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Development of a sensitive liquid chromatography method coupled with a tandem mass spectrometric detection for the clinical analysis of vinflunine and 4-*O*-deacetyl vinflunine in blood, urine and faeces

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Abstract

A sensitive and specific liquid chromatographic method coupled with tandem mass spectrometric detection was set up and fully validated for the simultaneous quantification of vinflunine (VFL) and its pharmacologically active metabolite, 4-*O*-deacetyl vinflunine (DVFL). The two compounds, as well as vinblastine (used as internal standard), were deproteinised from blood and faeces, analysed on a cyano type column and detected on a Micromass Quattro II system in the positive ion mode after ionisation using an electrospray ion source. In blood, linearity was assessed up to 200 ng/ml for vinflunine and 100 ng/ml for 4-*O*-deacetyl vinflunine. The lower limit of quantification was validated at 250 pg/ml for both compounds. In other biological media, the linearity was assessed within the same range; the limit of quantification was adjusted according to the expected concentration levels of each compound. This method was first developed in order to identify the structures and to elucidate the metabolic pathway of vinflunine. Thanks to its high sensitivity and specificity, the method has enabled the quantification of vinflunine and 4-*O*-deacetyl vinflunine in blood at trace levels, and has contributed to the knowledge of vinflunine metabolism by monitoring up to 10 metabolites.

Keywords: Vinflunine; 4-*O*-Deacetyl vinflunine; LC–MS/MS; Human; Metabolites

1. Introduction

Vinflunine is the first bi-fluorinated, tubulin-targeted agent obtained by Pierre Fabre Medicament through a semi-synthesis process using super acidic chemistry [1]. This original approach led to a new generation of vinca alkaloids from which vinflunine was selected on the basis of its high level of in vivo antitumour activity against experimental tumour models [2].

Vinflunine, synthesised and developed by Pierre Fabre Medicament, is currently in phase III trials in bladder, non-small cell lung and breast cancers [3]. As part of the clinical development of this drug, extensive pharmacokinetic investigations were carried out in animal and in human. Two bioanalytical methods have been successively developed to measure both vinflunine (VFL) and its active metabolite 4-*O*-deacetyl vinflunine (DVFL) [4] in blood and excreta. The first method used a high-performance liquid chromatography (HPLC) followed by UV detection for the quantification of both compounds after a liquid–liquid extraction from blood and urine [4]. This sensitive (2 ng/ml in blood) and reproducible method has been largely used in preclinical tests and during the phase I clinical studies [4–6]. Few additional peaks to those of VFL and DVFL were observed in human samples. They were named and assigned to potential metabolites of VFL, then monitored through the clinical studies. In order to identify the chemical structures of these potential VFL metabolites, a LC–tandem mass spectrometry (MS/MS) method was set up first to identify the different metabolites and subsequently to quantify VFL and DVFL in human blood, urine and faeces.

2. Experimental

2.1. Chemical and reagents

Methanol, acetonitrile and formic acid from Acros Organic (Geel, Belgium) were HPLC grade. Ammonium acetate was

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provided by UCB (Leuven, Belgium). Deionised water was obtained from a Milli-Q system, Millipore (Brussels, Belgium). Vinflunine bitartrate and 4-*O*-deacetyl vinflunine sulfate were obtained from Pierre Fabre Medicament (Castres, France). Vinblastine sulfate, used as internal standard (IS), was purchased from Sigma Chemical Co. (Saint Louis, MO, United States of America). Control blood matrix was from Haematology Unit of Hospital Department (Liège, Belgium) while urine and faeces were from healthy volunteers. These matrices were stored at *ca.* −80 °C.

2.2. Instruments

Chromatographic analysis was performed by using a HP1100 system (Hewlett-Packard, Waldbronn, Germany) equipped with an automatic thermostated injector set at 10 °C, vacuum degaser and a thermostated column compartment.

The detection was achieved by using a triple stage quadrupole Quattro II equipped with the orthogonal Z-spray electrospray interface and the acquisition data processor Masslynx 3.5 software from Micromass (Altrincham, UK).

2.3. Analytical conditions

HPLC separations were carried out on a Spherisorb CN column (100 mm × 4.6 mm i.d., dp = 3 μm) set at 40 °C and protected with a Spherisorb CN pre-column (10 mm × 2 mm i.d., dp = 5 μm), both from Varian Chrompack (Middleburg, The Netherlands).

The mobile phase was a mixture of ammonium acetate buffer, 40 mM, obtained with 3.1 g of ammonium acetate in 1000 ml of water, and adjusted with a solution made of formic acid (adjusted to pH 3.0) and acetonitrile (55:45, v/v). The mobile phase was filtered through a 0.45 μm membrane filter before the run. The column was initially activated for at least 2 h with acetonitrile, then with acetonitrile–water (40:60, v/v) and finally with the mobile phase at a flow rate of 0.2 ml/min.

Separation was achieved by isocratic solvent elution at a flow rate of 0.65 ml/min by direct introduction in splitless mode into the electrospray source. Electrospray ionisation was performed implying the detection of positive ions in the multiple reaction monitoring (MRM) mode. Argon, used as collision gas, was regulated at 3×10^{-3} mbar with a collision energy of 50 eV. The interface temperature was set at 400 °C and the source temperature at 150 °C with the cone voltage maintained at 40 V for all transitions.

