Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 192–197

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Rapid assay of rufinamide in dried blood spots by a new liquid chromatography–tandem mass spectrometric method

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ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form 8 July 2010
Accepted 13 July 2010
Available online 22 July 2010

Keywords:
Rufinamide
Liquid chromatography–tandem mass spectrometry
Epilepsy
Anticonvulsant

ABSTRACT

Rufinamide (RUF) is a new antiepiletic drug with efficacy in several types of seizures. The aim of this study was to evaluate the use of dried blood spot (DBS) specimens to determinate RUF levels during treatment. Therapeutic drug monitoring of RUF could be useful in routine clinical practice. Advantages of DBS include short collection time, low invasiveness, ease and low cost of sample collection, transport and storage. The analysis was performed in selected reaction monitoring (SRM) mode. The calibration curve in matrix was linear in the concentration range of 0.008–0.8 mg/L (0.48–47.60 mg/L in DBS) of rufinamide with correlation coefficient value of 0.996. In the concentration range of 0.48–47.6 mg/L, the coefficients of variation in DBS were in the range 1.58–4.67% and the accuracy ranged from 89.73% to 107.32%.

The sensitivity and specificity of tandem mass spectrometry allow now high throughput rufinamide analysis. This new assay has favourable characteristics being highly precise and accurate. The published HPLC–UV methods also proved to be precise and accurate, but required not less than 0.2–0.5 mL of plasma and are therefore unsuitable for sample collection in neonates in whom obtaining larger blood samples is not convenient or possible.

1. Introduction

Rufinamide (RUF) [1-(2,6-diflurophenyl) methyl-1H-1,2,3-triazole-4-carboxamide] is a triazole compound and has a chemical formula of C10H8F2N4O. In experimental models, RUF has been shown to modulate sodium channels, prolonging their inactivation phase, thereby limiting the firing of sodium-dependent action potentials in neurons, and resulting in a membrane stabilizing effect [1,2].

Rufinamide has been approved by EMEA and FDA for the adjunctive treatment of Lennox–Gastaut syndrome and, in the US, for the adjunctive treatment of partial-onset seizures with and without secondary generalization in adults and children aged 4 years and older.

Several aspects of the pharmacokinetics (PK) of RUF have been elucidated [3,4] but others remain to be investigated in formal studies. In particular, although it is established that the relationship between plasma concentration at steady state and administered dose of RUF is nonlinear due to reduced bioavailability with increasing doses [5], this relationship has not been studied systematically. Furthermore, although RUF is now currently used in paediatric patients, available evidence with regard to potential differences in its PK profile in children is fragmentary. Other aspects of RUF PK that require further investigation relate to interactions with concomitantly administered AEDs. Preliminary observations suggest that plasma RUF concentration correlate positively with clinical response, suggesting that monitoring the plasma levels of the drug may aid in the individualization of dose [5].

Repeated blood sampling, however, may be a cause for discomfort, particularly in children.

Up to now RUF has been mostly assayed by liquid chromatography (HPLC) with UV detection [6,7]. However, these methods have common problems of long run time and a large amount of plasma consumption to be sensitive enough for determination of drug levels. In addition, UV detection is limited owing to the diffi-

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doi:10.1016/j.jpba.2010.07.015

