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New sensitive liquid chromatography method coupled with tandem mass spectrometric detection for the clinical analysis of vinorelbine and its metabolites in blood, plasma, urine and faeces

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Abstract

A new sensitive and specific liquid chromatographic method coupled with tandem mass spectrometric detection was set up and validated for the simultaneous quantitation of vinorelbine, its main metabolite, 4-*O*-deacetylvinorelbine and two other minor metabolites, 20′-hydroxyvinorelbine and vinorelbine 6′-oxide. All these compounds, including vinblastine (used as internal standard) were deproteinised from blood, plasma and faeces (only diluted in urine), analysed on a cyano column and detected on a Micromass Quattro II system in the positive ion mode after ionisation, using an electrospray ion source. Under tandem mass spectrometry conditions, the specific product ions led one to accurately quantify vinorelbine and its metabolites in all biological fluids. In whole blood, linearity was assessed up to 200 ng/ml for vinorelbine and up to 50 ng/ml for the metabolites. The limit of quantitation was validated at 250 pg/ml for both vinorelbine and 4-*O*-deacetylvinorelbine. In the other biological media, the linearity was assessed within a same range and the limit of quantitation was adjusted according to the expected concentrations of each compound. This method was initially developed in order to identify the metabolite structures and to elucidate the metabolic pathway of vinorelbine. Thanks to its high sensitivity, this method has enabled the quantitation of vinorelbine and all its metabolites in whole blood over 168 h (i.e., 4–5 elimination half lives) whilst the previous liquid chromatographic methods allowed their measurement for a maximum of 48–72 h. Therefore, using this method has improved the reliability of the pharmacokinetic data analysis of vinorelbine.

Keywords: Validation; Vinorelbine; Deacetylvinorelbine; Hydroxyvinorelbine; Vinorelbine oxide

1. Introduction

Vinorelbine (nor-5′-anhydrovinblastine bitartrate, marketed under the name Navelbine by Pierre Fabre Medicament), is a semi-synthetic vinca alkaloid compound which was introduced as an antineoplastic agent. Different bioanalysis methods: radioimmunoassay technique [1] and several high-performance liquid chromatography (HPLC) methods [2–6] were successively developed to measure vinorelbine (VRL) in plasma. More recently, a HPLC procedure was validated in blood to quantify both VRL and its main metabolite 4-*O*-deacetylvinorelbine (DVRL) [7].

The development of an oral form of vinorelbine is ongoing. Its application required accurate informa-

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tion on the VRL metabolism and consequently a more sensitive and specific analytical tool. Therefore, an LC–tandem mass spectrometry (MS–MS) method was set up firstly to identify the formed metabolites and secondly for the quantitation of VRL, DVRL and two other metabolites in human blood, plasma, 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 provided by UCB (Leuven, Belgium). Deionised water was obtained from a Milli-Q system, Millipore (Brussels, Belgium). Vinorelbine bitartrate, 4-*O*-deacetylvinorelbine sulfate, 20′-hydroxyvinorelbine bitartrate, vinorelbine 6′-oxide base and vinblastine sulfate were obtained from Pierre Fabre Medicament (Castres, France).

2.2. Instruments

Chromatographic analysis was performed by using a HP 1100 system (from Hewlett-Packard, Waldbronn, Germany) equipped with an automatic thermostated injector, vacuum degaser and a thermostated column compartment.

The detection was performed by using a triple stage quadrupole Quattro II equipped with the orthogonal Z-spray-electrospray interface and the acquisition data processor Masslynx 3.2 software from Micromass (Altrincham, UK).

2.3. Analytical conditions

HPLC separations were carried out on a Spherisorb CN column (100×4.6 mm I.D., 3 μm particle size) from Varian Chrompack (Middleburg, The Netherlands) set at 40°C with a Spherisorb CN pre-column (10×2 mm I.D.).

The mobile phase was a mixture of acetate buffer, 40 m*M*, obtained with 3.1 g of ammonium acetate in 1000 ml of water and adjusted with formic acid to pH 3.0–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 3 h with the mobile phase at a flow-rate of 0.5 ml/min. Back pressure of the system was 1500 p.s.i. (1 p.s.i.=6894.76 Pa).

