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# Metabolism pathway of vinorelbine (Navelbine®) in human: Characterisation of the metabolites by HPLC–MS/MS

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**Abstract**

The biotransformation of vinorelbine (VRL), an anti-neoplastic vinca-alkaloid derivate already marketed for nonsmall cell lung cancer and advanced breast cancer as an i.v. form and currently registered in several countries as an oral form, was investigated in human. Biological specimen from several human sources constituted the material for the metabolic identification in human. An isocratic liquid chromatographic system composed of 40 mM ammonium acetate (pH 3) and acetonitrile was used for separation of the potential metabolites of VRL. Tandem mass spectrometry with positive electrospray ionisation was used to enable the structural identification of the metabolites. A total of 17 metabolites (12 directly obtained from VRL and 5 involving sequential step pathways) were characterised with proposed structures for most of the metabolites. All metabolites went through phase I reactions by the way of deacetylation, dealkylation, oxidation and hydroxylation. No conjugates were observed. Despite the high number of metabolites quantified, VRL was the major compound observed whatever the matrix. Most of the metabolites rapidly disappeared from blood, except 4-*O*-deacetyl vinorelbine which was slowly cleared. Most of the enzymatic pathways involved in the metabolites strongly suggested the major role of cytochrome P450 in the biotransformation of vinorelbine.


*Keywords:* Vinorelbine; HPLC–MS/MS; Metabolites; Human

## 1. Introduction

Vinorelbine (VRL), nor-5′-anhydrovinblastine is a semi-synthetic vinca-alkaloid developed by Pierre Fabre Medicament (Navelbine®) that exhibits antimitotic activity [1]. An intravenous form is marketed worldwide for nonsmall cell lung cancer and advanced breast cancer and an oral form is currently registered in several countries for the same indications. Very little was known about vinca-alkaloid metabolism, although some of them have been marketed for more than 40 years [2]. Until recently, and because of the complexity of its chemical structure, little was known on the metabolism of vinorelbine. Literature review identified two potential metabolites [3]. The first one, 4-*O*-deacetylvinorelbine (DVRL), only detected in human urine [4] was observed and later quantified in plasma [5]. This metabolite attracted high interest since its pharmacological and toxicological activity seemed to be similar to that of the parent drug [6]. The second metabolite, vinorelbine 6′-oxide (NOVRL), which was described as inactive and non-toxic [4], had never been detected either in blood or in urine. Other structurally related but still unidentified peaks were observed in human urine [7] or in isolated human hepatocytes [8]; they were also strongly suspected to be linked to the metabolic pathway of VRL. In vitro experiments allowed separation of several new peaks in human in both microsomes and hepatocytes [9]. Structural identification was difficult because of the small amount of metabolites produced in these incubations. However, hydroxylation, oxidation and demethylation reactions were postulated, confirming the previous findings observed in rat bile where five new metabolites formed by hydroxylation and oxidative ether formation were identified [10].

The development of an oral form of VRL, as a line extension of the intravenous form required a full pharmacokinetic program including metabolism. Thanks to a new, highly sensitive HPLC method [5], DVRL was quantified for the first time in blood whatever the route of administration, and its pharmacokinetics

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was largely discussed [11–13]. Knowledge of the metabolism pathway is a key issue in the development of an oral form since some metabolites can be generated in the gastro-intestinal tract. Using radiolabelled compound, an extensive exploration of vinorelbine metabolism was carried out in animal before initiating a similar study in human [14]. It should be considered that the investigation of VRL metabolism has also been limited by the lack of a sensitive and specific analytical method [7,15]. Therefore, a more sensitive and reliable liquid chromatography tandem mass spectrometry (LC–MS/MS) tool was set up for the metabolite identification and quantification in human biological media [16].

The opportunity of obtaining bile or faeces human samples together with blood and urine was expected to radically improve the in vivo metabolism knowledge of VRL in human. Liquid chromatography with either triple quadrupole or hybrid quadrupole ToF mass spectrometry provided structural data for metabolite identification.

## 2. Experimental

### 2.1. Chemical and reagents

Vinorelbine ditartrate (Fig. 1), 4-*O*-deacetylvinorelbine sulfate; vinorelbine 6′-oxide base, 20′-hydroxyvinorelbine ditartrate and vinorelbine 3,6-ether ditartrate were synthesised at the Research Centre of Pierre Fabre Medicament laboratories (Castres, France). Methanol and acetonitrile (Acros Organics Geel, Belgium) were of HPLC grade. Formic acid (Acros Organics Geel, Belgium) and ammonium acetate (UCB, Belgium) were of analytical reagent grade. β-D-Glucuronidase/arylsulfatase, purified fraction from Helix Pomatia (ref. 127698) was obtained from Roche, Germany. Dry nitrogen (quality N45) was from Air Liquide (Liège, Belgium).

### 2.2. Biological specimen

Blood, plasma, urine, faeces and bile were obtained from patients treated with vinorelbine either through an i.v. infusion or an oral administration. All the biological samples were collected into silicone coated glass tubes and stored at −80 °C until their processing.

### 2.3. Sample preparation

Two sample preparation procedures were used for this experimental work depending on the biological medium.

#### 2.3.1. Solid-phase extraction

Oasis™ HLB columns (3 ml volume) were conditioned with approximately 6 ml of methanol, 3 ml of water and 3 ml of 40 mM ammonium acetate buffer (pH 5.9). Urine and bile samples (0.5–1 ml) were diluted with 1 ml of 40 mM ammonium acetate buffer (pH 5.9) made of 3.1 g of ammonium acetate in 1000 ml of water adjusted to pH 5.9 with formic acid and applied to the extraction column.

After a washing step (1 ml of water), elution was performed using two volumes of acetonitrile. The organic phase was dried under a gentle nitrogen stream and re-dissolved into 200 μl of a 40 mM ammonium acetate buffer pH 5.9 solution.

#### 2.3.2. Deproteinisation procedure

To 0.5 ml of blood or plasma and 100 mg of faeces, 1 ml of methanol was added. The mixtures were sonicated, shaken for 10 min and finally centrifuged. In order to prevent the evaporation under dryness, 200 μl of 40 mM ammonium acetate buffer (pH 3) solution (3.1 g of ammonium acetate in 1000 ml of water adjusted to pH 3 with formic acid) was added to the supernatant. Then, the organic phase was evaporated under a gentle stream of dry nitrogen and re-dissolved with 1 ml of a 40 mM ammonium acetate buffer (pH 3) solution.