2.4. Assay procedure

2.4.1. Preparation of stock and reference solutions

Stock solutions for either calibration curves or quality controls (QC) samples were independently prepared by two scientists using silicon-coated glass tubes (Venoject, Terumo) in order to avoid any adsorption of VFL on the containers, and then compared for accuracy [7,8]. Stock aqueous solutions (1 mg/ml expressed as free-base) of VFL and DVFL were prepared separately by weighing the appropriate amounts of compounds and dissolving them in 40 mM ammonium acetate buffer, pH 3.0.

These stock solutions were then diluted in ammonium acetate buffer 40 mM, pH 3.0 as serial dilutions in order to obtain at least seven final reference solutions of each compound for the calibration curves, and four reference solutions for the QC samples. A stock solution (1 mg/ml) of vinblastine (IS) was similarly prepared and reference solutions were obtained by appropriate dilutions that were consistent with the calibration range of each medium. All the stock solutions were stored at +4 °C for 1 month and the reference solutions were prepared twice monthly from the stock solutions and stored under the same conditions.

2.4.2. Preparation of calibration curves

To mimic the reference solution volumes added to each calibration standard, 50 μl of 40 mM ammonium acetate buffer were added to unknown samples. All calibration standards were freshly prepared on the day of analysis. A volume of 500 μl of human control blank sample was spiked with 25 μl of VFL and DVFL reference solutions to obtain concentrations ranging from 0.25 to 200 ng/ml and 0.25 to 100 ng/ml for VFL and DVFL, respectively, in blood samples. The same dilution ratio was used in urine and faeces using 1 ml and 100 mg of the respective matrices to obtain the following calibration ranges: 20–5000 ng/ml in urine and 2–1000 μg/g in faeces. Then, the spiked samples were processed according to the sample preparation procedure detailed in Section 2.5.

2.4.3. Preparation of QC samples

At least three final concentrations of QC samples were prepared within the calibration range, with one at the expected lower limit of quantification (see Table 2). The QC samples were processed according to the preparation procedure described in Section 2.5. QC samples were prepared in bulk in silicon-coated glass tubes at regular intervals and stored at −80 °C until use.

In blood, 500 μl of VFL and DVFL reference solutions were diluted in human control matrix with a 1/100 ratio. The same dilution factor was used for other two matrices, and enough volume of QC bulk was prepared to cover the studies.

2.5. Sample preparation

2.5.1. Sample preparation for blood

In a silicon-coated glass tube, 0.5 ml aliquots of sample previously thawed at room temperature and briefly shaken, were diluted with 0.5 ml of 40 mM ammonium acetate buffer, pH 3.0. After the addition of 25 μl of IS solution (0.25 μg/ml) and a brief mixing (*ca.* 30 s), the samples were deproteinised with 1 ml of methanol and 5 ml of acetonitrile. The tubes were sonicated for 1 min, then shaken for 10 min on a back and forth shaker and finally centrifuged for 10 min at 2400 × *g* at +4 °C . Then, 200 μl of 40 mM ammonium acetate buffer, pH 3.0 was added in the tube before evaporation under a gentle nitrogen stream at *ca.* +40 °C until about 500 μl of solution remained in the tube. Finally, 1 ml of 40 mM ammonium acetate buffer, pH 3.0, was added. The tubes were briefly mixed. An aliquot of 25 μl was injected into the LC system.

2.5.2. Sample preparation for urine

Samples were first thawed at room temperature, briefly shaken and centrifuged over 5 min at 2400 × *g* at +4 °C . Then, in a silicon-coated glass tube, 25 μl of IS solution (3 μg/ml) and 4 ml of 40 mM ammonium acetate buffer, pH 3.0, were added to 10 ml sample aliquots. The mixture was sonicated for 1 min and centrifuged for 10 min at 2400 × *g*. Aliquots of 25 μl were injected into the LC system.

2.5.3. Sample preparation for faeces

Faeces control and biological samples were lyophilised using a Virtis model 112 lyophilisator. Then, an aliquot of 100 mg was weighted in a silicon-coated glass tube. A volume of 50 μl of IS solution (50 μg/ml), 1 ml of methanol and 4 ml of 40 mM ammonium acetate buffer, pH 3.0, was added. The mixture was sonicated then shaken for 10 min on a back and forth shaker and finally centrifuged for 10 min at 2400 × *g* at +4 °C. The supernatant layer was transferred to a silicon-coated glass tube. The extraction process was carried out twice using 1 ml of methanol and 4 ml of 40 mM ammonium acetate buffer, pH 3.0. The two supernatant layers were combined and mixed for 1 min. Then, 100 μl of this mixture was diluted to 10 ml with 40 mM acetate buffer, pH 3.0. The tubes were briefly mixed. An aliquot of 25 μl was injected into the LC system.

In blood and urine, when VFL or DVFL concentrations were found outside the calibration range, appropriate dilution of samples with control matrices was prepared before reprocessing the samples.

