolimus-free patient samples were spiked with concentration levels of everolimus at the level of 8 μg/l. The ratio area was compared to standards without extraction at the same nominal concentrations after reconstitution [16].

2.14.5. Selectivity method

In order to investigate whether interfering endogenous compounds were present, surplus venous EDTA blood, from everolimus-free routine samples of 10 different patients taking immunosuppressive medication, was analysed using the blood spot method. The blood spots were extracted without internal standard.

2.15. Stability of DBS

The stability of the DBS samples was tested using quality controls. These quality controls were stored at different temperatures (4, 20, 32, 60, and 70 °C) and were measured at different points in time. The results were compared with the results of freshly made blood spots.

2.16. Comparison of DBS and venous samples from a patient

In Maastricht University Hospital's out-patient department, both venous samples and blood spot samples were collected from a patient on different occasions. Upon arrival in the laboratory, the blood spots were inspected visually.

Criteria for inclusion were:

(1) The pre-defined 8 mm circle must be homogenously and symmetrically filled.
(2) Both sides of the paper must have the same red colour.

Assays were performed within 5 days. Blood spots of 30 μl were also prepared from the venous patient samples in our laboratory. The DBS sample created by the patient was compared to the one obtained by the laboratory and also to the venous blood samples. The patient provided informed consent for the use of their samples in this study. Further, this study was approved by the local medical ethics committee.

3. Results

3.1. Routine assay for everolimus in venous blood

The assay in the venous blood was linear from 1 to 100 μg/l with a working range of 1–30 μg/l. Intraday precision and accuracy were 3.0, 4.4, 2.4% and 87.1, 97.0 and 97.6%, respectively ($N = 6$). Interday precision and accuracy were 7.6, 6.6, 6.2% and 101.0, 97.4 and 105.0% ($N = 9$), respectively at 3.5, 10.5 and 18.2 μg/l (Recipe Clinical check-controls).

The limit of quantification was 1 μg/l. Our laboratory participated in the international everolimus proficiency testing scheme. Results of the last nine samples showed satisfactory accuracy and precision, i.e. 106.9 and 6.5%.

3.2. Method development

One of the most difficult parts during the method development was to achieve a high and reproducible recovery from the sampling paper. A variety of different methods were evaluated and according

Table 1
Influence of impregnation on precision using different sample preparation methods.

NR method	Concentration (μg/l)	Precision $N = 5$ (%)	
		Without impregnation	Impregnation
1	2	27	38
	8	16	9
	30	19	11
2	2	18	15
	8	14	11
	30	11	13
3	2	31	10
	8	4	8
	30	9	8

to Blessborn et al. [13] we try to modify the paper by impregnation where we used different solvent combinations from acids to quaternary ammonium compounds and plasma protein solutions. The best combination was the mixture of plasma–protein, formic acid and ammonium acetate solution. Primary experiments showed that formic acid and ammonium acetate had influence on the extraction recovery and plasma proteins on the precision of the method. The mixture was used in a precision study between the three sample preparation methods.

Blood samples of everolimus free kidney transplant patients spiked with 2, 8, and 30 μg/l of everolimus were applied to both impregnated and non-impregnated paper. Precision of $N = 5$ was calculated for all the concentration levels. The results of the influence of each method on impregnation are shown in Table 1.

Impregnation had no remarkable influence on the precision of methods 1 and 2. The best results were obtained by using impregnation in combination with method 3 (filter filtration method).

Like Blessborn et al. [13] our strategy was to decrease the hydrogen bond activity of the –OH groups at the cellulose matrix by treating the sampling paper with the mixture of plasma–protein, formic acid and ammonium acetate solution. Still this is not enough for everolimus and it is necessary to pull the extraction liquid more vigorous through the blood spot.

3.3. Method validation DBS samples

Accuracy, precision, lower limit of quantification and detection limit. The accuracy is shown in Table 2 as inter-assay, intra-assay and trueness. For all three concentrations, the coefficients of variation and trueness were less than 10%.