### 2.4. Metabolite identification

The LC–MS/MS system consisted of a HP1100 system (Hewlett-Packard, Waldbronn, Germany) and a Quattro II triple stage quadrupole mass spectrometer, controlled by Masslynx 3.2. software (Micromass, Altrincham, UK). The chromatographic separation was achieved on a Spherisorb CN column (100 mm × 4.6 mm i.d., 3 μm) using an isocratic elution mode at a flow rate of 0.65 ml/min. The mobile phase was a mixture of 40 mM ammonium acetate buffer (pH 3) and acetonitrile (55:45, v/v). The mass spectrometer was operated in the positive electrospray mode of ionisation. The following conditions were selected in order to achieve the highest sensitivity: capillary voltage: 3.5 kV, cone voltage ranging from 50 to 100 V, source temperature: 150 °C, collision energy: 2–5 eV, and collision gas cell pressure: $3 \times 10^{-3}$ mbar. Data were collected by MS1 under full-scan mode (*m/z* 100–1000) at a scan rate of 500 Da/s and 1 U mass resolution. Product ion scan mode was applied to collect the signals required to identify the chemical structures of the respective metabolites.

The relative retention time (RRT) of each metabolite was calculated using the ratio of their respective retention times to the corresponding experimental value recorded for the unchanged parent drug. Interpretation of the product ion mass spectra (MS/MS) of each metabolite was conducted in reference to the parent drug and some available chemical reference products.

To improve the structural information on some metabolites, a quadrupole-time of flight hybrid mass spectrometer (Q-ToF) fitted with a Z-spray ion interface (Micromass, Altrincham, UK) and a lock spray option was used. The separation of vinorelbine metabolites was achieved by HPLC on a Spherisorb CN column (100 mm × 4.6 mm i.d., 3 μm) using a gradient elution. Mobile phase A consisted of 0.1% formic acid in 2 mM ammonium acetate buffer; mobile phase B consisted of 0.1% formic acid in acetonitrile. The initial elution condition was 0% of B maintained for 30 min and increased to 90% of B over 1 min and 0% of B the next 9 min. The flow rate was 0.2 ml/min. The electrospray source was operated in the positive mode of ionisation with a capillary voltage of 2.5 kV, a cone voltage of 45 V and a source temperature

of 135 °C. Argon was used as collision gas and the collision energy was fixed, respectively to 10 eV in ToF MS and 30 eV in ToF MS/MS modes. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine–enkephalin was used as the lock mass (*m/z* 556.2771) at a concentration of 2 pg/μl and flow rate of 10 μl/min. Data were collected in a centroid mode from 90 to 965 *m/z* with a LockSpray frequency of 5 s, and data averaging over 5 scans.

### 2.5. Search for glucurono- or sulfo-conjugates

Enzymatic hydrolysis was performed on both urine and bile specimen treated with a diluted β-D-glucuronidase/sulfatase (1/100, v/v) mixture. Selected urine over 48 h and bile from 1 to 24 h post-administration (500 μl) were diluted with 500 μl of 1 M sodium acetate buffer (pH 4.3) before the addition of 100 μl of the glucuronidase–sulfatase reagent. The mixture was incubated at *ca.* 37 °C for 12 h. The samples

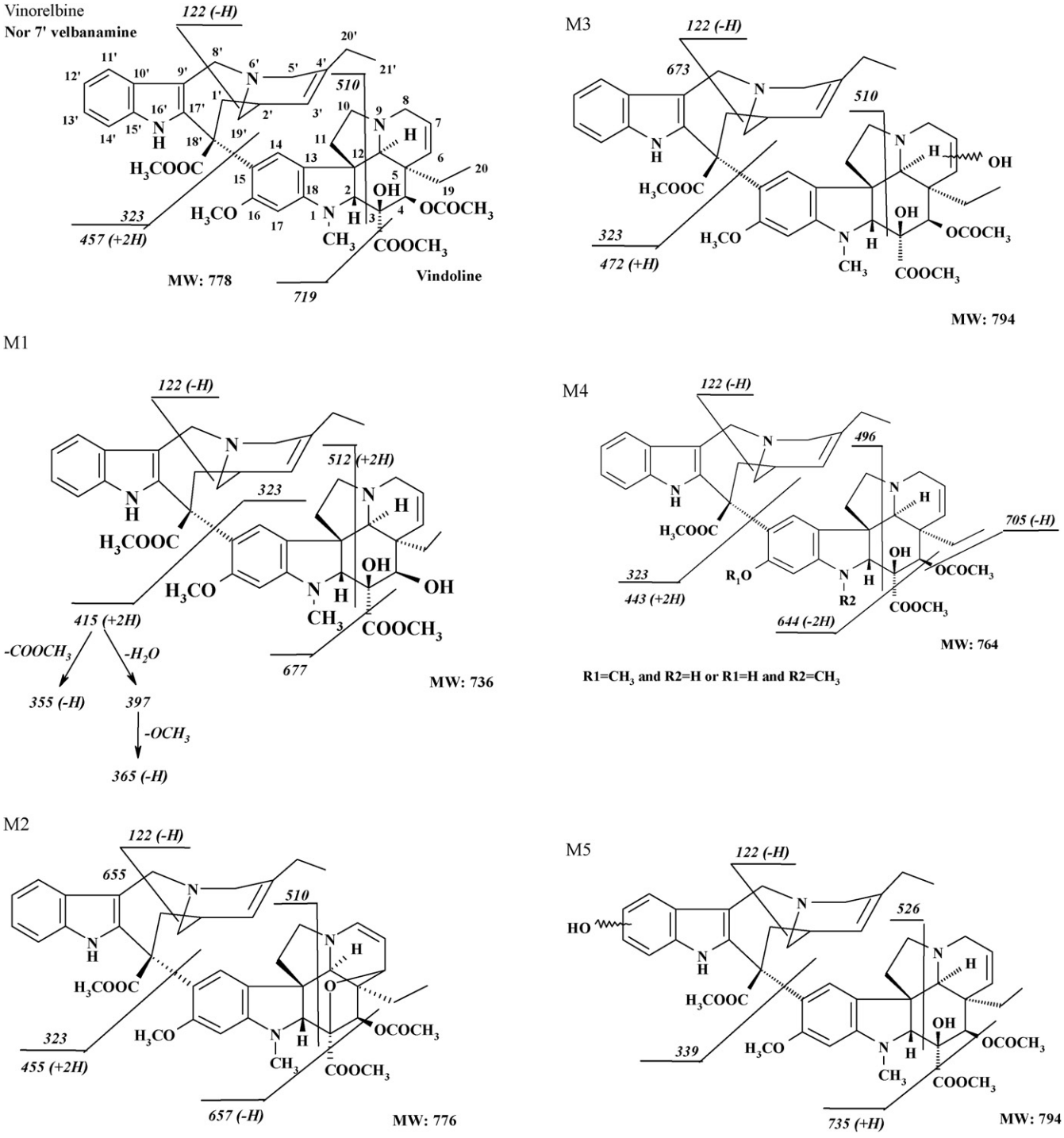
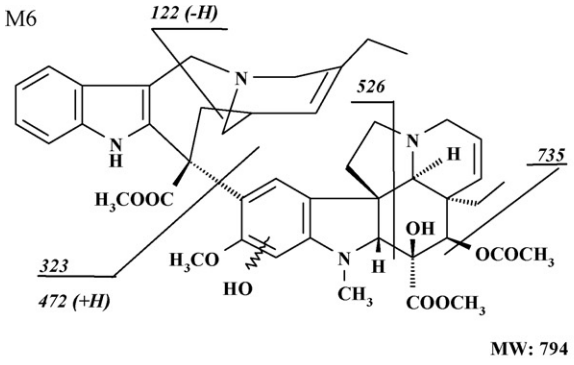
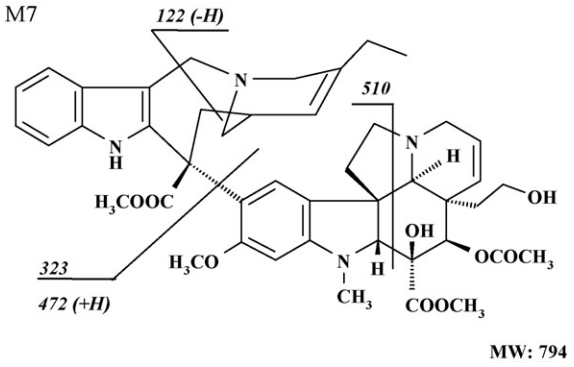
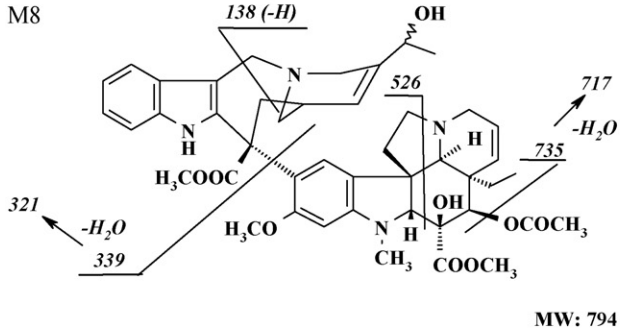
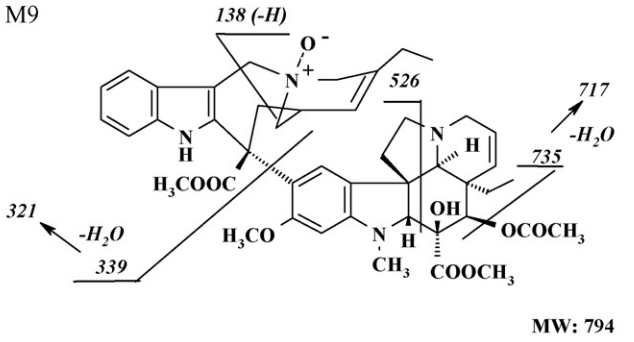