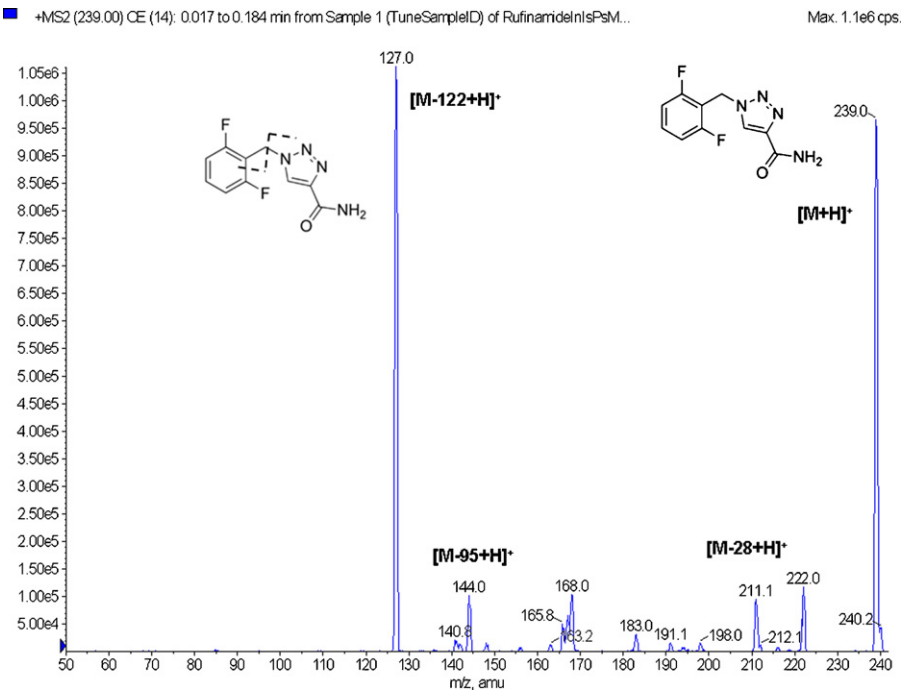

Fig. 1. Product ion scan of rufinamide.

culty of finding specific wavelength apt to avoid disturbances due to matrix effects. MS/MS detection is sensitive and effectively eliminates interference from other endogenous components.

The use of dried blood spot (DBS), for the collection and analysis of human blood, dates back to the early 1960s when Robert Guthrie used the specimens to measure phenylalanine in newborns for the detection of phenylketonuria [8]. This novel application for collecting blood led to the population screening of newborns for the detections of some inborn errors of metabolism [9]. To date, many other clinical applications for measurement of biological markers on DBS have been reported [10,11]. Starting from recent years, DBS has been used as sample collection technique for pharmacokinetic studies [12–16].DBS sampling has several advantages including the small blood volume required, easy and cheap shipment, minimally invasive sample collection and long-term stability during storage.

As a part of pharmacokinetic study of RUF used off-label as an add-on antiepileptic in severe epileptic encephalopathies of children aged less than 4 years, we developed rapid assay of the drug in DBS by a liquid chromatography-tandem mass spectrometric method.

We demonstrated that minimal sample preparation, with no derivatization steps, high sensitivity/specificity, high throughput and minimal instrument maintenance make this method a good candidate for PK studies in small children.

2. Experimental

2.1. Standards

Chemical standard of RUF was supplied by *Eisai* Co., Ltd, NJ, USA. Stock solution of 1000 mg/L was made in acetonitrile:water (70:30, v/v). Successive dilutions were made using HPLC grade water. All solutions were stored in a freezer at −20 °C. All chemicals and solvents were of the highest purity available from commercial sources and used without any further purification.

2.2. Sample preparation

Blood spot samples were stored at 4 °C in a sealed plastic bag containing desiccant until analysis. One 3.2 mm diameter disk (containing about 3.3–3.4 μL of blood) was punched

Table 1
Demographic data of 14 patients.

Pt	Sex	Age	Diagnosis	RUF dose (mg/kg/day)	Concomitant AED at PK determination
1	M	26 months	EE	30	TPM, PB, PHT
2	F	10 months	EE	23	LZP, PB
3	F	26 months	EE	41	MDZ i.v., PB, CLB, TPM
4	M	6 months	EE	30	NZP
5	M	18 months	EE	20	CLB, STP
6	M	8 months	EE	28	TPM
7	F	22 months	EE	38	TPM, PB
8	M	3 years 10 months	SFE	27	CBZ
9	M	12 years 4 months	EE (LGS)	48	LTG, LVT, TPM, CLZ
10	M	2 years 4 months	EE	28.5	CBZ
11	M	15 years 8 months	SFE	35	VPA, CLB, PHT
12	F	23 years	SFE	28.5	LTG, TPM
13	M	10 years 4 months	EE (LGS)	18	VPA, CLB, TPM
14	M	3 years 10 months	EE	32	VPA

CLB: clobazam; CZP: clonazepam; EE: epileptic encephalopathy; i.v.: intravenous infusion; LGS: Lennox–Gastaut syndrome; LGT: lamotrigine; LZP: lorazepam; NZP: nitrazepam; PHT: phenytoin; PB: phenobarbital; SFE: symptomatic focal epilepsy; STP: stiripentol; TPM: topiramate; VPA: valproic acid.