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 $3 \cdot 10^{-3}$ 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. Preparation of the calibration curves and quality control (QC) samples

2.4.1. Preparation of stock and reference solutions

Stock solutions for either calibration curves or QC samples were independently prepared by two different scientists using silicon coated glass tubes (Venoject, Terumo) in order to avoid adsorption of VRL on the containers [7]. Stock aqueous solutions (1 mg/ml) of VRL, DVRL, 20′hydroxyvinorelbine (20′OH-VRL) and vinorelbine 6′-oxide (NO-VRL) were prepared separately by weighing the appropriate amount of compounds and dissolving them in 40 m*M* acetate buffer, pH 3.0. These stock solutions were then successively diluted and finally pooled in order to obtain at least seven final reference solutions of all compounds for the calibration curves and four reference solutions for the QC samples.

A stock solution of vinblastine (VBL, internal standard) was similarly prepared every 3 weeks and a reference solution of 2 μg/ml (1000 μg/ml in faeces) was obtained by dilution. All the solutions were stored at +4°C and the reference solutions were prepared weekly from the stock solutions.

Reference solutions were prepared separately for either standard calibration or QC samples.

2.4.2. Preparation of calibration curves

The spiked samples were processed according to the described sample preparation procedure. All the calibration samples were prepared freshly on the day of analysis.

2.4.3. Preparation of QC samples

Aliquots of 0.5 ml (0.1 g in faeces) of human control samples were spiked with both VRL and metabolites reference solutions mixtures in order to achieve at least four final concentrations within the calibration range, but including a QC at the expected limit of quantitation (see Table 1). The QC samples were also processed according to the hereafter reported sample preparation procedure. Series of QC were regularly prepared in one batch, aliquoted and then stored at −80°C until daily use.

2.5. Sample preparation

2.5.1. Sample preparation for blood and plasma

In a silicon coated glass tube, 0.5 ml aliquots sample were deproteinised with 1 ml of methanol. Then, 25 μl of vinblastine reference solution (internal standard) and 5 ml of acetonitrile were successively added. The tubes were sonicated, then shaken for 10 min on a back and forth shaker and finally centrifuged for 10 min at 2400 *g*. Then, 200 μl of 40 m*M* ammonium acetate buffer, pH 3.0 was added before evaporation under a gentle nitrogen stream. Finally, 1 ml of 40 m*M* ammonium acetate buffer, pH 3 was added. The tubes were briefly mixed. An aliquot of 25 to 40 μl was injected into the HPLC system.

2.5.2. Sample preparation for urine

In a silicon coated glass tube, to 1-ml sample aliquots were added 250 μl of VBL solution and 4 ml of 40 m*M* acetate buffer, pH 3. The mixture was sonicated and centrifuged for 10 min at 2400 *g*. An aliquot of 25 to 40 μl was injected into the HPLC system.

2.5.3. Sample preparation for faeces

In a silicon coated glass tube, to 0.1-g sample aliquots were added 50 μl of VBL solution, 1 ml of methanol and 4 ml of 40 m*M* ammonium acetate buffer, pH 3. The mixture was sonicated and centrifuged for 10 min at 2400 *g*. The extraction process was carried out twice. Then, 100 μl of the supernatant layer was transferred to another tube, where 10 ml of 40 m*M* acetate buffer, pH 3 was added. The

Table 1
Intra-assay precision for QC samples ($n=4$)