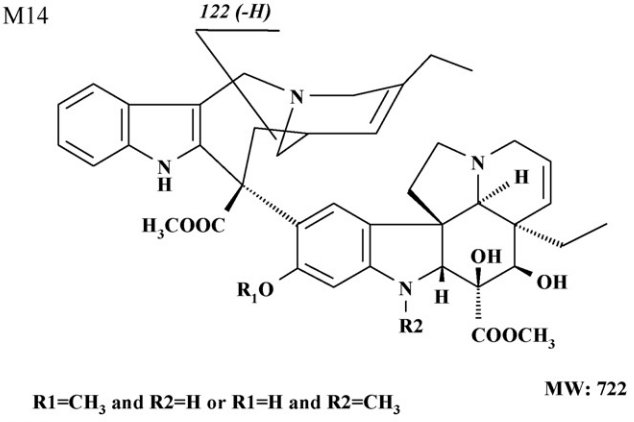

Fig. 1. Structures and typical fragmentations of vinorelbine and metabolites.

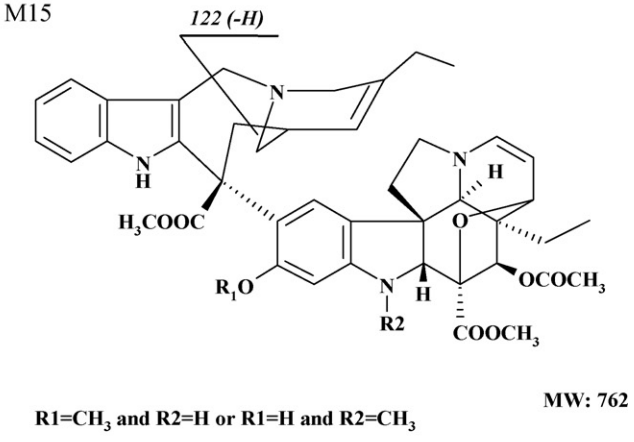


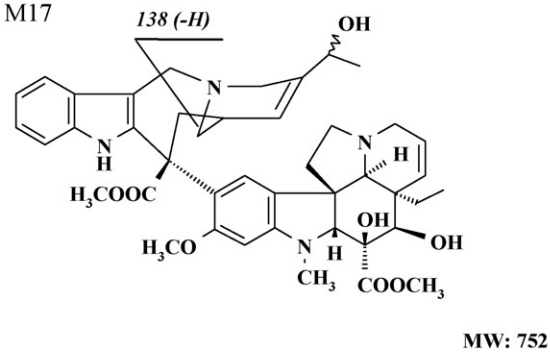




Fig. 1. (*Continued*).

were then processed by solid-phase extraction as described above.

Furthermore, and after an appropriate tuning of the MS source, losses spectra of sulfate (80 Da) or glucuronic acid (176 Da) moieties were searched for throughout the mass spectrometric analysis, on crude faeces samples collected between 24 and 48 h after VRL administration.

## 3. Results

### 3.1. Identification and characterisation of metabolites

The following description of the metabolites is developed according to the IUPAC numbering recommendations. The structures and the relevant fragmentations supporting their identification are presented in Fig. 1.

#### 3.1.1. Vinorelbine (parent drug)

The electrospray ionisation (ESI) spectrum of VRL (Fig. 2) showed a protonated molecular ion at *m/z* 779. Specific fragmentated ionic species were identified at *m/z* 457 and 323; they belong to the vindoline and nor-7′-velbanamine moiety, respectively. The key fragment ion *m/z* 122 resulted from a specific cleavage arising in the bonds between C-8′ and C-9′ and between C-1′ and C-2′ of the velbanamine part of the molecule. This typical cleavage could be definitely confirmed from closely related compounds exhibiting additional atoms on this substructure (data not shown). Finally, fragment ions *m/z* 719 [M–(–$COOCH_3$)] and *m/z* 510 were also characteristic ions from this molecule (Fig. 1).

The spectral ToF analysis of VRL generated a *m/z* of 779.4030, suggesting an elemental composition of $C_{45}H_{55}N_4O_8$ with a mass accuracy of 1.3 ppm. The molecular weight and elemental composition of VRL were used as a baseline for comparison with some metabolites. These data were illustrated in Table 2.

