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Structural Characterization of Mono- and Dihydroxylated Metabolites of Paclitaxel in Rat Bile Using Liquid Chromatography/Ion Spray Tandem Mass Spectrometry

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The capability of high performance liquid chromatography/ion spray mass spectrometry (HPLC/ISP-MS) and HPLC/ISP-tandem mass spectrometry (HPLC/ISP-MS/MS) were investigated to achieve mass separation as well as structural characterization of taxol metabolites directly in rat bile, without their previous isolation. HPLC/ISP-MS yielded information on the molecular weights of several hydroxylated derivatives while HPLC/ISP-MS/MS allowed the on-line structural characterization of all metabolites present in different ratios in rat bile.

The approach led to the extraction of nine metabolites and their distinction from the other endogenous contaminants. These metabolites were recognized as three dihydroxytaxols, four monohydroxytaxols, one deacetyltaxol and one containing the taxane ring. Among the derivatives, we were able to identify four new metabolites of paclitaxel belonging to the dihydroxy and monohydroxy series, never previously detected. HPLC/ISP-MS/MS enabled the classification of all di- and monohydroxy isomers.

These results demonstrate that the high sensitivity of this method, based on the combined use of tandem mass spectrometry with chromatographic separation, can be considered as offering a valid approach to the detection of new taxol derivatives directly in biological fluids. © 1997 by John Wiley & Sons, Ltd.

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Paclitaxel (taxol ®), tax-11-en-9-one,5β, 20-epoxy-1,2α, 4,7β, 10β, 13α-hexahydroxy-4,10-diacetate-2-benzoate-13-(α-phenylhippurate), Fig. 1(a), is a taxane diterpene amide, that was first extracted from the stem bark of the western yew, *Taxus brevifolia* and identified as a potential antineoplastic agent in 1971.[1] This natural product has proven to be one of the most promising anticancer agents now available. Several clinical studies have been performed in a variety of human neoplastic diseases, including the treatment of ovarian cancer, breast cancer, lung cancer, head and neck cancer.[2–6]

Due to the potency of paclitaxel, there are high demands for the development of an analytical method which is supportive of pharmacokinetic and metabolic studies.[7,8] To this end, several high performance liquid chromatography (HPLC) assays have been already reported in different biological matrices. In particular Monsarrat *et al*. found nine metabolites in rat bile using HPLC analysis.[9,10] Three compounds have been isolated by semi-preparative HPLC columns and structurally characterized by NMR analysis and mass spectrometry, using desorption chemical ionization (DCI) and fast atom bombardment (FAB) as ionization methods. The major metabolites obtained were two hydroxytaxol isomers, one exhibiting the hydroxyl group on the *para* position of the phenyl function at position C-3' of the side chain, the other at the *meta*-position of the benzoate group at the C-2 atom of the taxane ring. Other minor metabolites have been characterized using the protonated molecule mass values obtained by FAB mass spectrometry; one of these appeared to be Baccatin III. In the analytical study reported by Rizzo *et al.* one potential metabolite has been detected but not identified in human plasma and urine after liquid–liquid extraction followed by solid-phase extraction.[11] With the introduction of a new sensitive HPLC technique, described by Huizing *et al*.,[12] which employs photodiode array detection (PDA) combined with a solid-phase extraction, eleven possible metabolites were found in plasma. Three of them had the paclitaxel chromophore and hydroxylation was suggested to play an important role in their metabolism.

Recently mass spectrometry has been recognized as the most appropriate analytical technique to perform structural studies on paclitaxel molecules because the drug and all its detected derivatives follow a specific fragmentation pathway which cleaves the molecules into two major fragment ions, considered as markers in biological fluids. It is possible to take advantage of this mass spectrometric behavior to establish the main features of taxol metabolism, because the hydroxylation process takes place on these two major fragment ions.

While ionization techniques such as DCI, FAB and electrospray (ES) ionization need a separation procedure to isolate the various taxane derivatives,[13–15] the direct coupling of the chromatographic device to the mass spectrometer allows the identification of major