Compound	Blood		Plasma		Urine		Faeces	
	C_{theor} (ng/ml)	RSD (%)	C_{theor} (ng/ml)	RSD (%)	C_{theor} (ng/ml)	RSD (%)	C_{theor} (μg/g)	RSD (%)
VRL	0.25	7.5	n.c.	n.a.	n.c.	n.a.	n.c.	n.a.
	0.5	4.4	0.5	7.8	2.5	4.7	2	9.4
	10	0.2	10	6.6	50	2.0	50	5.3
	50	5.6	50	6.6	250	5.5	250	5.8
	200	4.1	200	8.5	1000	3.0	1000	6.8
DVRL	0.25	3.4	n.c.	n.a.	n.c.	n.a.	n.c.	n.a.
	0.5	9.3	0.5	5.2	2.5	2.9	2	5.9
	2.5	5.3	2.5	3.9	12.5	3.2	50	3.4
	10	5.9	10	5.2	50	7.4	250	2.1
	50	3.6	50	6.7	250	2.8	1000	5.3
20′OH-VRL	0.5	10.9	0.5	8.2	2.5	4.0	n.c.	n.a.
	2.5	3.1	2.5	6.7	12.5	2.6	n.c.	n.a.
	10	7.3	10	4.4	50	6.5	n.c.	n.a.
	50	2.8	50	3.3	250	3.3	n.c.	n.a.
NO-VRL	2.5	5.3	2.5	11.7	12.5	3.5	n.c.	n.a.
	10	4.8	10	9.1	50	5.0	n.c.	n.a.
	50	5.1	50	2.9	250	0.7	n.c.	n.a.

n.c.: Not calculated, n.a.: not applicable.

tubes were briefly mixed. An aliquot of 25 to 40 μl was injected into the HPLC system.

When VRL or its metabolites concentrations were out of range of the calibration curve, appropriate dilution of samples with control urine was carried out before reprocessing the samples.

2.6. Recovery

Recoveries of VRL, DVRL, 20′OH-VRL, NO-VRL and VBL were calculated in blood and faeces, comparing the HPLC signals obtained from spiked biological samples with those obtained from directly injected similar concentrations in aqueous solutions.

2.7. Accuracy, precision and linearity

Within- and between-run validation provided accuracy and precision of the method in each medium. For the within-run validation, at least four replicates at each QC concentration value were processed. The between-run validation was carried out over 3 days. Mean errors and SD values were calculated from the theoretical and experimental concentrations, in order to determine the precision and accuracy of the method.

In blood and faeces, the linearity was explored on six replicates at each calibration concentration value. The same descriptive parameters were calculated.

2.8. Data analysis and daily run acceptance

The calibration curves were calculated through a linear least-squared regression model with a weighting factor of $1/C$, where C is the concentration of the calibration standards. QC and test biological samples were calculated from the equation of the regression.

The daily run was accepted if no more than a maximum of two QC sample values were out of range (out of six QCs randomly located into the run protocol), and not at the same QC level. Individual QC sample values were accepted if they were ±15% for the high and middle level and ±20% for the low QC level [8].

2.9. Limit of quantitation (LOQ)

The LOQ of the method was defined as being the concentration level which presented an error of 20% or less, between theoretical and calculated values during the between-run analysis.

3. Results

3.1. Mass spectra

The pseudo molecular ions $[M+H]^+$ of VRL and DVRL were recorded at m/z 779 and 737, respectively. After collisionally induced dissociation (CID) of $[M+H]^+$, the product ion spectrum showed a strong fragment ion at m/z 122. This key fragment ion which was attributed to a part of the catharanthine moiety of the molecule was the most abundant product ion and therefore was used to monitor VRL and DVRL in the MRM mode (Figs. 1 and 2).

For the two other metabolites, the pseudo molecular ion $[M+H]^+$ was recorded at m/z 795 resulting from a shift of 16 Dalton (oxide group for NO-VRL and alcoholic group for 20′OH-VRL). The corresponding product ion spectrum showed a strong fragment ion at m/z 138 which was used to monitor the two compounds in the MRM mode (Figs. 3 and 4).

In LC–MS–MS, the internal standard (I.S.) should match the structure of the analyte as closely as possible. Since the synthesis of vinca alkaloids is too complex, isotope-labelled analytes could not be obtained. Therefore, vinblastine, an analog of vinorelbine was used. The multi-reaction monitoring transition 811→224 was followed.

3.2. Chromatograms – specificity

Whatever the biological medium, no endogenous interfering peaks were observed in any extracted blank sample at the respective retention times of VRL, DVRL, 20′OH-VRL, NO-VRL and VBL. Thus, high specificity concerning endogenous compounds could be guaranteed and achieved when operating with the triple-quadrupole system.