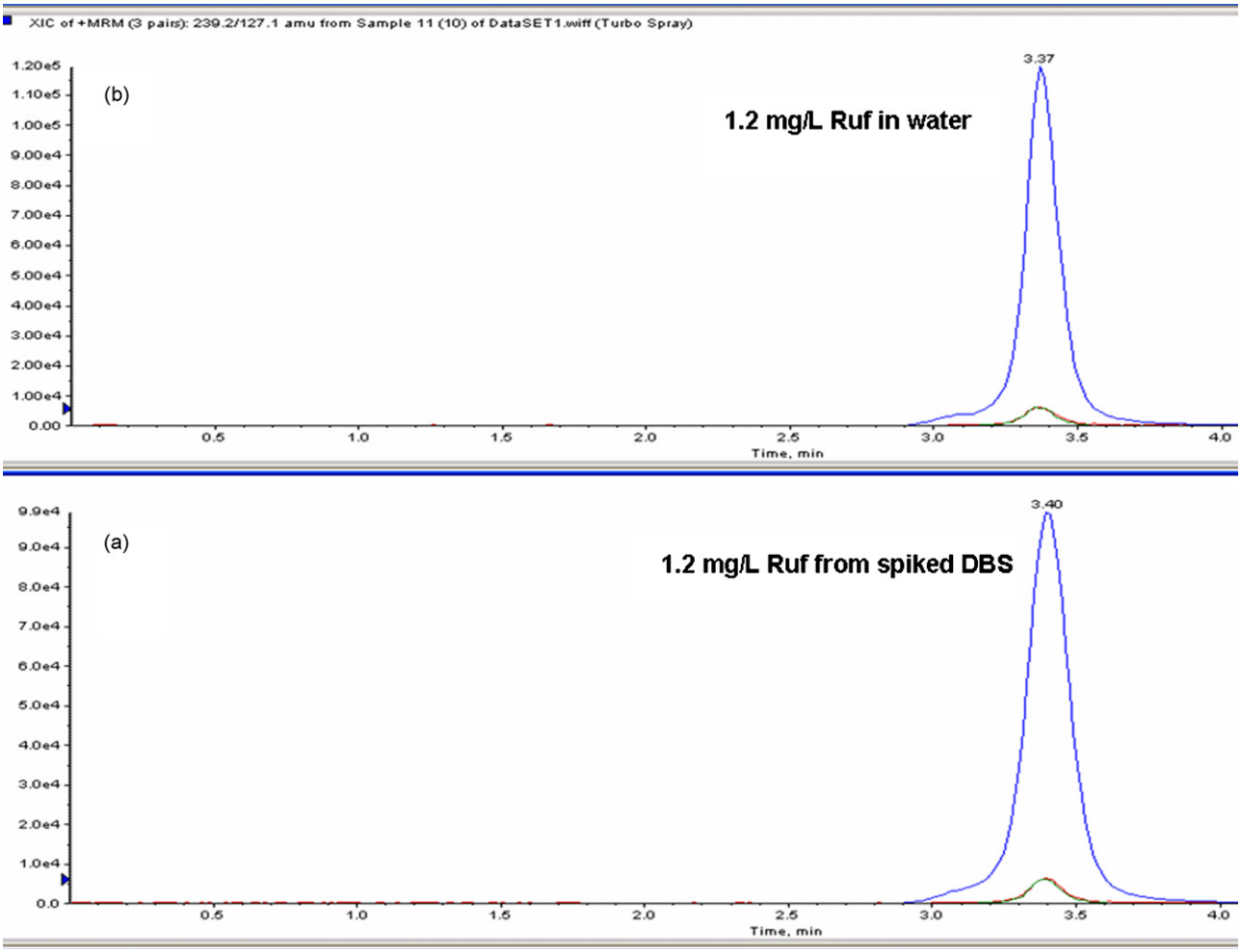

Fig. 2. Extract ion chromatogram from DBS spiked with 1.2 mg/L of RUF versus a RUF standard solution in water at the same concentration.

from each DBS sample and extracted with 200 μL 30–70 of water/acetonitrile + 0.05% of formic acid solution. Samples were put in an orbital shaker and kept at 37 °C for 25 min.