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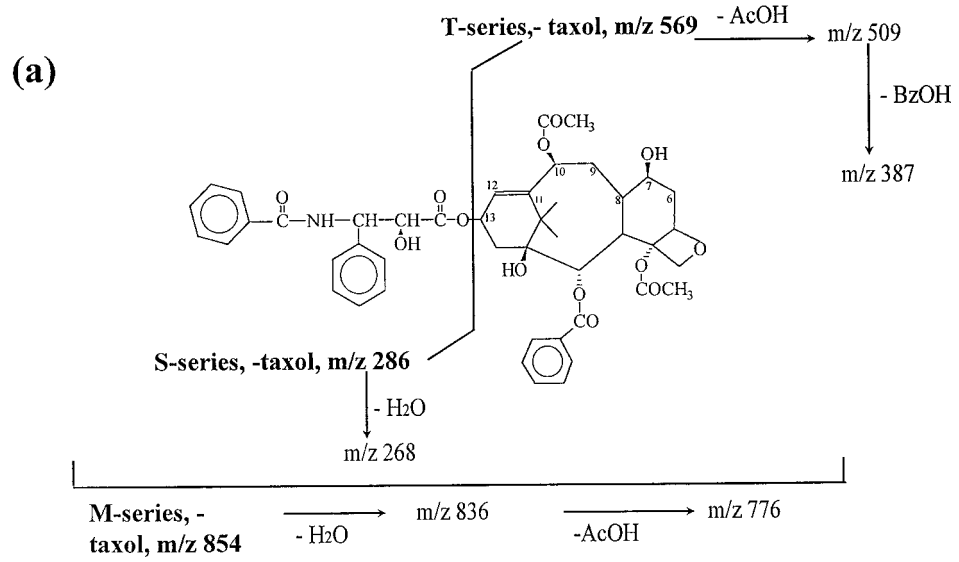
metabolites. Monsarrat *et al.*[16] described a new method, developed for the analysis of seven separated compounds from different biological samples, such as cell culture medium, plasma, bile and urine. This method can be applied for the analysis of human plasma and urine and recognizes the 6α-hydroxypaclitaxel in biological fluids on the basis of its retention time.

The aim of the present study was to perform the structural characterization of paclitaxel metabolites directly in rat bile using a chromatographic separation directly linked to mass spectrometry without performing a separation process of every compound by semi-preparative HPLC column. The capacity for discrimination between taxol derivatives using PHLC/ion spray (ISP)-MS as well as HPLC/ion spray-tandem mass spectrometry (MS/MS), which provides extreme selectivity, was investigated. These analyses were performed directly on the entire eluent from a conventional microbore HPLC column. HPLC/ISP-MS had the potential to yield information on molecular weights of several hydroxylated derivatives and HPLC/ISP-MS/MS allowed the immediate structural characterization of all these metabolites, present in different proportion in rat bile, without the traditionally used solid-phase extraction procedure that can compromise the detection of hydrophilic compounds, such as sulfated and glucuroconjugated derivatives.

EXPERIMENTAL

Reagents and chemicals

Paclitaxel was used as supplied by the Bristol-Myers Squibb Company (Wallinford, CT, USA). Ammonium acetate was of analytical grade and purchased from E. Merck (Darmstadt, Germany). Acetonitrile of HPLC



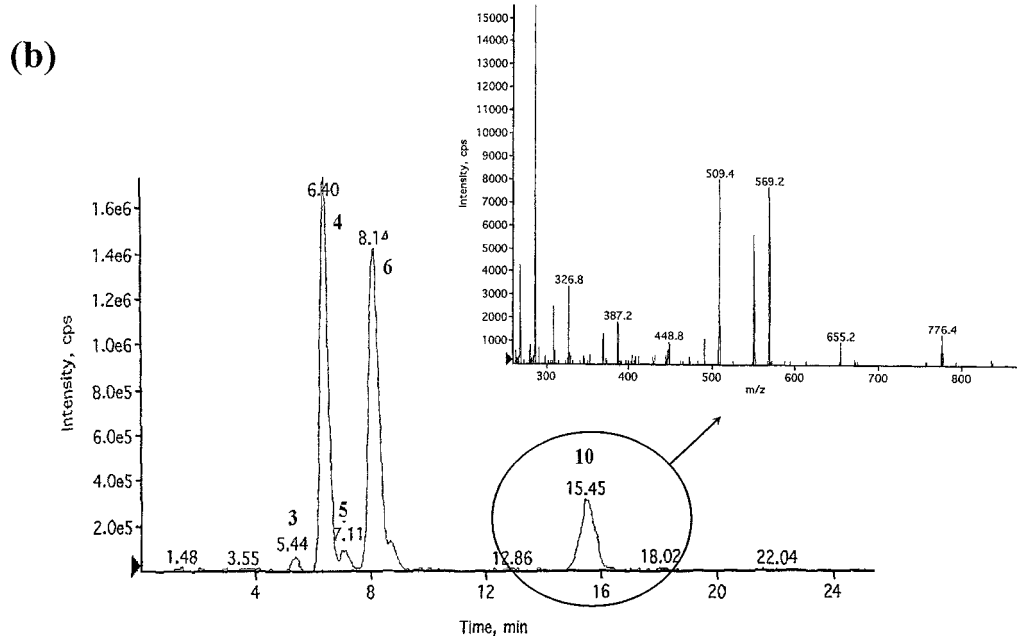


Figure 1. (a) Scheme of fragmentation pattern of taxol (peak 10), (b) CAD spectrum of taxol obtained from the selected molecular ions at *m/z* 870.4 and 871.4 (E_{lab} 30 eV; collision gas nitrogen, 8 mTorr).

grade was obtained from Carlo Erba, Milan, Italy. Filtered and deionized water, obtained from a Milli-Q Plus system (Millipore, Milford, MA, USA), was used throughout the study.