A typical chromatogram from a blood QC sample at 0.5 ng/ml is illustrated in Fig. 5. All the com-

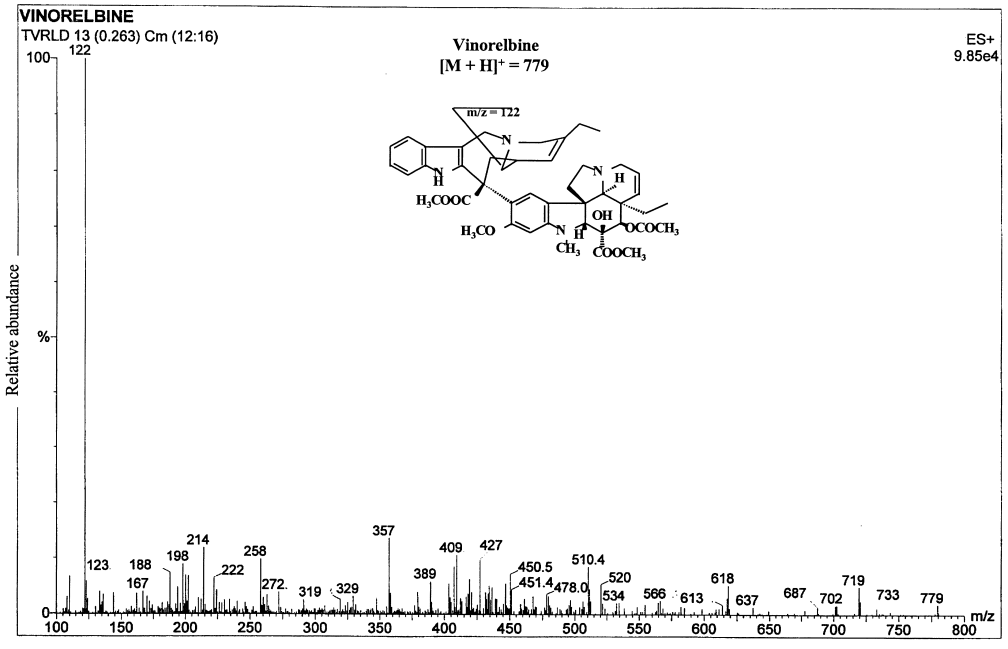

Fig. 1. Product ion spectrum and structure of vinorelbine.

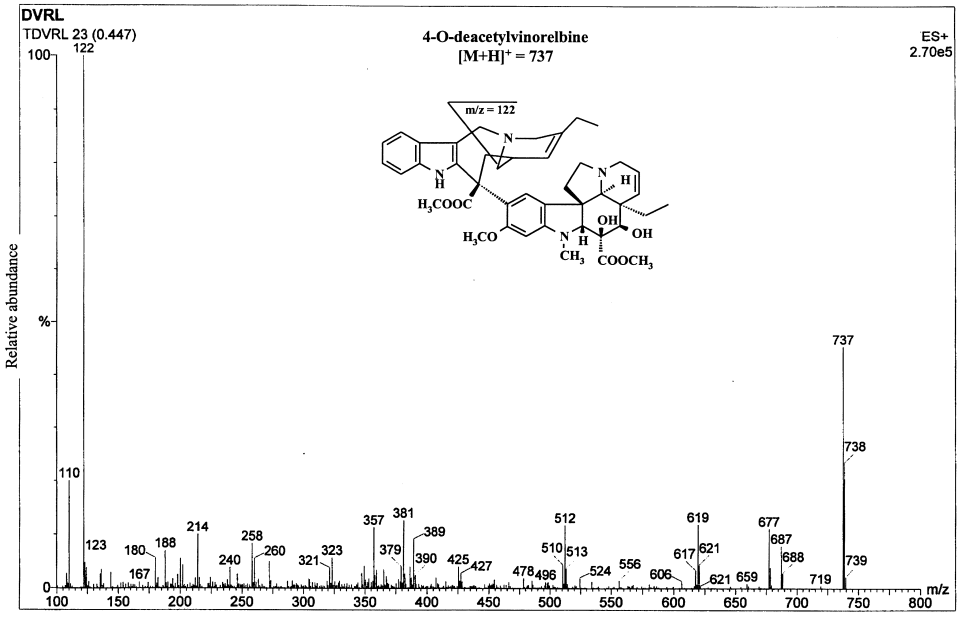

Fig. 2. Product ion spectrum and structure of 4-*O*-deacetylvinorelbine.