For the setting-up of this study, a pooled mixture of blood samples was spiked with RUF and 20 μL were put on filter paper (903®, Whatman GmbH, Dassel, Germany).

We tested 10 DBS from healthy controls and 16 spots from 14 patients with confirmed epilepsy to whom RUF was administered (Table 1). In two out of 14 patients, sampling was made twice in 2 different days but at the same drug dosage. The age of the patients ranged from 6 months to 23 years (mean 5.5 ± 6.7 years; median 26 months). Eleven patients had a diagnosis of epileptic encephalopathy and three of symptomatic focal epilepsy. In all individuals RUF was used add-on medication. Median daily dose of RUF was 30 mg/kg/day (18–48 mg/kg/day). The procedure was approved by the review board of our institution.

2.3. Validation procedures

Calibration curve was prepared in duplicate by spotting on filter paper spiked human control blood to obtain concentrations of 0, 0.48, 1.19, 2.38, 11.90, 23.80 and 47.60 mg/L. Intra-day variation was assessed from two replicates of three different concentrations of RUF in 10 different runs. Inter-day precision and accuracy data were evaluated by using a daily prepared calibration curve and four different RUF concentration on 10 different days.

Average recovery of RUF from DBS samples was determined by comparing responses with those obtained by direct injection of the same amount of drug at three different concentrations (2.38, 11.90 and 23.80 mg/L) ($n = 5$ at each concentration). To calculate the linear regression, the ratio of peak sizes was plotted against the drug concentration in micrograms per milliliter.

The selectivity of the method has been established by the analysis of 10 different dried blood spot from controls.

The short-term stability study on DBS samples was evaluated up to 1 month after storage at room temperature, −20 and 4 °C.

2.4. Mass spectrometry

The samples were measured using an Applied Biosystems-Sciex (Toronto, Canada) API 4000 bench-top Triple-Quad Mass Spectrometer equipped with the TurboIonSpray source operated in MRM under positive ion mode. The capillary voltage of the mass spectrometer was set to 5500 V, the "turbo" gas flow was 10 L/min of air heated at 350 °C (nominal heating-gun temperature). The following transitions were monitored: m/z 239.1 > 127.1 (quantifier) and m/z 239.1 > 211.1 (qualifier) and m/z 239.1 > 144.1 (qualifier). Optimal CE and CXP were found at 25, 13, 15 V and 11, 12, 11 V respectively. The resulting DP was +50 V for all transitions.

The quantitation experiments were undertaken by using a Series 1100 Agilent Technologies (Waldbronn, Germany) CapPump coupled to an Agilent Micro ALS autosampler, both being fully controlled from the API 4000 data system. Liquid chromatography was performed using a Phenomenex Synergi Fusion-RP 80A 4 μm, 2 mm × 150 mm HPLC column (Phenomenex Italia, Anzola Emilia, Italy). Column flow was 0.2 mL/min using an aqueous solu-