Treatment of animals

Male Sprague–Dawley rats (CD-COBS, Charles River, Italy) were used for determination of drug biliary excretion. Rats were anaesthetized with 3.5 mL/kg Equithesin and the bile duct was externalized and cannulated with a medical grade clear polyethylene tube (0.5 mm i.d.), and a control bile sample was collected from each rat for 10 min. before drug administration. Two rats were then injected via the caudal vein with taxol (30 mg/kg) and bile was collected continuously for 3 hr after treatment. Due to the low solubility of taxol in water, taxol was dissolved in cremophor + ethanol + 5% glucose in water (1 : 1: 8; v : v : v).

Sample pretreatment

The bile was collected every hour from $\frac{1}{2}$ hr to 3 hr and four samples were labeled A, B, C and D. An aliquot of 300 μL was picked up from every sample, diluted with acetonitrile 1 : 1 (v : v) and then centrifuged (13 000 × g). The supernatant liquid was evaporated to dryness under nitrogen.

The residue, dissolved in 100 μl using 0.02 M ammonium acetate buffer and acetonitrile 65:35 was directly injected into the microbore column of the liquid mass spectrometer, protected by an in-line pressure filter (Perkin Elmer, Norwalk, CT, USA). The entire effluent from the column of the control and pooled bile samples were analyzed by mass spectrometry.

The same samples, labeled A, B, C and D, were then extracted following the solid-phase extraction (SPE) procedure, as reported by Willey *et al.*[17] which employs Sep-pak-CN-cartridge columns (Waters Associates, Milford, Massachusetts, USA). They were analyzed by HPLC/ion spray-tandem mass spectrometry and compared to the other non-extracted samples in order to establish the possible loss of hydrophilic metabolites, e.g. sulfated and glucuroconjugated derivatives. To this end, aliquots of bile diluted in 0.9% NaCl, were incubated for 3 hr with *B*-glucuronidase (*Escherichia Coli*, type VII from Sigma Chemical Co (St Louis, MO, USA), 5000 units mL, final and with aryl-sulfatase (*E. Coli*, type VII from Sigma) 200 units mL, final. These samples were analyzed under the same conditions to exclude the production of such taxol derivatives during the metabolic process.

High-performance liquid chromatography/ISP-tandem mass spectrometry

All HPLC/MS and HPLC/MS/MS experiments were performed on a triple quadrupole mass spectrometer API 300 (Perkin Elmer-Sciex, Toronto, Canada), operating in the positive-ion mode. The instrument was equipped with an atmospheric pressure ionization (API) source and with an ion spray interface. An Apple™ Macintosh® System 7.5.3 was used for instrument control, data acquisition and data processing. Polypropylene glycol was used to calibrate the instrument and to adjust the resolution of 0.7 mass unit over the *m/z* range 800–1200 for HPLC/MS spectral acquisition and one mass unit over the *m/z* range 260–900 for HPLC/MS/MS spectral acquisition.

The instrument was interfaced to an HPLC System (Perkin Elmer, Norwalk, CT, USA), including a Series 200 LC quaternary pump. The analyses of rat bile samples 3 h after the beginning of the experiment were achieved on a 15 × 1.0 mm i.d. column packed with 5-μm Vydac 218 TP stationary phase (Separation Group, Hesperia, CA, USA) equipped with an in-line pressure filter assembly of 2 μm porosity. The mobile phase used for the chromatographic separation was 0.02 M ammonium acetate buffer (pH = 5.0) and acetonitrile 65 : 35 (v : v) at a flow rate of 70 μL/min under isocratic conditions, with an injection volume of 5 μL.

The rat bile samples were analysed by HPLC/MS using the ionspray needle operated at +5600 V and the cluster-breaking orifice voltage at 28 V. The full-scan mass spectra were obtained while continuously scanning from *m/z* 800 to 1200 with a step size of 0.1 u and a dwell time of 0.5 ms.

Product-ion HPLC/MS/MS scans were performed on the same rat bile samples and the MS/MS measurements were based on collision-activated dissociation (CAD) experiments made through the closed-design Q2 collision cell operating with a collision energy (E_{lab}) of 30 eV, using nitrogen as collision gas at a pressure of 8 mTorr (1 Torr = 133.3 Pa). The MS/MS mass spectra were obtained with a step size of 0.2 u and dwell time of 0.5 ms.