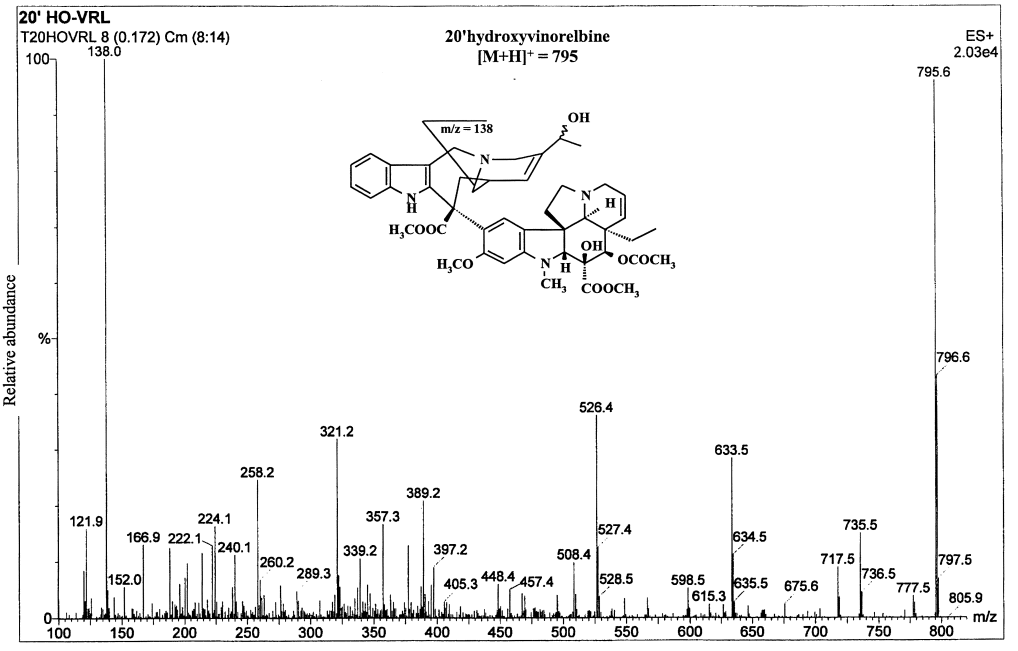

Fig. 3. Product ion spectrum and structure of 20′-hydroxyvinorelbine.