2.6. Validation

Biological samples were quantified using the ratio of peak area of VFL or DVFL to that of IS.

The calibration curves were calculated through a linear least-squares regression model with a weighing factor of $1/C$, where C is the concentration of the calibration standards. Concentration in the QC and unknown biological samples were quantified from the regression equation.

In blood, the linearity was explored on eight replicates at eight calibration concentration levels.

Within- and between-run validation provided accuracy and precision of the method in blood using QC samples quantified towards calibration curves processed in duplicate. For the within-run validation, at least five replicates at four QC concentration level were processed. The between-run validation was carried out over 3 days. For urine and faeces, and in agreement with the FDA guidance for industry [13], only a partial validation was performed consisting of a within-run validation using five QC samples in urine and four in faeces at each concentration level. Mean errors and SD values were calculated from the theoretical and experimental concentrations, in order to estimate the precision and accuracy of the method.

The lower limit of quantification (LLOQ) of the method was the lowest concentration level with an imprecision (expressed as RSD) and an error of 20% or less between theoretical and observed values during the within- or between-run analyses.

Recoveries of VFL, DVFL and VBL were calculated in blood by comparing the MS signals obtained from spiked biological samples (five replicates at three concentration levels) with those recorded from similar concentrations injected from directly prepared aqueous solutions.

The short-term stability of VFL and DVFL during the storage in the autosampler at 10 °C was assessed in blood by a repeated injection of six QC samples processed with at least three concentration levels after 48 h interval.

3. Results and discussion

3.1. Analytical issues

An experience of more than 15 years in bioanalysis of vinca alkaloid, and especially in the development of vinorelbine, has enabled to rapidly set up bioanalytical tools for the pharmacokinetic assessment of vinflunine. As a result, a LC–MS/MS method could be developed for qualitative and quantitative metabolism issues. The analytical conditions previously used for vinorelbine [11] were applied to VFL. The pseudo-molecular ions $[M+H]^+$ of VFL and DVFL were recorded at $m/z = 817$ and 775, respectively. After collisionally induced dissociation (CID) of these specific ionic species, each product ion spectrum showed an abundant fragment ion at $m/z = 160$. This key fragment ion, which was attributed to a part of the nor-7′-velbanamine moiety of the molecule, was predominant and therefore selected to monitor VFL and DVFL in the MRM mode (Figs. 1 and 2). In LC–MS/MS, the ideal internal standard should match the structure of the analytes as closely as possible. Since the synthesis of vinca alkaloids is too complex, isotope labelled analytes could not be obtained. Therefore, vinblastine, an analogue of vinflunine, was used. The MRM transition $m/z = 811 \rightarrow 224$ was followed to trace this compound. Once the chromatographic conditions were properly adjusted to obtain a consistent separation, ESI-MS–MS in the positive mode was required to achieve the highest sensitivity. Special attention was paid to the extraction procedure in order to supply relatively clean extracts so that a large number of samples could be daily processed. Liquid–liquid extraction, successfully used in the LC-UV method [4] set up for both VFL and DVFL, was not considered to be the most suitable for an efficient separation of several metabolites with different degrees of polarity.

Moreover, this process was previously demonstrated to extract only 50% of the metabolic pool of vinorelbine [11]. Therefore, a new solid-phase extraction (SPE) procedure was set up as an appealing method before the LC–MS/MS analysis. This method definitely offered some decisive advantages, such as less solvent consumption and on-line automation. However, and because it was developed in blood and faeces, it also required an additional clean-up step to prevent any formation of particles that could jeopardize a reproducible elution of the compounds [8]. In fact, deproteinisation was demonstrated to be the best procedure in terms of easiness, standardisation and reliability in the accurate quantification of VFL and DVFL in the three biological media. Potential interference from