RESULTS AND DISCUSSION

Presence of several mono- and dihydroxy metabolites of taxol in rat bile

A separation method, based on reverse-phase HPLC/atmospheric pressure chemical ionization (APCI) mass spectrometry has been previously developed[16] using seven reference taxol derivatives considered as major drug metabolites selected from different biological matrices, such as bile, plasma, urine and cell-culture medium. This pool of taxoid compounds, including epitaxol, has been analyzed and the full APCI/MS spectra have been reported. Human plasma and urine samples have also been analyzed and 6α-hydroxytaxol and epitaxol identification was made by comparing their chromatographic retention times and their APCI/MS spectra with those of authentic reference compounds.

In the present work, the capability of HPLC/ISP-MS and HPLC/ISP-MS/MS to perform mass separation as well as structural characterization of taxol metabolites directly in rat bile, without a prior isolation of every drug derivatives, was investigated.

HPLC/ISP-MS/MS, due to its extreme selectivity, enabled the structural analysis of all taxol derivatives, including both major and minor metabolites. This method, allows one to identify which part of the molecule is involved in the phenyl hydroxylation process, representing the most relevant metabolic process.

Taxol is essentially composed of two parts, the side

chain S- at C-13 and the taxane ring T- that produce, under mass spectrometric analysis, the S- and T-series fragment ions as previously observed by Blay *et al.*[18] and as is shown in Fig. 1. The most important S-series fragment ions, which form by neutral loss from the protonated molecule of taxol, are characterized by the *m/z* values 286 and 268. The intense T-series fragment ions, corresponding to the taxane ring, have *m/z* values of 569 and 509. This fragmentation pattern has been used to detect the presence in rat bile of nine metabolites of taxol.

Figure 2(b) (HPLC/ISP/MS) shows the relative retention times (min.) of the nine peaks present in bile samples obtained without any extraction step in order to detect hydrophilic metabolites. They were: Peak 1(4.33), 2(4.92), 3(5.44), 4(6.21), 5(7.40), 6(8.33), 7(9.14), 8(10.10), 9(13.03), 10(15.05). These peaks were undetectable in the control bile sample, as shown in Fig. 2(a). Peaks 1, 2 and 8 have been recognized as due to dihydroxylated taxol derivatives with the same molecular weight (at *m/z* 886.6). In Figs 3(b), 3(c) and 3(d) the related MS/MS spectra are reported. The peaks 3, 4, 5 and 6 correspond to monohydroxylated taxol derivatives with the same molecular weight at *m/z* 870.6 (see TIC scan, Fig 3(a)). Figure 4(a) shows the TIC scan of the *m/z* 870.4 and 871.4 ions. The corresponding MS/MS spectra are reported in Figs 4(b), 4(c) and 5(a), 5(b). We established that among the dihydroxylated derivatives, peak 2 corresponds to a new metabolite with two hydroxyl groups, both on the side chain, S-, as shown in Fig. 6. This feature is particularly important because the structure–activity studies with taxol emphasize the importance of the side chain and its hydroxylation at C-13 for biological activity.

Among these metabolites the peak numbered 7 was in agreement with deacetyl-taxol characterized by the protonated molecular ion $[MH]^+$ of 812, peak 9 corresponded to a derivative which possess the fragment ions of the taxane ring (*m/z* 569, 509) whose structure was not determined and the peak numbered 10 corresponded to unmodified taxol. None of the other peaks can be assigned to hydrolytic metabolites or baccatin III. None of the chromatographic peaks, which exhibited molecular ions corresponding to glucuroconjugated or sulfated derivatives in the solvent front showed the characteristic fragment ions of the taxane ring under MS/MS conditions; for this reason, these metabolites can be excluded from the metabolic pathway in rat bile.

Combined LC/MS/MS analyses for the structural characterization of the three dihydroxylated derivatives of taxol in rat bile; peaks 1, 2 and 8

The three peaks, 1(4.33), 2(4.92) and 8(10.10) reported in the total ion current (TIC) chromatogram (Fig. 2(b)) of rat bile samples, have the same MH^+ peak, at *m/z*