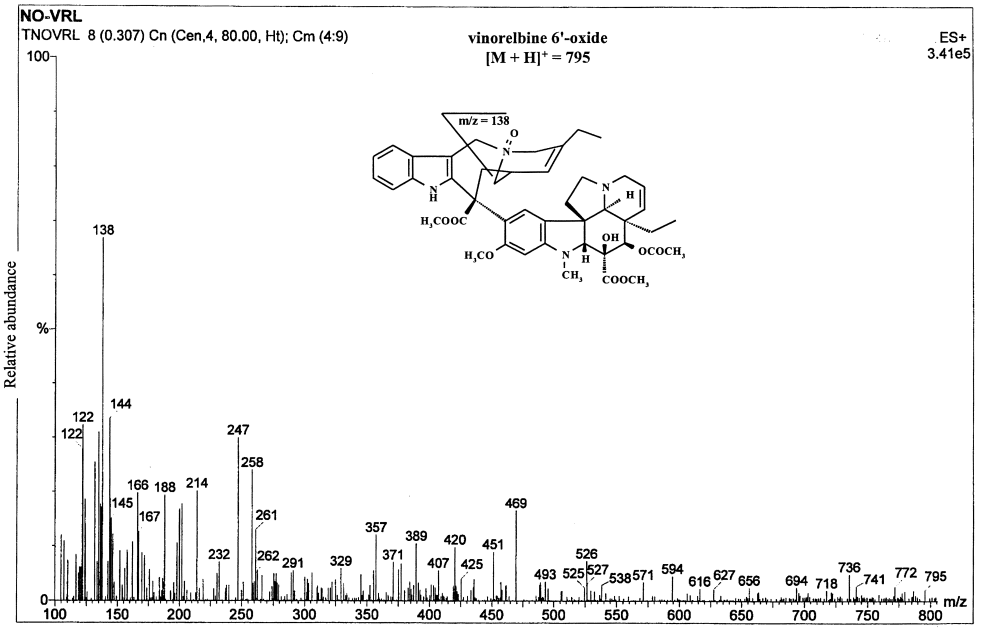

Fig. 4. Product ion spectrum and structure of vinorelbine 6′-oxide.

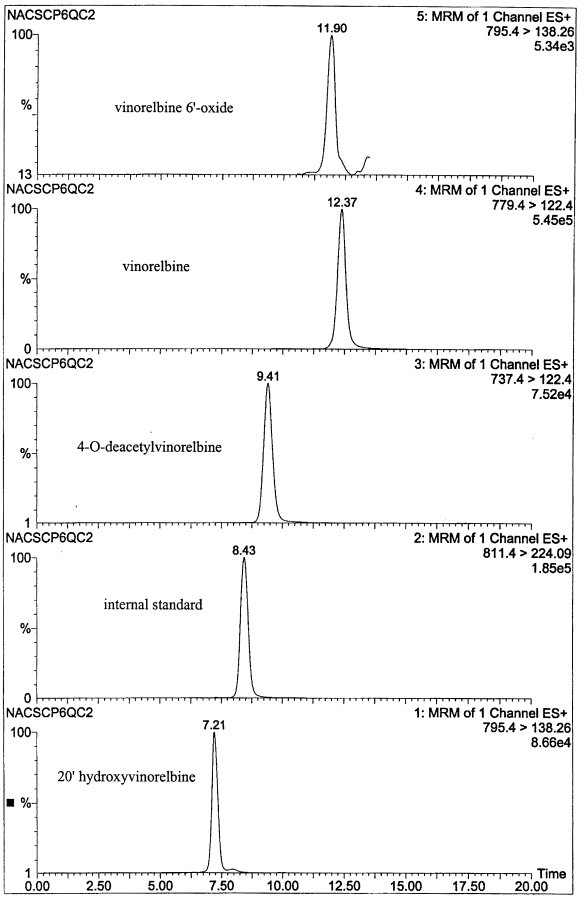

Fig. 5. Typical chromatogram from a blood control sample spiked with 0.5 ng/ml of vinorelbine and its metabolites. Time scale in min.

pounds were rapidly eluted (less than 13 min). Relative retention times of the five compounds were approximately as follows: 20′OH-VRL, 0.6; VBL, 0.7; DVRL, 0.8; NO-VRL, 1.0; VRL, 1.0.

3.3. Performance of the method

The best fit of the calculated concentrations towards the theoretical values was observed with the weighted $1/C$ least-squared regression analysis. In whole blood and plasma, calibrations were validated up to 200 ng/ml for VRL and 50 ng/ml for the three metabolites. In urine and faeces, respective calibrations were validated up to 1000 ng/ml and 1000 μg/g for VRL and 250 ng/ml and 1000 μg/g for the metabolites.

The mean extraction recoveries were higher than 80% in blood for VRL and its three metabolites and close to 100% in faeces for both VRL and DVRL. The extraction yields of VBL was estimated higher than 94% in both media.

The within- and between-run reproducibilities of the method for blood, plasma, urine and faeces are summarised in Tables 1 and 2. Whatever the biological fluid, the within-run RSDs were always below 9.5% and the between-run RSDs below 12.7% for both VRL and DVRL (mean bias$<$10.2%) and below 11.8% and 18.3% for 20′OH-VRL and NO-VRL (mean bias$<$16.1%).