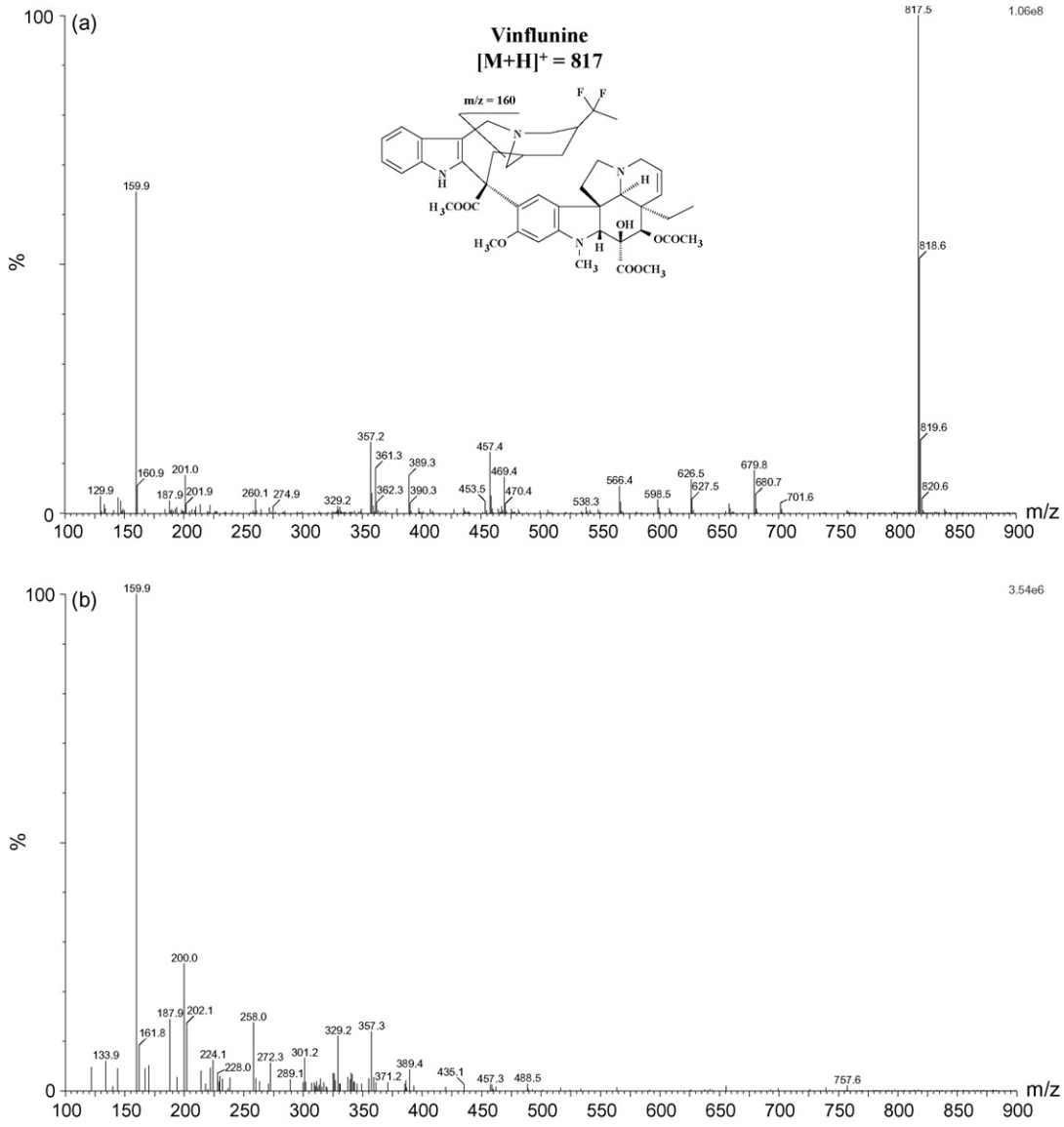

Fig. 1. Parent ion spectrum (a) and product ion spectrum (b) of vinflunine.

endogenous compounds was investigated by analysing human blood, urine and faeces from five different sources. Typical chromatograms from each matrix are shown in Figs. 3 and 4. No interference from endogenous substances was observed at the retention time of the analytes and IS. Same conclusions were addressed for urine and faeces (data not shown). Thus, high specificity concerning endogenous compounds could be guaranteed when operating with the triple-quadrupole system under the MRM mode. All compounds were eluted within 15 min. Relative retention times of the three compounds were approximately: VBL, 0.7; DVFL, 0.8; VFL, 1.0 (see Figs. 3–5).

3.2. Performance of the method

At the time of validation, the guidelines were those recommended in the international consensual strategy on method validation, further adapted during a harmonization conference in 1990 and later published in 1992 [12]. Nevertheless, reproducibility was assessed on quality control samples even if the guidelines were recommended to validate on calibration curves only. This validation procedure was more in agreement with FDA guidance for industry published later [13], but the QC levels did not exactly fit with the targets recommended by this guidance.

The calibration curves in blood were linear over the concentration range 0.25–200 ng/ml of VFL and 0.25–100 ng/ml of DVFL (see Table 1). The precision expressed as RSD% ranged from 1.9 to 5.6% and 1.1 to 10.4% for VFL and DVFL, respectively, while the accuracy ranged from 94.8% to 98%. In urine and faeces, calibrations were linear up to 5000 ng/ml and 1000 μg/g for both compounds, respectively, with an accuracy higher than 88% in urine and 86% in faeces.

The mean blood extraction recoveries estimated at three concentration levels ranged from 98.4 to 103% for VFL (RSD < 3.8%) and 94.8 to 100% for DVFL (RSD < 9.4%). The