In order to estimate at which concentration LOQ could be found, a precision profile of the calibration curve was made by analyzing each concentration five times on 3 days. Fig. 3 demonstrates the precision profile of the method ($n = 15$). It also indicates that the LOQ was between 1 and 2 μg/l.

The LOQ of the method was investigated by analyzing the 30 μl of spiked fresh venous blood at a concentration of 1 and 2 μg/l.

Table 2
Accuracy of assay at three concentrations of everolimus.

	QC 3.0 μg/l	QC 8.0 μg/l	QC 24 μg/l
Inter-assay (n)	7	7	7
Mean (μg/l)	2.86	7.86	22.24
S.D. (μg/l)	0.23	0.74	0.88
CV (%)	8.2	5.4	4.0
Trueness (%)	95.3	98.2	92.7
Intra-assay (n)	6	6	6
Mean (μg/l)	3.04	8.13	23.87
S.D. (μg/l)	0.23	0.39	0.82
CV (%)	7.74	4.74	3.42
Trueness (%)	101.2	101.7	99.5

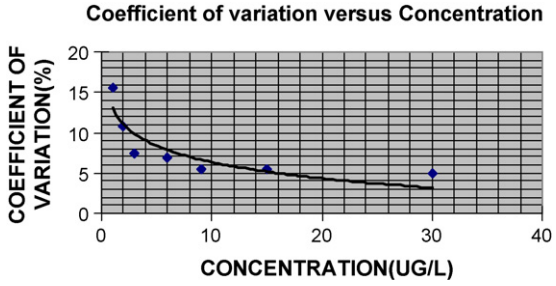

Fig. 3. Precision profile of everolimus.

Table 3
Precision at limit of quantitation.

Concentration level (μg/l)	Precision (%)	Trueness
1	15.6	126.8
2	10.8	110.4

Analyses were conducted in duplicate over 6 days. Table 3 displays the results of samples spiked with 1 and 2 μg/l. According to the guidelines [15], the limit of quantification is 2 μg/l.

The limit of detection estimated by three times noise was 0.5 μg/l. The proposed therapeutic range for everolimus in kidney allograft patients in the first year following the transplant, with reduced exposure to cyclosporine, is 3–8 μg/l with an apparent tolerable upper concentration limit of 12 μg/l [18]. The accuracy of the method was found to be adequate in this concentration range thus, making TDM possible.

3.4. Linearity

The calibration curve was linear from 2 to 30 μg/l. The mean regression coefficient of the calibration line was $R^2 = 0.98732$, S.D. = 0.00243 ($n = 6$).

3.5. Extraction recovery

Measurements were done with and without impregnation, in combination with the filter filtration method. The results are shown in Table 4. Extraction without impregnation was obviously less than with impregnation. These results indicate that impregnation is required in order to attain a more substantial recovery.

3.6. Matrix effects

Matrix effects were measured by comparing the results of six everolimus-free patient blood extracts spiked with 8 μg/l everolimus with six standards in an extraction liquid of 8 μg/l. The recovery ratio was 105.9% ± 2.7% indicating that there is no matrix effect.

3.7. Selectivity

Analysis of everolimus-free blood from 10 different kidney transplant patients, who were taking immunosuppressive medication at the time of this study, showed no interfering peaks for everolimus or the internal standard.

Table 4
Extraction recovery everolimus at 8 μg/l.

Concentration level	8 μg/l
With impregnation	76.5% ± 5.4% ($N = 5$)
Without impregnation	60.8% ($N = 2$)

Table 5
Stability study everolimus in DBS at different temperatures.