#### 3.1.2. Major drug-related peaks

The relative retention times (RRT) and mass spectra of M1 and M2 were identical to those obtained, respectively with the pure substances DVRL (RRT = 0.78) and vinorelbine 3,6-ether (RRT = 0.92) (Fig. 3).

Metabolite M1 was characterised by a specific transition 737-122 in MRM mode (Fig. 4). The fragmentation pathway of DVRL with its characteristic ion located at fragmented *m/z* 677 [M–(–$COOCH_3$)] confirmed a 42 Da shift from the parent drug as the result of the hydrolysis reaction occurring on C-4 (*i.e. m/z* 457 shifted to 415). This positive and formal identification from the reference compound provided relevant information that could be used for the interpretation of spectra generated by less abundant metabolites. In addition, the product ion mass spectrum from *m/z* 415 signal exhibited diagnostic ions which included *m/z* 397, 365, 355 and 188 justified from the fragmentation pattern shown in Figs. 1 and 2.

The full-scan mass spectrum of M2 showed the molecular ion at *m/z* 777 as the most abundant ion. The product ions at *m/z* 323 and 122 where consistent with an unchanged nor-7′-

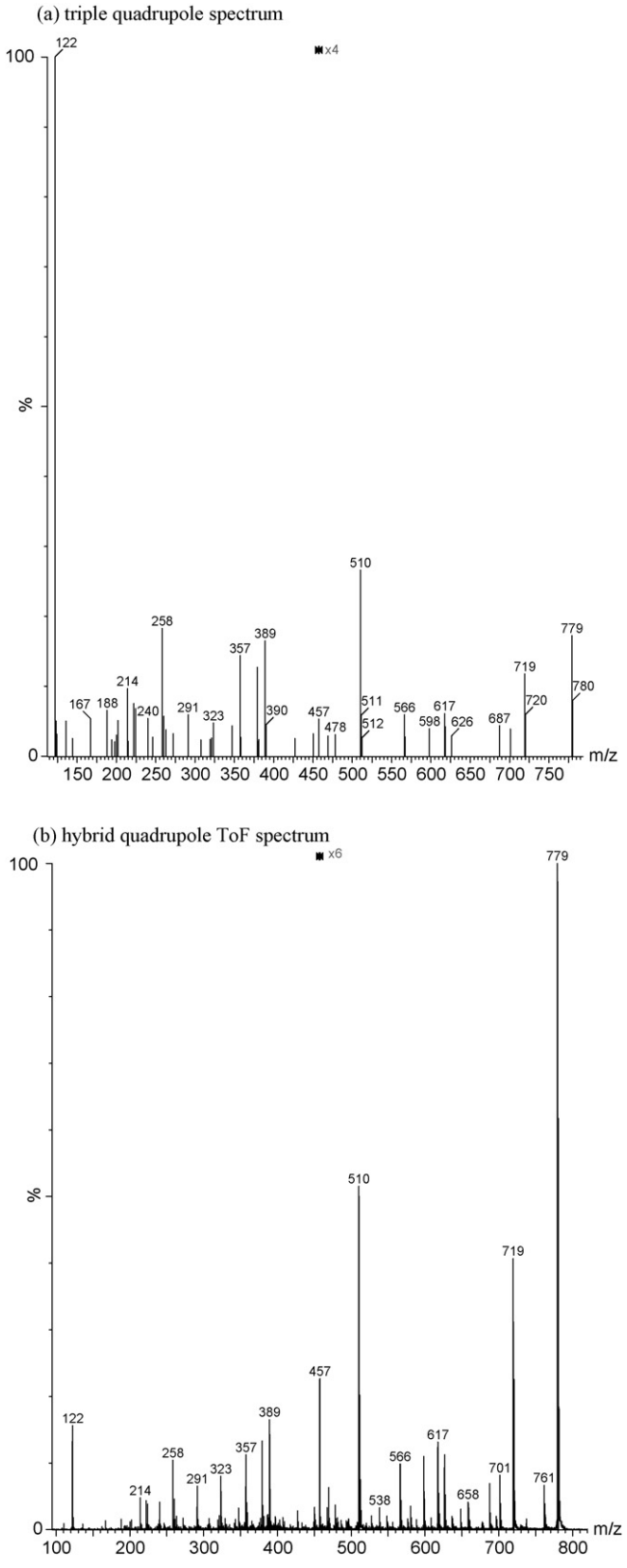



Fig. 2. ESI full-scan spectrum of vinorelbine.

Table 1
LC–MS/MS characteristics of VFL and DVRL in human biological fluids

| Identification | Metabolite name | RRT | Ion transition | Blood or plasma[a] | Urine[a] | Faeces or bile[a] |
|---|---|---|---|---|---|---|
| M1 | DVRL | 0.78 | 737→122 | + | + | + |
| M2 | VRL 3,6-ether | 0.92 | 777→122 | + | + | + |
| M3 | HydroxyVRL isomer #2 | 0.76 | 795→122 | + | + | + |
| M4 | DesmethylVRL | 0.91 | 765→122 | + | + | + |
| M5 | HydroxyVRL isomer #1 | 0.68 | 795→122 | + | + | + |
| M6 | HydroxyVRL isomer #3 | 0.71 | 795→122 | Tr | - | Tr |
| M7 | HydroxyVRL isomer #4 | 0.88 | 795→122 | - | - | Tr |
| M8 | 20'-HydroxyVRL | 0.62 | 795→138 | Tr | - | + |
| M9 | VRL 6'-oxide | 0.97 | 795→138 | Tr | - | Tr |
| M10 or M10bis | Hydroxy-M9 or Hydroxy-M8 | 0.54 | 811→138 | - | - | Tr |
| M11 | Metabolite 777 (a) | 0.66 | 777→122 | - | - | Tr |
| M12 | Metabolite 777 (b) | 0.84 | 777→122 | - | - | Tr |
| M13 | Metabolite 777 (d) | 1.38 | 777→122 | Tr | Tr | Tr |
| M14 | Desmethyl-M1 | 0.69 | 723→122 | - | - | Tr |
| M15 | Desmethyl-M2 | 0.83 | 763→122 | - | - | Tr |
| M16 | Desmethyl-M13 | 1.23 | 763→122 | - | - | Tr |
| M17 | 20'-Hydroxy M1 | 0.49 | 753→138 | - | - | Tr |

–: Not detected; Tr: trace level. [a]The main metabolites quantified in the biological media are displayed in grey area, whereas the minor ones detected as a trace are shown in parentheses.

velbanamine moiety while the ion at *m/z* 455 hypothesised the loss of two hydrogens from the vindoline moiety (Fig. 4). Other relevant and informative signals were from *m/z* 655 [M-122], 510 and 657. This last fragment was likely observed from the loss of $–OCOCH_3$ and $–COOCH_3$ on the vindoline moiety. As a result of the loss of two hydrogen atoms from an intermediate oxidation step of vinorelbine arising on C-6, an internal cyclisation occurring between C-3 and C-6 was postulated. The di-hydroxylated product (on C-6 and C-3) underwent afterwards the departure of a molecule of water.