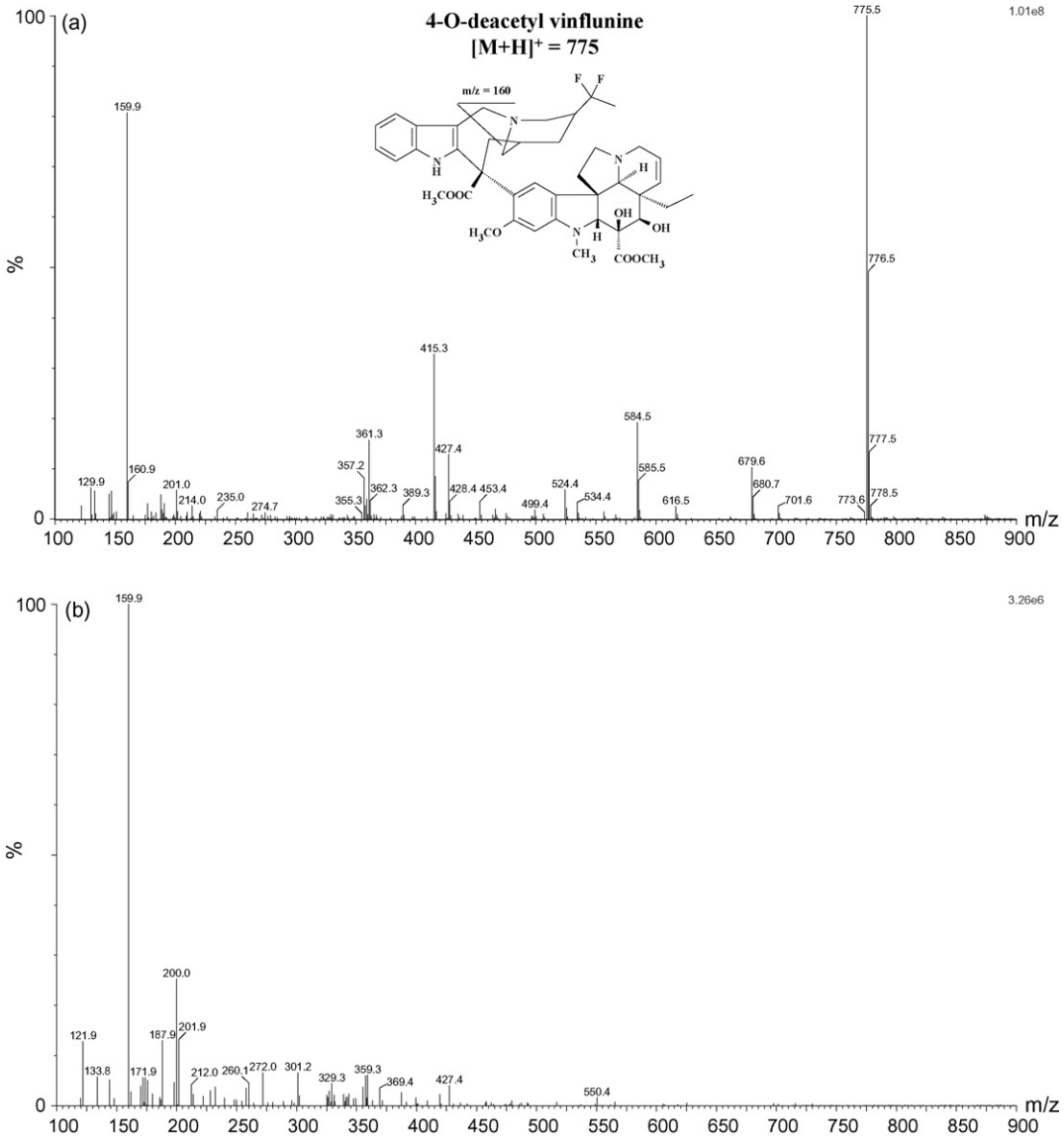

Fig. 2. Parent ion spectrum (a) and product ion spectrum (b) of 4-*O*-deacetyl vinflunine.

Table 1
Linearity of VFL and DVFL in human blood

Vinflunine			4-*O*-Deacetyl vinflunine		
Concentration found (mean ± SD) (ng/ml)	RSD (%)	Accuracy (%)	Concentration found (mean ± SD) (ng/ml)	RSD (%)	Accuracy (%)
0.25 ± 0.01	5.0	99.5	0.20 ± 0.02	10.4	100.6
0.51 ± 0.01	2.7	101.8	0.38 ± 0.04	7.3	95.3
4.78 ± 0.1	2.2	95.5	1.01 ± 0.05	4.5	101
10.0 ± 0.2	1.9	100.3	2.03 ± 0.08	4.2	101.3
24.7 ± 1.4	5.6	98.8	4.04 ± 0.17	4.1	101
51.1 ± 1.9	3.7	102.1	10.2 ± 0.2	1.7	101.8
105 ± 2.9	2.8	105.2	20.4 ± 0.5	2.2	102
210 ± 4.9	2.4	104.8	50.1 ± 0.5	1.1	100.2
495 ± 15.9	3.2	99.1	99.3 ± 4.4	4.4	99.3
989 ± 38.1	3.9	98.9			

$n = 8$ calibration curves.