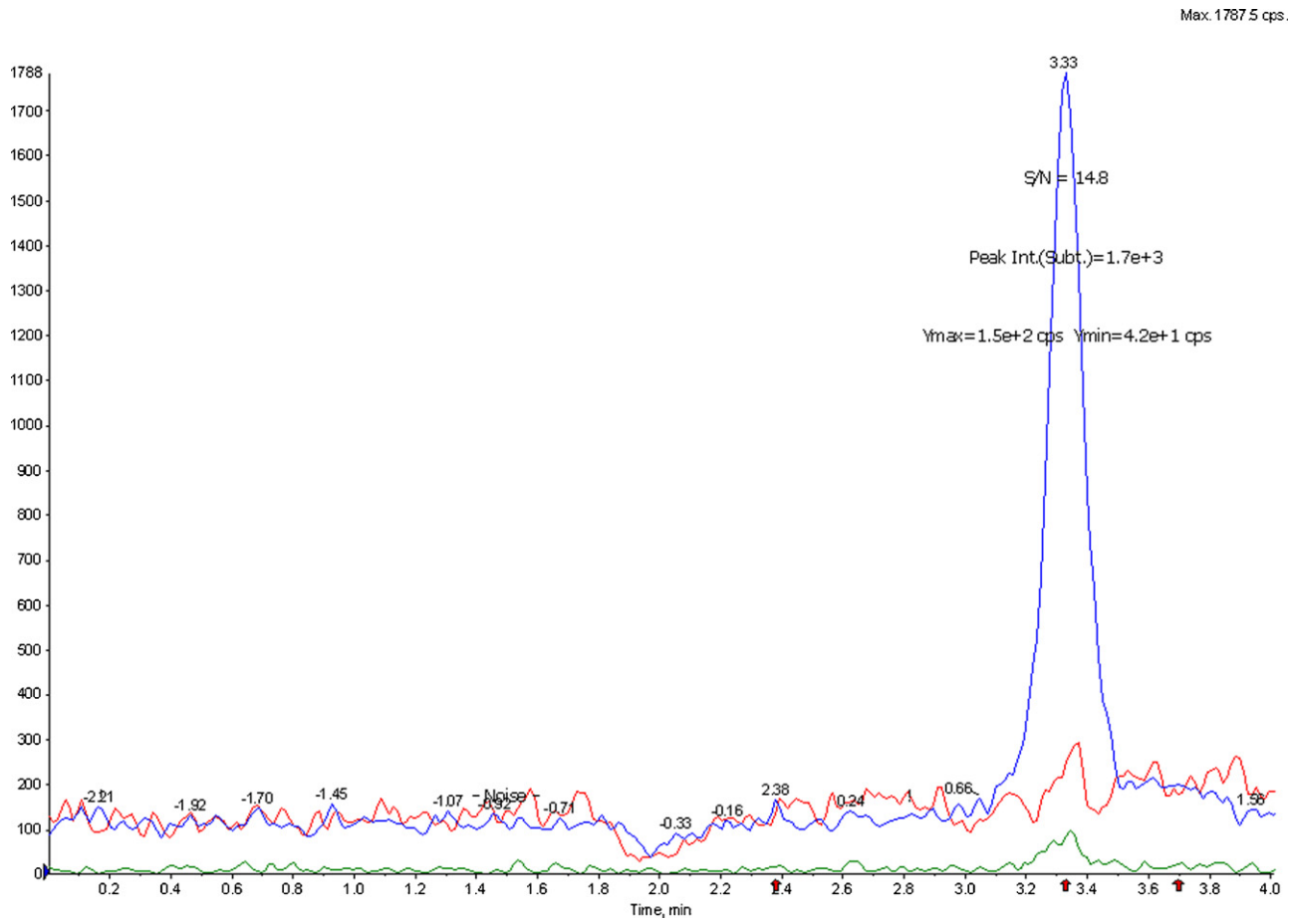

Fig. 3. Extract ion chromatogram of RUF obtained near the estimated detection limit and at signal-to-noise ratio of 14.8.

tion of 63% acetonitrile containing 0.05% formic acid. The eluent from the column was directed to the TurboIonSpray probe without split ratio. Five microliters of the extracted sample were injected for the LC–MS/MS experiments.

System control and data acquisition were performed with Analyst 1.4.1 software including the "Explore" option (for chromatographic and spectral interpretation) and the "Quantitate" option (for quantitative information generation). Calibration curves were constructed with the Analyst Quantitation program using a linear least-square regression non-weighted.