Metabolite M4 was characterised by a specific transition 765-122 in MRM mode; it eluted at a RRT of 0.91 (Fig. 3). The MS1 full-scan mass spectrum displayed an abundant ion at *m/z* 765 which was 14 amu lower than that of vinorelbine. Subsequent LC–MS/MS analysis generated product ions at *m/z* 705 [M–$OCOCH_3$ or M–$COOCH_3$], 644 [M–2(–$COOCH_3$)–H],

Table 2
Molecular mass and molecular formula of vinorelbine and some metabolites

| | Molecular weight (Da) | Molecular formula | Accurate mass found | Accurate mass calculated |
|---|---|---|---|---|
| Vinorelbine | 778 | $C_{45}H_{55}N_4O_8$ | 779.4030 $[M+H]^+$ | 779.4020 $[M+H]^+$ |
| DVRL (M1) | 736 | $C_{45}H_{55}NO_8$ | 737.3922 $[M+H]^+$ | 737.3928 $[M+H]^+$ |
| VRL 3,6-ether (M2) | 776 | $C_{43}H_{53}N_4O_7$ | 777.3874 $[M+H]^+$ | 777.3863 $[M+H]^+$ |
| HydroxyVRL isomer #2 (M3) | 794 | $C_{45}H_{55}N_4O_9$ | 795.3970 $[M+H]^+$ | 795.3969 $[M+H]^+$ |
| DesmethylVRL (M4) | 764 | $C_{44}H_{53}N_4O_8$ | 765.3870 $[M+H]^+$ | 765.3863 $[M+H]^+$ |
| HydroxyVRL isomer #1 (M5) | 794 | $C_{45}H_{55}N_4O_9$ | 795.3974 $[M+H]^+$ | 795.3969 $[M+H]^+$ |
| VRL 6′-oxide (M9) | 794 | $C_{45}H_{55}N_4O_9$ | 795.3980 $[M+H]^+$ | 795.3969 $[M+H]^+$ |

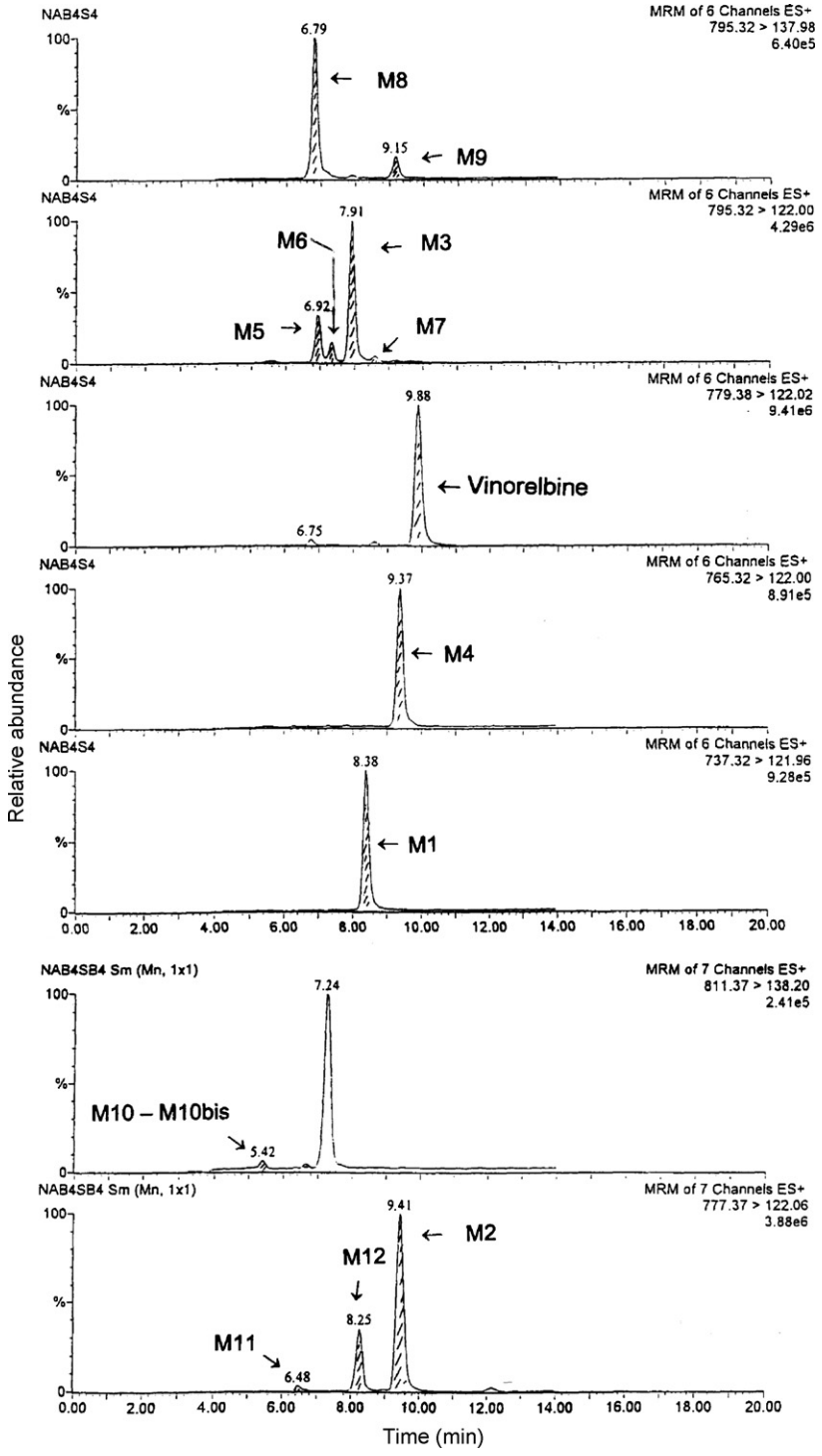



Fig. 3. ESI-MS/MS of vinorelbine and its metabolite in human faeces.
Peak at Rt 7.24 is vinblastine, used as internal standard for LC–MS/MS quantification [16]. Not discussed in this manuscript.

323 and 122 (Fig. 4). After ToF analysis, this peak produced an $MH^+$ ion of 765.3870. This generated an isotope fit value (iFIT) of 2.6 for the elemental composition $C_{44}H_{53}N_4O_8$ and a mass error of 0.9 ppm. This information combined with shifted ions (e.g. *m/z* 443, 496 and 644) and unshifted signals at *m/z* 122 and 323 allowed to postulate on a loss of a methyl group either on N-1 or C-16 resulting from an oxidative *N*-dealkylation or *O*-dealkylation process.