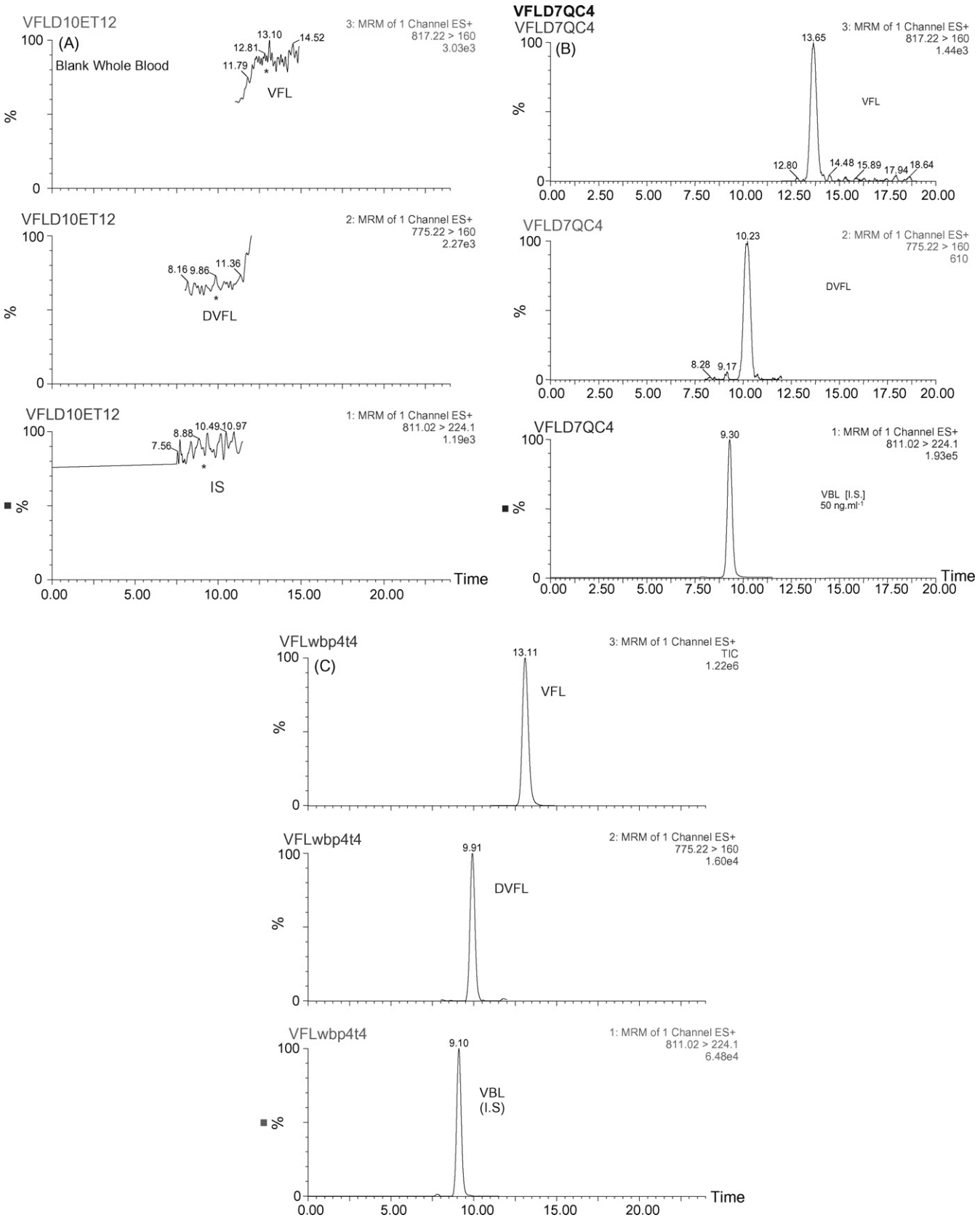

Fig. 3. Representative chromatograms from (A) double blank human blood, (B) a blood control sample spiked with 0.25 ng/ml of vinflunine and 4-*O*-deacetyl vinflunine, (C) blood sample obtained 2 h after a single i.v. dose of 250 mg/m^2 vinflunine in a patient (VFL: 542 ng/ml; DVFL: 13.3 ng/ml) – time scale in minutes.