Temperature (°C)	Result after storage time (days) compared to fresh made blood spot (%)							
	3	7	14	16	20	24	30	34
4		102 ($N=6$)		88 ($N=6$)	100 ($N=6$)		96 ($N=6$)	
Room		106	93			97		91
32		88	86			87		87
60	95% ($n=6$)							
70		76	77			73		61

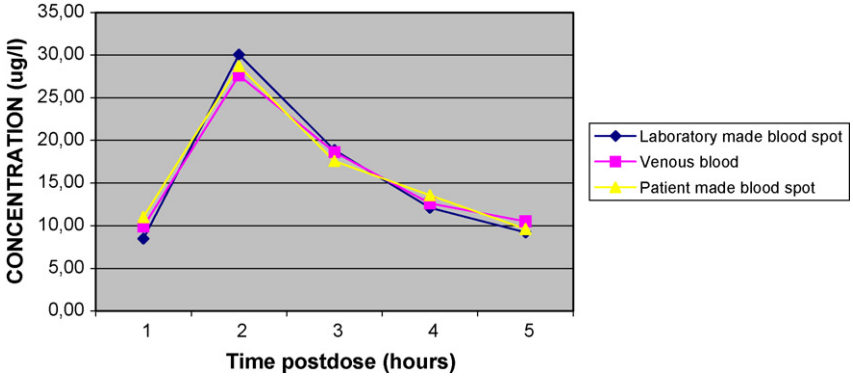

Fig. 4. Concentration time curves of everolimus.

3.8. Stability

The goals of the stability testing of DBS testing were:

(1) To investigate the stability of DBS samples during simulated transport conditions.
(2) To investigate the stability of calibration standards and controls of DBS in order to determine whether batch-wise preparation and subsequent storage at 4 °C would be possible.

Everolimus concentrations of a spiked sample of 15 μg/l were measured the day after sampling and then again after several days of storage under different temperature conditions. The results of the 60 and 4 °C were in six fold other results are in duplicate (Table 5).

The results show that the everolimus blood spots were stable for 3 days in a mailbox at 60 °C, and calibration curves and quality controls can be stored for 30 days at 4 °C. At room temperature and at 32 °C, all the results were within 15% of the nominal value. At 70 °C, the deviation was more than 15%, indicating that everolimus is not stable in a blood spot at this temperature.

3.9. Comparison of everolimus concentrations in blood spots and venous samples from a patient

The concentration time curves of everolimus after venous sampling, DBS sampling by patient, and DBS sampling by the laboratory are shown in Fig. 4.

Blood spot sampling of everolimus seems to give higher results than venous sampling but these differences are not significant.

4. Discussion

When developing the method for our assay of everolimus, we began by evaluating the method we had previously published on our work with tacrolimus [1]. Our preliminary findings were that extraction recovery at lower concentrations was greater than at higher concentrations. Optimization through prolonged shaking time, ultrasonic vibration, and the development of a new method based on Allanson et al. [2], gave higher recoveries of the upper concentration levels. Even still we discovered that the recovery depended on the concentration, and the precision did not meet our demands especially at lower concentrations.

Our findings during method development can be explained as follows:

In both our tacrolimus method [1] and the method posited by Allanson et al. [2], the solution is in direct contact with the surface of the blood spot and not in direct contact with the inner side. The migration of the extraction liquid at the inner side is rather slow and is characterized by diffusion. Everolimus stays on the surface at lower concentrations and permeates the blood spot at higher concentrations. This may explain why the extraction recovery at lower concentrations is greater than with higher concentrations. By extracting the liquid more vigorously by the use of the filter filtration method in combination with impregnation, the precision was lower than 15% and the extraction recovery increased by approximately 16%. Impregnation could deactivate sites of active adsorption on the paper and make desorption of components easier.

The validation process shows that accuracy between 2 and 30 μg/l corresponds with the guidelines. The LOQ and LOD are of clinical relevance meaning that pharmacokinetic studies are possible with this method.

DBS samples are stable for 34 days at 32 °C, and for 3 days at 60 °C. This stability study indicates that blood spots could be sent by mail, even in extreme weather conditions. Storage of DBS samples is possible for at least 1 month at 4 °C.

DBS sampling of everolimus appears to give higher results than venous sampling, but these differences were not significant. A more elaborate clinical study is therefore necessary.

5. Conclusion

We developed a new method for the analysis of everolimus using DBS sampling. We used impregnated sampling paper and filtration

of the blood spot. This method seems promising for the monitoring of renal transplant patients.

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