The iFIT value is calculated by the manufacturer's software. The software compared the isotope pattern of the obtained data with that predicted using an isotope model employing known isotopic abundance values. This allows an extra dimension of confidence [24].

M3 and M5 both gave $[M+H]^+$ ions at *m/z* 795 as the most abundant ion (*i.e.* 16 amu higher than that of vinorelbine). Both compounds were identified with specific 795-122 transition in

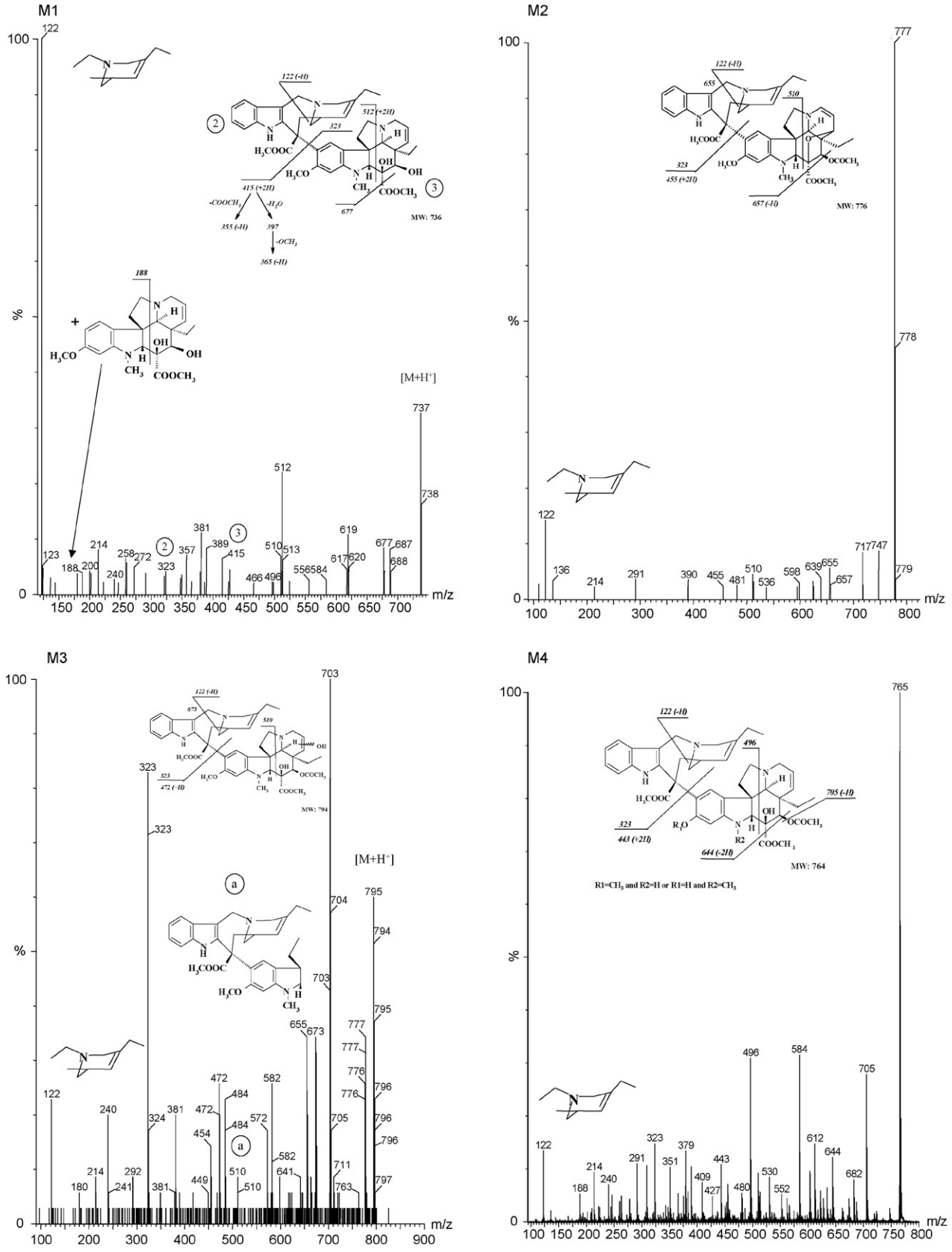



Fig. 4. ESI-MS/MS spectra of vinorelbine metabolites M1–M5 in human.

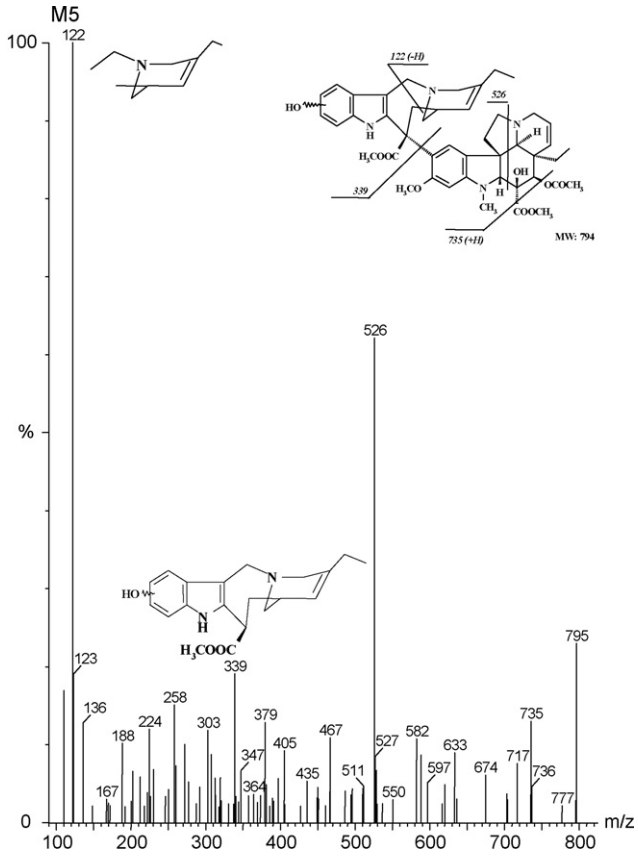



Fig. 4. (*Continued*).

MRM mode (Fig. 4). These two metabolites eluted at RRT of 0.76 and 0.68, respectively (Fig. 3). The 16 Da shift could only be attributed to an additional oxygen atom (as shown in Table 2). For M3, fragments at *m/z* 472 (also 473) and 510, as well as the absence of *m/z* 526 ion were consistent with the hydroxylation onto the vindoline moiety (Fig. 1). In addition, both MRM transitions: 526-122 and 510-122 were screened; only the latter provided an ion chromatographic signal. The most relevant proposed structure was a hydroxyl function located on one of the two carbon atoms linked to the original double bound at C-6/C-7. This C-6/C-7 hydroxylated product could lose a molecule of water which could by cyclisation induce the formation of M2 previously identified as vinorelbine 3,6-ether. The exact position of the hydroxyl group could not be fully ascertained.