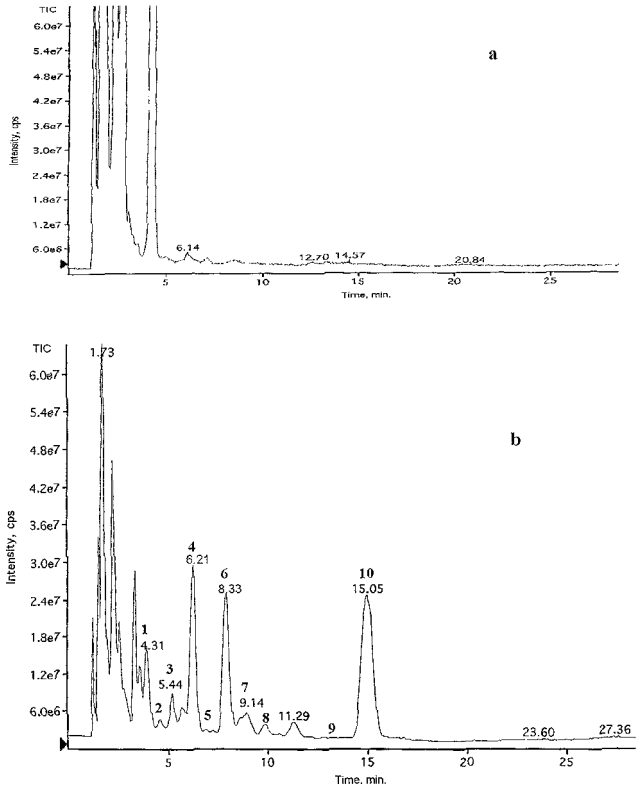


Figure 2. Analysis of paclitaxel metabolites in rat bile samples using HPLC/ISP-MS, with full scan analysis from *m/z* 800–1200. (a) TIC profile of untreated rat samples, (b) TIC profile of treated rat bile samples.

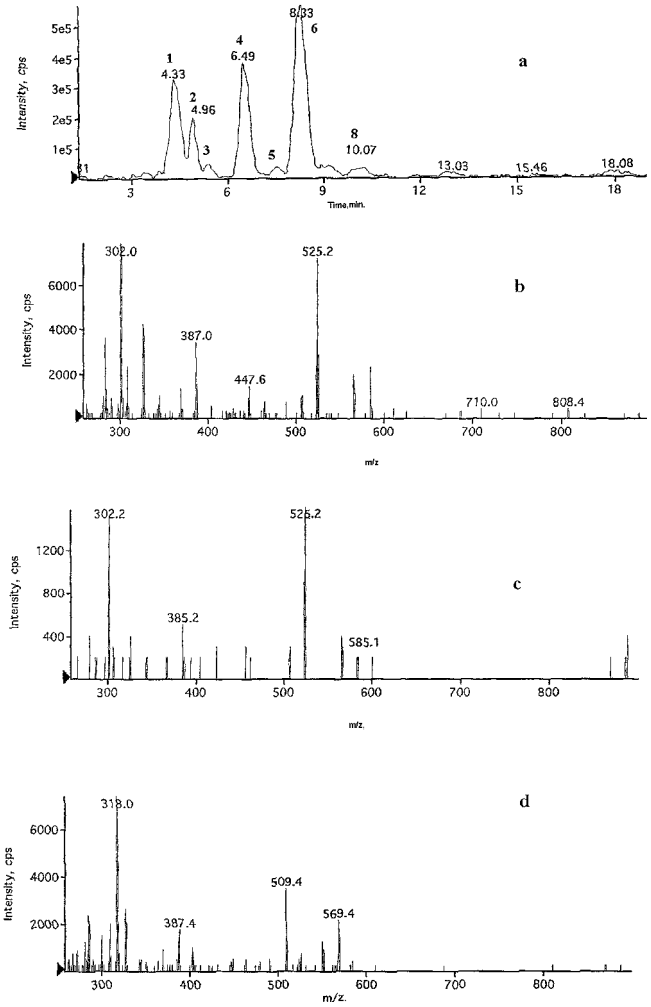


Figure 3. Analysis of mono- and dihydroxylated paclitaxel metabolites in rat bile samples using HPLC/ISP-MS/MS. (a) TIC profile of the selected molecular ions at *m/z* 886.4 and 887.4; (b) MS/MS spectrum of peak 1, (c) MS/MS spectrum of peak 8, (d) MS/MS spectrum of peak 2.