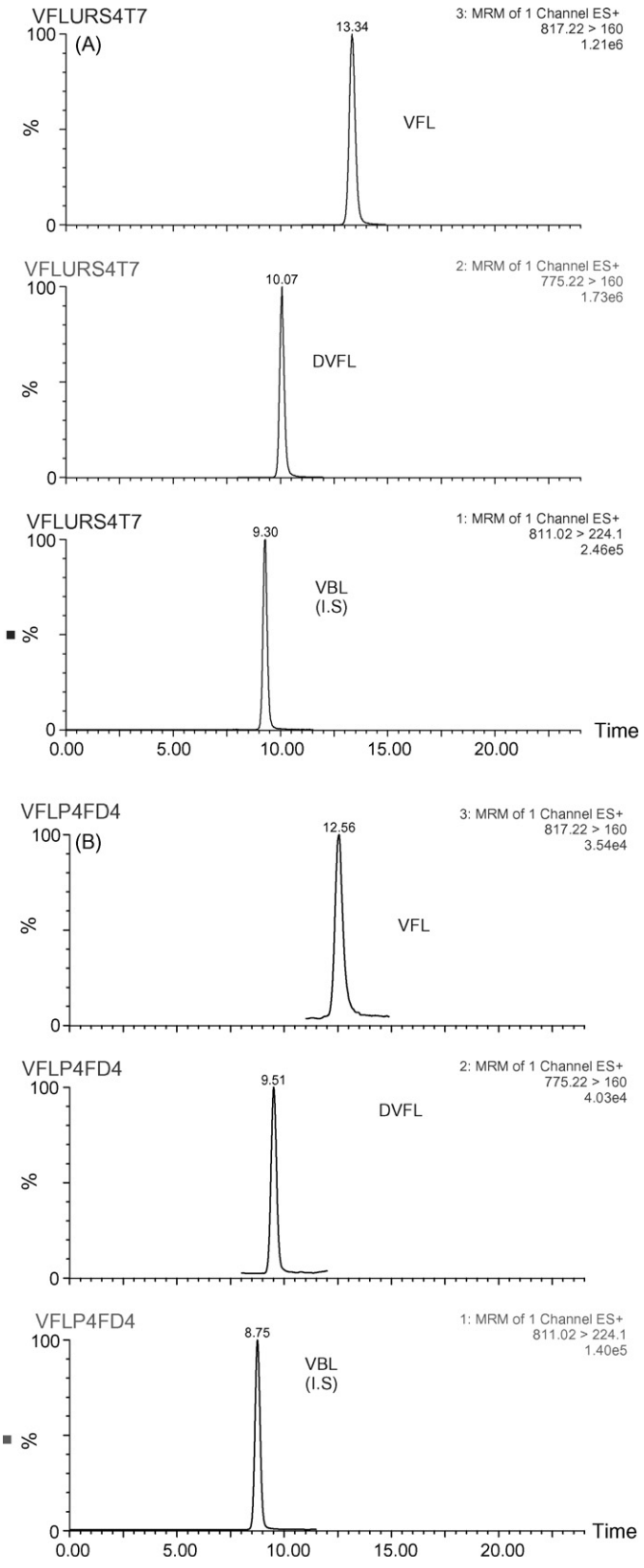

Fig. 4. Representative chromatograms from (A) an urine patient sample collected between 96 and 120 h (VFL: 1568 ng/ml; DVFL: 1973 ng/ml), (B) a faeces patient sample collected between 72 and 96 h (VFL: 4 μg/g; DVFL: 7 μg/g).

Table 2
Within-run precision for QC samples ($n = 4$)

Compound	Blood			Urine[a]			Faeces		
	Concentration found (mean ± SD) (ng/ml)	RSD (%)	Accuracy (%)	Concentration found (mean ± SD) (ng/ml)	RSD (%)	Accuracy (%)	Concentration found (mean ± SD) (μg/g)	RSD (%)	Accuracy (%)
VFL	0.27 ± 0.02	6.8	109	47.9 ± 2.4	5.0	95.9	n.c.	n.a.	n.a.
	10.8 ± 0.2	2.3	108.3	237 ± 9.5	4.0	95.0	46.9 ± 0.4	8.1	93.8
	51.0 ± 2.2	4.4	102	967 ± 39.9	4.1	96.7	238 ± 1.7	7.1	95.1
	206 ± 3.0	1.5	102	4716 ± 153	3.2	94.3	941 ± 6.4	6.6	97.6
DVFL	0.20 ± 0.01	7.0	101.2	49.1 ± 2.4	4.8	98.2	n.c.	n.a.	n.a.
	1.07 ± 0.04	3.8	107.3	233 ± 11.8	5.0	93.2	49.6 ± 0.4	7.6	99.1
	4.17 ± 0.1	2.9	104.4	939 ± 51.9	5.5	93.9	247 ± 0.9	3.5	98.6
	20.5 ± 0.3	1.7	102.4	4708 ± 250	5.3	94.2	1002 ± 6.4	6.4	99.8

n.c.: not calculated; n.a.: not applicable.
[a] $n = 5$ replicates.

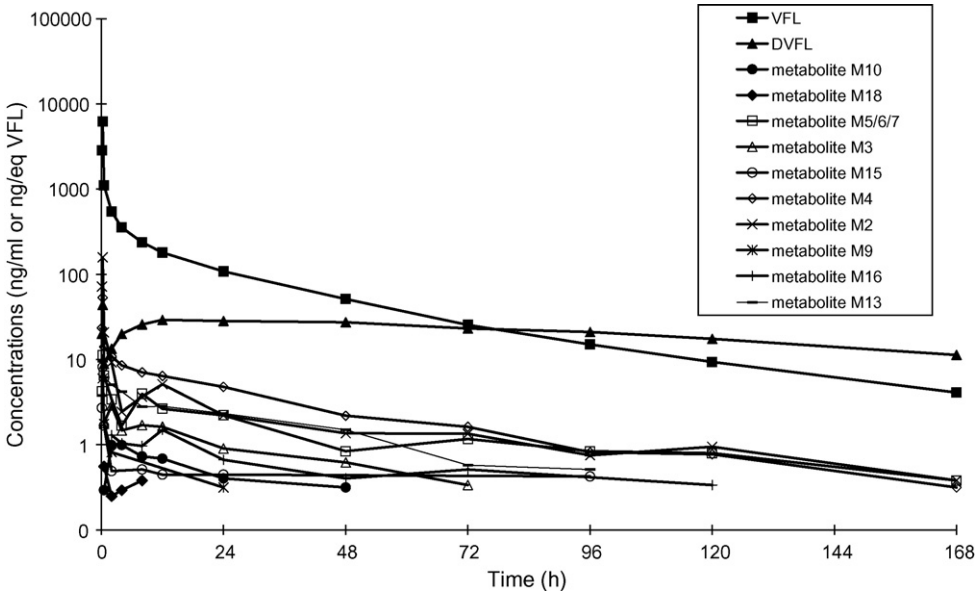

Fig. 5. Representative blood profile for vinflunine and its metabolites. Concentrations are expressed on log scale. Concentrations of metabolites are given for information and are expressed as Vinflunine equivalents.