The most likely structure of M5 involved the addition of a hydroxyl group on the aromatic ring of the nor-7′-velbanamine moiety. This resulted from the full-scan mass spectrum (Fig. 4) which exhibited a specific fragment ion located at *m/z* 339 (*i.e.* 16 amu greater than *m/z* 323) with an unchanged *m/z* 122 ion and most of the unchanged ions corresponding to the vindoline substructure. The presence of *m/z* 188 ion, which was also observed in the fragmentation of VRL and shown on the fragmented spectrum of DVRL, might aimed at excluding an oxidation on N-1. In the absence of NMR confirmation, M3 and M5 were, respectively labelled as hydroxyvinorelbine isomer #1 and isomer #2.

The identity of M8 and M9 were definitely matched with their respective chemical references. M8 and M9 both gave $[M+H]^+$ ions at *m/z* 795, whereas the MRM detection was *m/z* 795-138. The +16 Da shift attributed to an oxygen atom excluded a hydroxylation on the aromatic rings. M8 (RRT 0.62) was 20′-hydroxyvinorelbine and M9 (RRT 0.96) was vinorelbine-6′-oxide.

### *3.1.3. Minor drug-related peaks*

M6 and M7 were traced by means of the 795-122 transition in MRM mode. Two very weak peaks were detected at RRT 0.71 and 0.88, respectively. The product ion spectrum of M6 gave low abundant ions at *m/z* 735 [M–(–$OCOCH_3$)], 323, 472 and 122. Based on a fragment ion at *m/z* 472 (*i.e.* 456 + 16), the additional oxygen-related shift was tentatively attributed to an oxidation reaction occurring onto the vindoline moiety. Hence, either a hydroxylation occurring on the aromatic ring or an oxidation on N-1 that will consider the signal recorded at *m/z* 526 in the product ion spectrum of mass 795 could be hypothesised. Metabolite M7, found in bile samples only, was tentatively assigned as an alcoholic derivate on C-20. This would be in accordance with the characteristic signals corresponding to an unchanged velbanamine substructure which appeared unaffected as demonstrated by the product ions at *m/z* 122, 510 and 389 (as 510-122) (Fig. 1). Nevertheless, in the absence of NMR confirmation, M6 and M7 were putatively labelled hydroxyvinorelbine isomer #3 and isomer #4, respectively.

M11, M12 and M13, eluted at 0.66, 0.84 and 1.38 of RRT, respectively, all gave $[M+H]^+$ ion at *m/z* 777 (*i.e.* 2 U lower than that of VRL). The abundance was too low to generate full-scan mass spectra. Their actual presence was even difficult to

confirm as well as their involvement in the metabolic pattern of VRL. The only way to ascertain their presence was through the typical and specific MRM transition between *m/z* 777 and 122. The detection of adequate chromatographic profiles combined with the absence of any measurable peaks at the corresponding RRT values in drug free biological matrices were determinant to match these peaks with metabolites. These 3 compounds were designed as "metabolites 777 (a), (b) and (d)", respectively. Note that metabolite 777 (c) was previously and definitely assigned to M2.

The five following metabolites involved sequential pathway step: M10 (RRT 0.54), M14 (RRT 0.69), M15 (RRT 0.83), M16 (RRT 1.23) and M17 (RRT 0.49). They were produced at trace levels in the elimination media (faeces or bile). The full-scan mass spectrum of M10 showed an ion at *m/z* 811 (*i.e.* 32 U greater than that of VRL), consistent with a di-oxidation (either hydroxylation or *N*-oxidation) process.

The MRM transition 811–138 suggested an oxidation of either M8 or M9. In the absence of a formal structure elucidation of the oxidative process, M10 was tentatively identified as hydroxy M8 or hydroxy M9.

M14, M15, M16 and M17 were assigned to metabolites of VRL from analysis performed in the product ion spectrum. A parent ion analysis was first conducted on the highly specific 122 and 138 product ion species that provided the respective $MH^+$ values of these four products. M14 gave a positive signal to the transition *m/z* 723-122; thus suggesting the hypothesis of an oxidative de-alkylation on N-1 or C-16 reaction occurring on metabolite M1 (DVRL). M15 that MRM transition was 763-122 was likely a de-alkylation process from M2. M16 (763-122) was consistent with another de-alkylation pathway. From its RRT (longer then VRL but more polar than M13), it is reasonable to speculate that M16 may have been formed by metabolism of M13. The pseudo molecular ion of M17 was *m/z* 753 (*i.e.* 16 U greater than that of M1). In addition, its detection was based on 753-138 transition. Therefore, this metabolite was tentatively identified as 20′-hydroxy DVRL.

Neither glucurono- nor sulfo-conjugate of vinorelbine could be detected in faeces on basis of neutral loss scans performed on either 176 or 80 Da shift, respectively. Further direct proof of the absence of conjugates was evidenced after enzymatic hydrolysis of urine and bile samples. No difference on the signals associated with metabolites M3, M5–M8 and M10 was observed before and after hydrolysis.

## 4. Discussion

Although vinorelbine has been widely used for several years, its metabolic profile was scarcely investigated. In fact, a few metabolite structures have been reported in literature from the series of vinca-alkaloids. They resulted from oxidation of vindoline moiety and were mainly isolated after microbial transformation of vindoline [2,19]. The only relevant publication for metabolite identification in vivo was those from Yamaguchi et al. who definitely elucidated five metabolites from hydroxylation and oxidation pathway in rat. Nevertheless, neither these structures nor others have been reported in human. This consisted therefore on a novelty to characterise the pharmacokinetic behaviour of vinorelbine. The present study has demonstrated that vinorelbine has a diversified and important metabolic pathway. Seventeen metabolites were identified (13 structures were proposed) and the data were listed in Table 1. Twelve metabolites were directly formed from VRL as a one-step metabolism reaction, while five others involved a sequential step pathway (Fig. 5). Whatever is the biological medium, the major metabolites were definitely M1 to M5. No glucuronide or sulfate conjugates were observed.

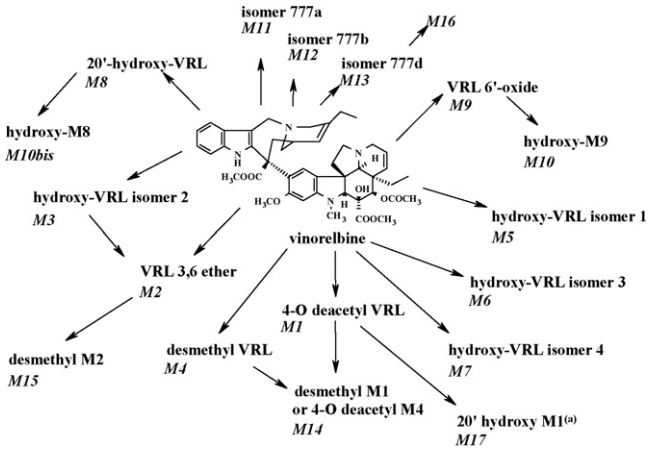



Fig. 5. Proposed metabolic pathway of vinorelbine in human.
(a): this metabolite can also result from M8 biotransformation.