3. Results and discussion

Fig. 1 shows the MS/MS spectrum obtained by fragmenting the precursor ion (239.1 Th) of RUF under the above described conditions. From these experiments, the resulting ion-pair transition for the quantitative experiment (SRM) is 239.1 > 127.1. We have chosen two additional transitions as qualifiers: 239.1 > 211.1 and 239.1 > 144.1. Fig. 2 shows an extract ion chromatogram from DBS spiked with 1.2 mg/L of RUF (a) versus a RUF standard solution in CH_3CN/water 70:30 at the same concentration (b). No interferences were revealed but a quenching of the intensity of the signal indicates that the assay performances are not completely independent on the sample matrix. Considering the sample dilution rate set at 60 times, the combined effect of all the components of the sample, other than the analyte, on the measurement of the quantity does not seems to be significant. Recovery data are summarized in Table 2. The recovery of RUF was between 90.6% and 92.7%.

Table 2
Extraction recovery of RUF from DBS ($n = 5$).

Expected concentration (mg/L)	Mean	Standard deviation	%CV
2.38	91.23	0.91	1
11.90	90.14	1.6	1.8
23.80	88.54	2.5	2.8

Table 3
Intra-day and inter-day imprecision of RUF measurements on DBS.

Expected concentration (mg/L)	Mean	Standard deviation	%CV	Accuracy
Intra-day ($n = 10$)				
0	0	0		
0.48	0.43	0.07	4.12	89.73
1.19	1.24	0.08	1.58	104.61
2.38	2.55	0.50	4.67	107.32
11.90	11.73	1.40	2.84	98.53
23.80	23.87	2.48	2.48	100.29
47.60	47.91	3.50	1.74	100.65
Inter-day ($n = 10$)				
0	0	0		
0.48	0.42	0.04	10.66	86.80
2.38	2.52	0.11	4.51	105.91
11.90	11.85	0.48	4.02	99.55
23.80	23.82	0.38	1.59	100.05

Intra-day and inter-day imprecision of the dried blood spot assays were determined as described in Section 2.

Table 4
Stability of RUF in dried blood spot at room temperature, +4 °C, −20 °C.

Expected concentration (mg/L)	Storage temperature	Average, analyses in triplicate for 30 days (mg/L)	DS	CV%	Accuracy
0.48	Room	0.48	0.07	13.59	100.78
2.38	Room	2.66	0.13	5.02	111.67
23.80	Room	25.41	2.17	8.54	106.78
0.48	+4 °C	0.55	0.09	16.24	115.50
2.38	+4 °C	3.08	0.14	4.53	126.00
23.80	+4 °C	25.23	0.48	1.89	106.00
0.48	−20 °C	0.53	0.02	3.65	110.67
2.38	−20 °C	2.67	0.07	2.72	112.33
23.80	−20 °C	24.83	1.17	4.73	104.33

RUF levels in DBS of samples were determined as described in Section 2. Values are the mean of duplicate measurements.

The chromatographic conditions selected were set in order to speed-up the running time (4 min) since specificity is provided by the MS/MS measurement. Sample dilution rate and injection volume (5 μL) were selected to avoid overloading the chromatographic column even after a high number of sample injections (0.28 μL of original blood per run). The injection sample volume was selected after several trials with different injection volumes. The outcome is that the column shows robust performances regardless of the salt or any other interfering component concentration in the specimen.

The non-weighted regression equation for our LC–MS/MS method was $y = 91{,}900x + 28{,}400$; the mean correlation coefficient for regression lines, generated on 10 different days was $R^2 = 0.9996$ (*SD*, ±0.0005; range 0.9988–0.9998). A correlation coefficient of >0.995 is generally considered as the evidence of an acceptable fit of the data to the regression line. The *y*-intercept is less than 3.5% (2.84%) of the response obtained for the analyte at the target level (considered the lower level of the normal range: 5 mg/L).

The calibrators were at concentrations of 0, 0.48, 1.19, 2.38 and 11.90 mg/L. For spiking studies, we evaluated linearity by analyzing supplemented 3.2 mm dried blood spots prepared at 0, 0.48, 1.19, 2.38, 11.90, 23.80 and 47.60 mg/L.

Considering all the examined samples, discrepancies between expected values and measured values were within 12%. In order to assess the robustness of the method, two replicates of six different concentrations of spike were used (0.24, 0.48, 1.19, 11.90, 23.80 and 47.60 mg/L) and each was processed ten times in 1 day, resulting an intra-day repeatability below 4.67% for all values. The inter-day repeatability obtained in four separate assays for 2 weeks was better than 10.70% (Table 3).