A good signal-to-noise ratio was observed at the LOQ indicating that the corresponding value could be safely reached. As an illustration, the LOQ was validated at 0.25 ng/ml in whole blood, 0.5 ng/ml in plasma, 2.5 ng/ml in urine and 2 μg/g in faeces for both VRL and DVRL. The between-run RSDs were lower than 12.7% for VRL and DVRL and lower than 18.3% for the two other metabolites.

4. Discussion

In 1991 an extensive program was initiated on vinorelbine, which required the development of a robust and accurate method for bioanalysis of VRL and its main metabolite, DVRL, in human biological fluids. This method, based on a two-step liquid–liquid extraction and a reversed-phase separation with UV detection has been largely used since 1993 in animal and human studies, and more particularly in blood with an LOQ of 2.5 ng/ml for both compounds [7]. Both intravenous and oral formulations of vinorelbine evidenced few peaks in patients which were attributed to metabolites of VRL, then named and monitored all along the clinical studies. Obviously, only more specific analytical tools could check the purity of each peak by means of an accurate identification process conducted to elucidate the chemical structures of these metabolites. Therefore, a new analytical method was developed using LC–MS–MS for qualitative and quantitative investigations of VRL metabolism.

Separation of VRL, its metabolites and vinblastine

Table 2
Inter-assay precision for QC samples ($n=12$)

Compound	Blood		Plasma		Urine		Faeces	
	C_{theor} (ng/ml)	RSD (%)	C_{theor} (ng/ml)	RSD (%)	C_{theor} (ng/ml)	RSD (%)	C_{theor} (μg/g)	RSD (%)
VRL	0.25	6.8	n.c.	n.a.	n.c.	n.a.	n.c.	n.a.
	0.5	11.6	0.5	7.8	2.5	10.9	2	12.2
	10	0.6	10	3.6	50	3.2	50	1.2
	50	3.7	50	3.3	250	3.5	250	3.1
	200	4.1	200	8.7	1000	6.7	1000	4.7
DVRL	0.25	12.6	n.c.	n.a.	n.c.	n.a.	n.c.	n.a.
	0.5	3.8	0.5	5.2	2.5	10.8	2	8.2
	2.5	8.0	2.5	3.9	12.5	8.9	50	6.9
	10	8.8	10	5.2	50	2.2	250	7.0
	50	8.2	50	6.7	250	6.4	1000	7.0
20′OH-VRL	0.5	18.2	0.5	18.0	2.5	13.0	n.c.	n.a.
	2.5	9.0	2.5	10.6	12.5	8.3	n.c.	n.a.
	10	8.4	10	10.6	50	0.1	n.c.	n.a.
	50	13.6	50	6.5	250	10.9	n.c.	n.a.
NO-VRL	2.5	7.7	2.5	7.2	12.5	0.8	n.c.	n.a.
	10	13.6	10	6.6	50	8.5	n.c.	n.a.
	50	12.3	50	9.8	250	3.4	n.c.	n.a.

n.c.: Not calculated, n.a.: not applicable.

(used as internal standard) was achieved by a reversed-phase HPLC method using the same conditions as those previously detailed in our HPLC–UV method [7]. Several MS–MS conditions were tested. Electron impact ionisation (EI) produced very low abundance of the molecular ions M^+, as already documented for other vinca alkaloids [9]. On the other hand, both atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) MS–MS interfaces could be used for VRL related compounds, but ESI-MS–MS in the positive mode is actually required to achieve the highest sensitivity.

Once the chromatographic conditions were properly adjusted, the extraction procedure was explored. Liquid–liquid extraction (widely described by several authors [2,3,10,11] and successfully used in our initial HPLC method [7] for both VRL and DVRL) was not considered to be the most suitable for an efficient separation of the different metabolites. We actually demonstrated that only 50% of the metabolic pool was really extracted from matrices. Then, we developed a solid-phase extraction (SPE) procedure on the basis of those described by Vendrig et al. [12,13]. Biological matrices were extracted on Oasis HLB column pre-conditioned with methanol and acetate buffer under acidic conditions.