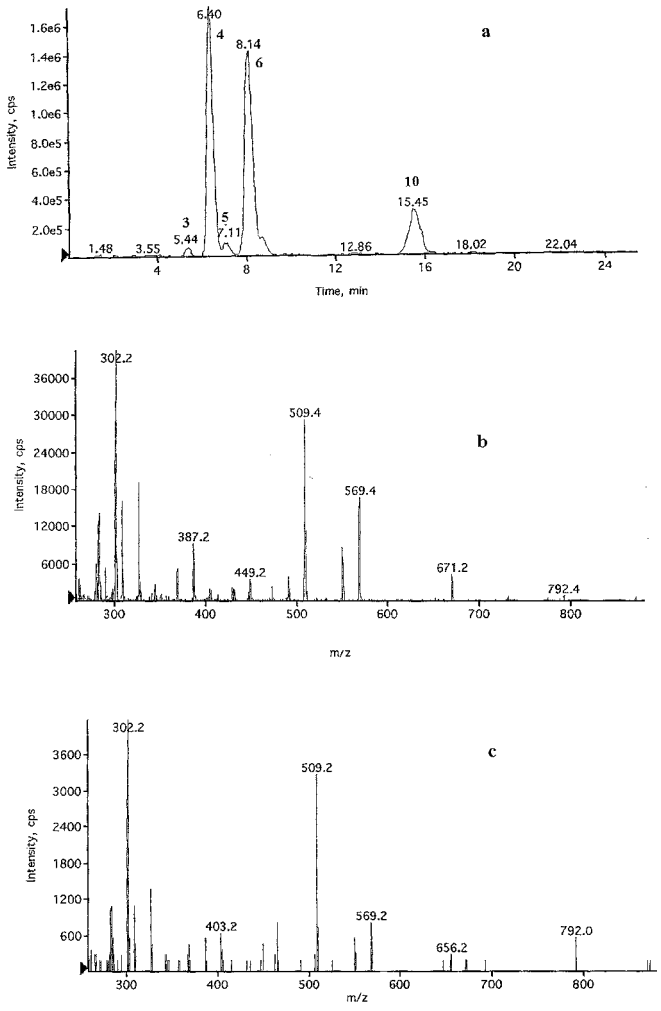


Figure 4. Analysis of dihydroxylated paclitaxel metabolites in rat bile samples using HPLC/ISP-MS/MS. (a) TIC profile of the molecular ions at *m/z* 870.4 and 871.4, (b) MS/MS spectrum of peak 4; (c) MS/MS spectrum of peak 5.

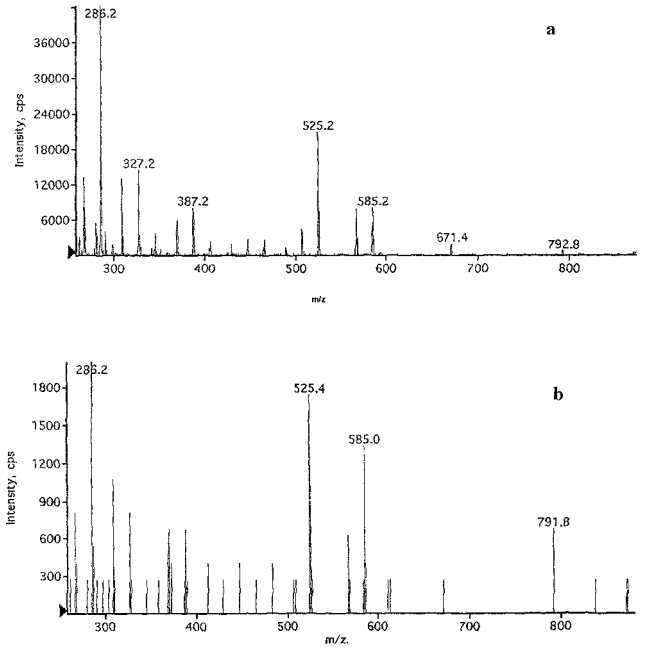


Figure 5. (a) MS/MS spectrum of peak 4; (b) MS/MS spectrum of peak 3.

886.6 with the related fragment ions at *m/z* 868.6 [MH $-$ H_2O]$^+$ and *m/z* 808 [MH $-$ AcOH $-$ H_2O]$^+$.

This molecular mass information establishes the presence of three isomeric compounds with molecular weight 32 mass units, larger than that of taxol. This observation is not, in itself, sufficient to establish that these compounds are dihydroxylated isomers structurally related to taxol molecule. In fact, the observation of an intense MH$^+$ ion, even if well separated from the chemical noise, is not enough to determine the presence of taxol analogs or the position of additional -OH groups. It is, however, possible to acquire the structural information details using the characteristic fragment ions in the lower mass range. The abundance of such fragment ions can be promoted by using higher orifice voltages or by using MS/MS experiments. The first approach can be used if a separated and purified mixture of metabolites is analyzed, as Monsarrat *et al.*[16] have been previously described; but it is impractical when a biological fluid containing several contaminants and endogenous products needs to be analyzed. In this latter case, only tandem mass spectrometric experiments (LC/MS/MS) permit one to explore the entire mass range of interest, from *m/z* 100 to 1200, because the selection of a specific precursor ion has been performed using the first mass analyzer.

The TIC chromatogram of the selected molecular ion at *m/z* 886.6, shown in Fig. 3(a), reports peaks 1 (Rt 4.33), 2 (Rt 4.92) and 8 (Rt 10.10) together with peaks 3, 4, 5 and 6 because this experiment detects simultaneously the ammonium adduct ions of the monohydroxylated metabolites (*m/z* 887.6), whose separated TIC chromatogram is reported in Fig. 4(a) and discussed in the next paragraph.