With the proposed parameters, the estimated limit of detection (signal-to-noise ratio >5) in DBS was 5 μg/L (0.30 mg/L in the original blood, 60 times diluted) (Fig. 3), the limit of quantitation (signal-to-noise ratio >15) was 10 μg/L (0.6 mg/L in the original blood, 60 times diluted). No deterioration in column efficiency was observed after the analysis of 200 DBS samples.

For 16 blood samples, collected from 14 patients, haematocrit (HCT) was measured so that the volume of plasma in each DBS could be determined and then used to obtain closely comparable plasma and dried blood spot values. RUF is reported to be evenly distributed between plasma and blood cells [17]. We observed a good correlation between RUF plasma and RUF levels measured in the corresponding DBS both if real HCT ($R^2 = 0.9815$) (Fig. 4A) and theoretical HCT (mean per age) ($R^2 = 0.9763$) (Fig. 4B) were used. HCT is a critical parameter that needs to be considered. Ignoring its impact could lead to serious error in drug level determinations from DBS compared with plasma, especially if HCT levels are exceedingly distant from average. The formula listed below was used to correlate RUF concentration between plasma and DBS: $DBS_{conc} \times (100/100 - HT) = Plasma_{conc}$.

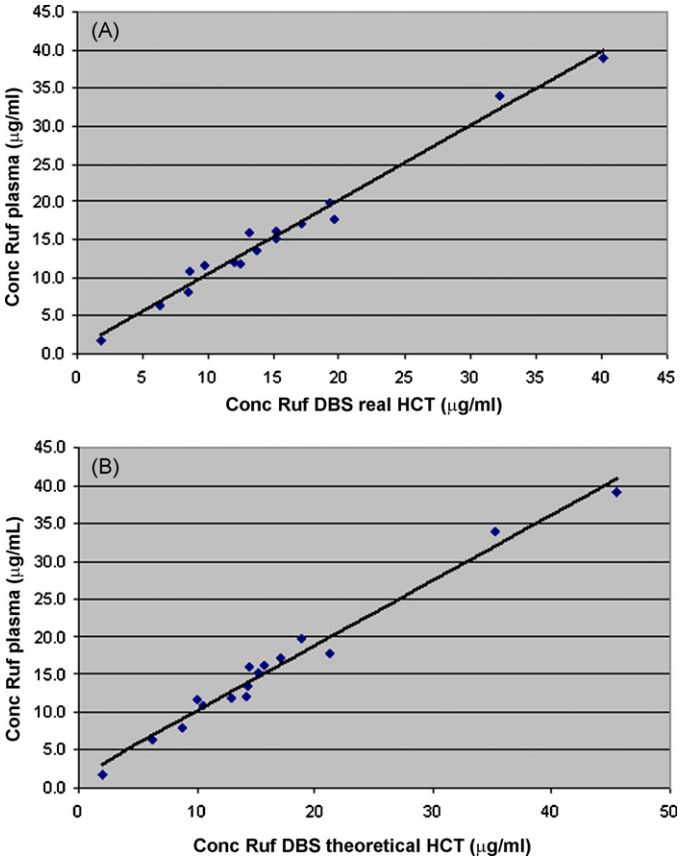

Fig. 4. Relationship between RUF levels in plasma and corresponding dried blood spots. The correlations of values in plasma fraction *vs*. those measured in DBS considering the real haematocrit (A; $y = 0.971x + 0.7924$; R^2 0.9815) and in DBS considering the theoretical haematocrit (B; $y = 0.866x + 1.5288$; R^2 0.9763) are shown. Values represent the mean of duplicate measurements.

We observed that spotted paper samples could be stored at room temperature for at least 4 weeks before assay when sealed in plastic bags containing desiccant. The overall results in determining stability at different temperatures are reported in Table 4.

The use of dried blood spots offers an easy way of collecting, storing, and shipping samples and is feasible to carry out in the monitoring of drug therapy for paediatric patients because blood can be obtained from heel or finger pricks sampling.

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