This method, applied to blood, urine and bile specimens, allowed the isolation and the consecutive identification of the metabolites' structures leading to the determination of the metabolic pathway of vinorelbine [14]. Although SPE was fully suitable and offered some decisive advantages (less solvent consumption, on-line automatisation), it also required an additional clean-up step which also precludes the formation of particles which can block the columns as previously observed in both blood and faeces analysis [11]. Finally, we demonstrated that deproteinisation was the best procedure in terms of easiness, standardisation and reliability; this process is characterised by a 90% quantitative extraction recovery for the VRL metabolic pool.

Chemical synthesis of vinca alkaloids compounds is quite complex and most of the metabolites were not available as reference chemicals. As such, they had to be quantified referring to the unchanged VRL compound.

Since 20′OH-VRL and NOVRL had been previously suggested as potential metabolites [15,16] and because they were initially produced within the chemical series synthesis, validation of these two compounds was performed simultaneously with VRL and DVRL. All validation criteria were fulfilled for all compounds. A significant advantage of this method was that it reached a tenfold higher sensitivity in whole blood (250 pg/ml for both VRL and DVRL) than the previously developed HPLC method [7].

The performance of this new LC–MS–MS method is illustrated in the pharmacokinetic profile displayed in blood (see Fig. 6). This medium was considered as relevant since VRL evidenced a high affinity for blood cells, particularly platelets [17]. As a result of the specificity and increased sensitivity of this method, VRL and six metabolites were detected and quantified over 168 h (i.e., 4–5 elimination half-lives) [18] compared with the previous HPLC methods which enabled the quantitation of vinorelbine up to 48 h [19].

From the clinical data available through this new method, it was demonstrated that NO-VRL was scarcely detected and, even then, only at trace levels in the elimination media. Furthermore, this method provided relevant information on DVRL: this metabolite was of interest because its pharmacological activity was similar to that of the parent drug [20]. However, depending on the biological medium, it was either undetected or scarcely detected [16]. With this new hyphenated methodology, we were able to confirm that DVRL was a metabolite quantified for a long time in blood and largely eliminated in bile [14].

5. Conclusion

This LC–MS–MS methodology was aimed at analysing vinorelbine and its metabolites in human biological fluids. The method was demonstrated to be accurate, reliable and sensitive. Together with the previous HPLC method, it has been frequently used since 1998 for quantitative determination, depending on the goals of clinical trials.

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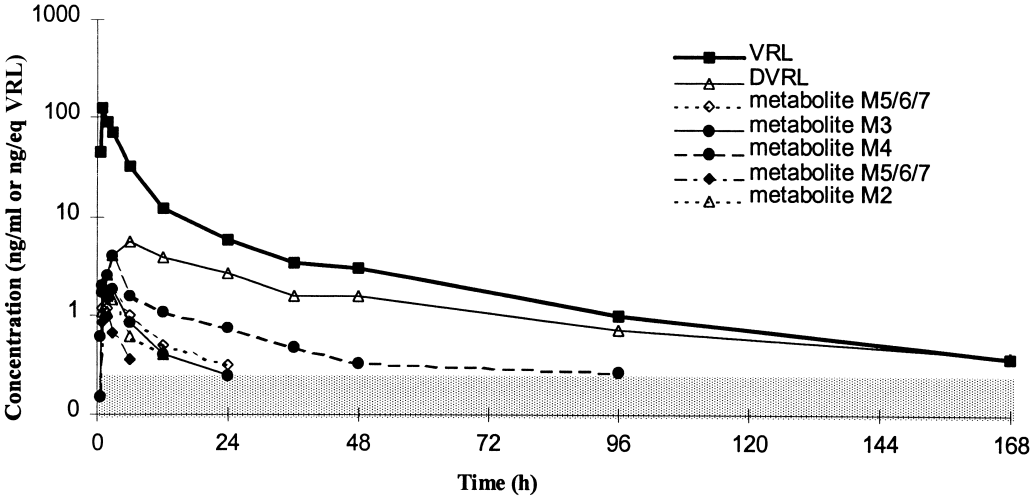

Fig. 6. Example of a blood profile for vinorelbine and its metabolites. Concentrations are expressed on log scale. The grey area represents the limit of quantitation (below 0.25 ng/ml).

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