The collision-activated decomposition (CAD) experiments on the dihydroxylated metabolites produced the MS/MS spectra which are reported in Fig. 3(b), (c) and (d) respectively. The *m/z* values reported in these spectra are analyzed in comparison with the *m/z* values of unmodified taxol. The HPLC/MS/MS spectrum of taxol shows the characteristic fragment ions at *m/z* 569, 509 and 387 corresponding to the taxane ring T, T-AcOH, and T-AcOH-BzOH as shown in Fig. 1, where the fragmentation pattern is described. These ions relate to the T-series. The fragment ions at *m/z* 286 and 268 correspond to the side chain S and to (S-H_2O).

The HPLC/MS/MS spectrum of peak 1 shows T-series fragment ions and S-series fragment ions, both increased by 16 mass units, giving the characteristic ions at *m/z* 585, 525 for the T-series and at *m/z* 302 for the S-series. The observation of these fragment ions is consistent with a taxol analog having one hydroxyl group on the side chain and one on the taxane ring and called S–T-series-dihydroxytaxol (Fig. 6). The fragment ions appearing in the MS/MS spectrum of peak 1 are consistent with the already known 6α-3'-*p*-hydroxytaxol described in previous report.[21]

The HPLC/MS/MS spectrum of peak 2 shows similar T-series fragment ions to those of and an S-series fragment ions increased by 32 mass units over that of taxol, giving the characteristic ions at *m/z* 569, 509 for the T-series and at *m/z* 318 for the S-series. The observation of these fragment ions is consistent with a new taxol analog having two hydroxyl groups on the

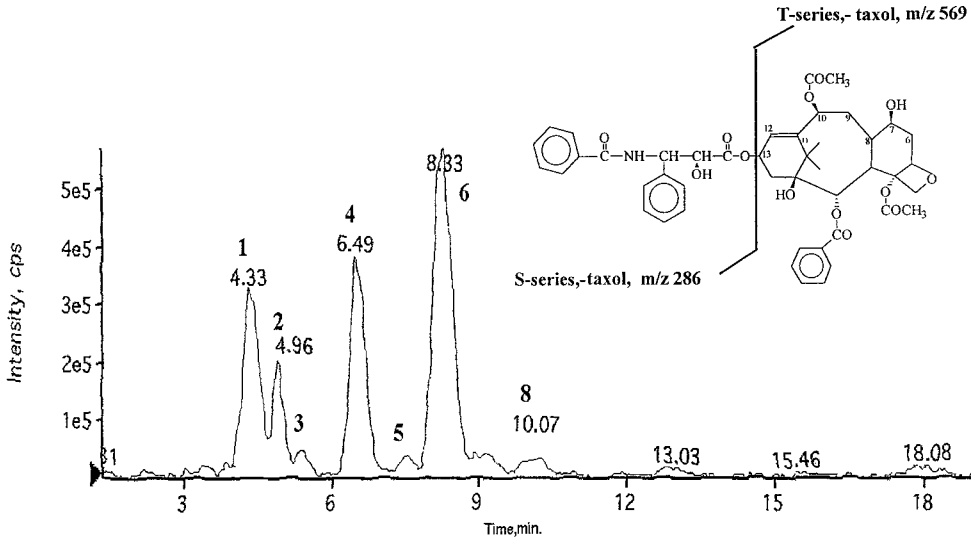
side chain and an unmodified taxane ring. This compound has been called S-series-hydroxytaxol (Fig. 6). The HPLC/MS/MS spectrum of peak 8 the same T-series fragment ions and S-series fragment ions as peak 1.

It is interesting to note that only the selectivity of this analytical approach makes possible the extraction of information on taxol metabolites from the multi-components present in the rat bile. All the data are summarized in Fig. 6.

Combined LC/MS/MS analyses for the structural characterization of the four monohydroxylated derivatives of taxol in rat bile; peaks 3, 4, 5 and 6

The four peaks, 3(5.44), 4(6.49), 5(7.40) and 6(8.33) appearing in the TIC chromatogram (Fig. 2(b)) of rat bile samples, have the same protonated molecular weight, MH^+, at *m/z* 870.6 with related fragment ions at *m/z* 852.4 $[MH\text{-}H_2O]^+$ and *m/z* 810.2 $[MH\text{-}AcOH\text{-}H_2O]^+$. These molecular ions were selected and CAD experiments were carried out. The TIC chromatogram of the hydroxylated compounds (*m/z* 870.6) is shown in Fig. 4(a). The peaks numbered 3, 4, 5 and 6 are reported together with peak 10 (15.05) because this experiment detects simultaneously the ammonium adduct of taxol (*m/z* 871.2). The HPLC/MS/MS spectra of peaks 4 and 5, reported in Fig. 4(b), (c) respectively, show T-series fragment ions similar to those of taxol and S-series fragment ions increased by 16 mass units, giving the characteristic ions at *m/z* 569, 509 for the T-series and *m/z* 302 for the S-series, as shown in Fig. 7. The observation of these fragment ions is consistent with a taxol analog having one hydroxyl group on the side chain and an unmodified taxane ring and called S-series-hydroxytaxol (Fig. 7).