Table 3
Between-run precision for QC samples in blood ($n = 12$)

Vinflunine			4-*O*-Deacetyl vinflunine		
Concentration found (mean ± SD) (ng/ml)	RSD (%)	Accuracy (%)	Concentration found (mean ± SD) (ng/ml)	RSD (%)	Accuracy (%)
0.25 ± 0.04	15.8	101.3	0.20 ± 0.01	5.1	98.8
10.5 ± 0.6	6.0	105.4	1.03 ± 0.4	11.0	103.6
50.7 ± 0.7	1.5	101.4	4.06 ± 0.3	6.2	101.4
203 ± 6.0	3.0	101.7	19.9 ± 1.4	6.9	99.5
			100 ± 3.7	3.7	100.2

mean extraction yield of IS estimated on 15 determinations was close to 93% (RSD = 11.9%).

The within-run and between-run precision and accuracy of the method are summarised in Tables 2 and 3. Whatever the biological fluid, the within-run RSDs were always below 8.5% and the between-run RSDs in blood remained below 16 and 12% for VFL and DVFL, respectively.

The between-run RSDs were lower than 15.9 and 5.2% for VFL and DVFL, respectively.

Dilution process was validated with a 1/10 factor in blood and up to 1/20 in urine. Stability of VFL and DVFL has been thoroughly explored in blood with the previously developed HPLC-UV method that combined UV detection [4]. The following findings were applicable to this new LC–MS/MS methodology: (1) at least three freeze-thaw cycles, (2) stability on 24 h at room temperature and at +4 °C and (3) stability for at least 24 months and 13 months at −80 °C stored for VFL and DVFL, respectively. As a consequence, only stability processed samples stored in the autosampler at +10 °C for at least 48 h was assessed for VFL and DVFL. As illustrated in Table 4, the concentration accuracy ranged from 100 to 105% for VFL and 94.2 to 114% for DVFL.

3.3. Clinical experience

One of the most significant advantages of the new method is the very low sensitivity obtained in blood for both VFL and DVFL. The sensitivity is 10-fold higher than the previously developed HPLC method [4] and 100-fold more than the LC–MS method recently proposed by Zhao et al. [10] in rat plasma. The high sensitivity of the method allows reliable measure-

Table 4
Stability of processed blood samples stored at 10 °C over at least 48 h

Concentration calculated (ng/ml)	Concentration found (mean ± SD) (ng/ml)	Remaining percentage (%)
Vinflunine		
0.25	0.25 ± 0.02	100
5	5.00 ± 0.3	100
50	52.7 ± 3.3	105
150	151 ± 7.8	101
4-*O*-Deacetyl vinflunine		
0.25	0.28 ± 0.01	114
5	4.71 ± 0.4	94.2
50	51.8 ± 2.2	104

ment of blood concentration. Blood was proved to be a relevant medium since a high affinity of VFL for blood cells has been evidenced in patients [14]. Moreover, the use of blood is an obvious benefice for pharmacokinetic sampling. Twice less volume of sample is required and no sample processing is necessary to separate plasma. A higher sensitivity of the method together with the possibility of using blood sampling instead of plasma improved the accuracy of the VFL and DVFL pharmacokinetic parameters.

The performance of this LC–MS/MS method is illustrated in the pharmacokinetic profile displayed in blood (see Fig. 5). Vinflunine and 4-*O*-deacetyl vinflunine were detected and quantified over 168 h (*i.e.* four to five elimination half-lives). Thanks to this method, structural identification of vinflunine and its metabolites has been performed. Mass spectra were acquired in positive ionisation mode and data-dependent scan was used for the identification of these structures in blood, urine and faeces [9]. Up to 10 additional metabolites to DVFL were observed in blood. Their structures mainly resulted from oxidation pathways. Chemical synthesis of vinca alkaloids compounds is quite complex and all these metabolites could not be obtained as reference chemicals. As such, concentration data were obtained through interpolation with unchanged VFL calibration curves parameters. Fig. 5 illustrates a rough estimation of the pharmacokinetic profile of each entity. Similar analytical performances were obtained in animal species (rat and monkey) especially with regard to the sensitivity (0.25 ng/ml in blood) and the intra-day and inter-day precision and accuracy (higher than 90%) during pharmacokinetic and toxicological studies (data not shown). The present method proved to be easy to handle and to transpose to other laboratories with the same performance. Together with the previous LC-UV method, it has been used for 10 years as the reference analytical tools for quantitative measurement of VFL and DVFL, the use of one or the other depending on the objectives of the clinical trials.

This LC–MS/MS method is expected to be the reference for further development of VFL.

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