The analytical process used for the metabolite identification has been thoroughly discussed elsewhere [16]. Briefly, ESI detection in the positive ion mode was selected for its high degree of sensitivity as it could be demonstrated on other related vinca-alkaloids such as vinblastine and vincristine [17]. If solid-phase extraction was selected for its ability to produce clean samples, deproteinisation led to standardise the sample preparation from all biological media. Both samples pretreatment produced approximately 90% quantitative extraction recovery for the metabolic pool of VRL [14] which was a key issue for a complete metabolic profiling.

Formation of these metabolites can be explained by four proposed pathways: (1) the *O*-deacetylation of the vindoline moiety (M1), (2) the hydroxylation on aromatic rings (M3, M5–M8), (3) the *N*-oxidation (M9) and oxidative ether formation (M2) and (4) the dealkylation on the vindoline moiety (M4). All these enzymatic reactions generated primary metabolites that were good substrates for the formation of further biotransformed products (M10, M14–M17).

The specificity and sensitivity, which characterised the LC–MS/MS method, enabled to detect DVRL (M1) in all biological media (see Fig. 3 and Table 1), therefore confirming the clinical findings on plasma and blood [5,12,13]. Enzymatic oxidation was highly suspected to be involved in the biotransformation of VRL to M2 as reported for vindoline or vinblastine [18]. Similar structures were observed with other vinca-alkaloids [2,19]. The ether derivate M2, previously identified as vinorelbine-epoxide according to the biogenetic numbering [10] was also known to be a degradation product of

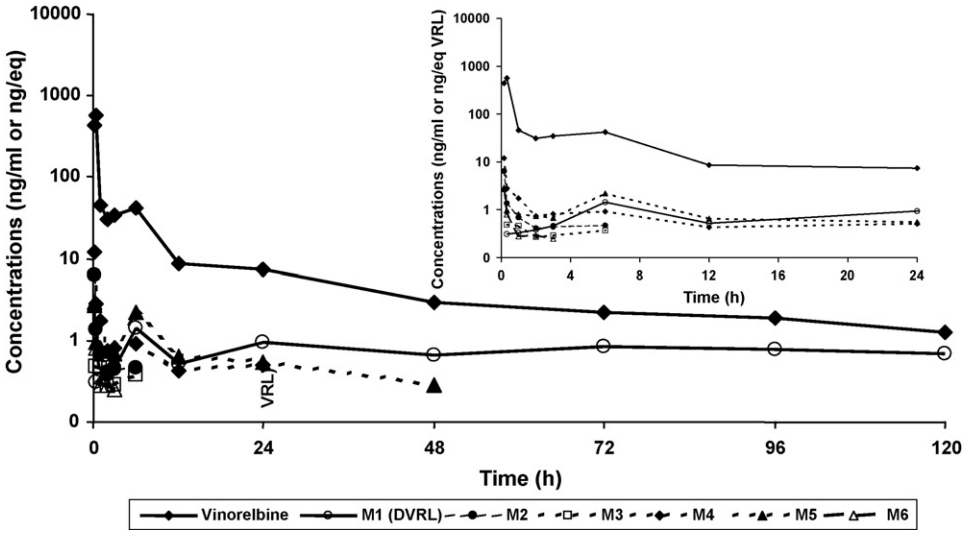



Fig. 6. Blood pharmacokinetic profile for vinorelbine and its metabolites.
Representative blood profile for vinorelbine and its metabolites. Concentrations are expressed on log scale (enlarged scale up to 24 h). Concentrations of metabolites are expressed as VRL equivalents.

VRL. Dealkylation postulated by a 14 Da shift led to the production of four metabolites of VRL, mainly those formed by sequential step pathway. This enzymatic reaction was previously identified in a study performed on human liver microsomes [9]. Vinorelbine 6′-oxide (M9) was scarcely observed (Figs. 3 and 5). This finding constituted a key issue in the controversial information about its contribution to the metabolism of vinorelbine [20,21]. Hydroxylation appeared to be a prominent pathway in human, generating at least six metabolites. M8 definitely identified from its chemical reference and two others have previously been identified in rat bile: M5 (as two isomers at C′-12 and C′-13) and M10 were found in both studies [10].

All but four structures were either elucidated or tentatively identified. For the four remaining metabolites (*i.e.* M11–M13 and M16), additional experiments conducted with a time-of-flight (ToF) mass spectrometer used to determine the accurate mass of the metabolites were not sufficient to confirm the structure of these compounds. Nevertheless, these metabolites were only detected in the elimination media and at trace levels and DVRL was the only metabolite that has shown at least the same activity of VRL. The metabolic pool consisting of all metabolites but DVRL was definitely pharmacologically inactive even with concentrations 4- to 10-fold higher than those of combined VRL and DVRL [22]. This was the main reason to argument to leave behind the remaining unanswered structural issues.

The enzymatic pathways involved in the metabolism of VRL supported the role of cytochrome P450. This was consistent with in vitro studies showing that CYP3A4 was the main isoenzyme involved in the metabolism of VRL [9,23].

The application of similar analytical conditions to those setup for the reference HPLC-UV method previously developed for the quantification of VRL and DVRL [5] from the clinical studies greatly facilitated the process required to match the unknown peaks with the new identified metabolites. Thus, previously observed P6 was M5, P9 was DVRL and P11 was M2 and M4.

Subsequent quantitative analysis was performed for VRL and of all these metabolites using MRM transition mode with typical and common fragment ions at *m/z* 122 and 138 [16]. Vinorelbine was the major compound observed in all tested biological specimen. In blood, a total of eight metabolites were detected (Table 1), all of them being quantified at low concentrations or at trace levels. All the metabolites were rapidly formed and cleared from blood except M1 (DVRL) which peaked lately (Fig. 6). In the elimination media, five metabolites were detected in urine whereas all 17 metabolites were observed in faeces and bile (Table 1).

In summary, this work constituted the first extensive structural identification of vinca-alkaloids from human biological fluids peculiarly bile or faeces that showed a large number of peaks. This work will improve the knowledge of the clinical pharmacokinetics of vinorelbine. Vinorelbine was metabolised in human via four primary metabolic pathways including deacetylation, hydroxylation, dealkylation and oxidation and by subsequent generation of secondary metabolites. The main products corresponded to DVRL, VRL 3,6-ether, desmethyl VRL and hydroxyl VRL isomers 1 and 2. All others were detected at trace levels in the biological media.

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