The HPLC/MS/MS spectra of peaks 3 and 4, shown in fig. 5(a) and (b) respectively, show the T-series fragment ions increased by 16 mass units and S-series fragment ions similar to those of the taxol molecule, giving the characteristic ions *m/z* 585, 525 for the T-series and *m/z* 286 for the S-series. The observation of these fragment ions is consistent with a taxol analog



Peak number(Rt)	Compound	T-series-fragment ions	S-series-fragment ions	△ mass vs. taxol
metabolite **1** (Rt 4.33)	S-T-series-dihydroxytaxol (6α - 3'p- dihydroxytaxol)	585, 525	302	T-series +16 amu S-series +16 amu
metabolite **2** (Rt 4.96)	S-series-dihydroxytaxol	569, 509	318	T-series +0 amu S-series +32 a mu
metabolite **8** (Rt 10.07)	S-T-series-dihydroxytaxol	585, 525	302	T-series +16 amu S-series +16 amu
	taxol	569, 509	286	

Figure 6. Analysis of dihydroxylated paclitaxel metabolites — Table of main fragment ions of metabolites 1, 2 and 8 obtained by HPLC/tandem mass spectrometry, using CAD experiments (E_{lab} 30 eV; collision gas nitrogen, 8 mTorr).

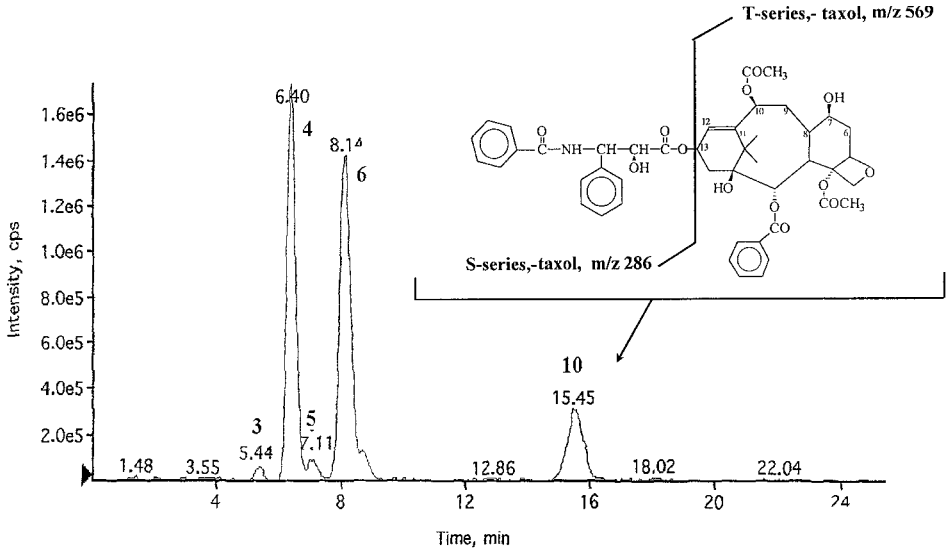
having one hydroxyl group on the taxane ring and an unmodified side chain (Fig. 7).

The fragment ions appearing in the MS/MS spectra of peaks 4 and 6 are consistent with the already known 3'-*p*-hydroxytaxol and 6α-hydroxytaxol, assuming that their sequence of elution is similar to that described in previous reports,[21] where these reference compounds were analyzed using same chromatographic conditions. The MS/MS spectra of peaks 3 and 5 have never previously been detected. They have been called T-series-, and S-series-hydroxytaxol respectively. All the data are summarized in Fig. 7.

The combined evidence from their retention times and the superimposable fragmentation spectra with those of compounds 4 and 6 strongly suggest that these taxol derivatives are isomers of *p*-hydroxyphenyl C_3 taxol and 6α-hydroxytaxol respectively. Confirmation was achieved by conducting additional HPLC/MS/MS experiments on rat bile samples heated to 140 °C for 70 hr, a procedure used to convert taxol into the 7-epitaxol isomer. In fact, previous reports[19,20] have suggested that epimerization at the C_7 position leads to a product less polar due to intramolecular hydrogen bonding between the 7α-hydroxyl group and the carbonyl oxygen at the C_4 position.

CONCLUSION

Taxol has been studied extensively both in the laboratory and in the clinic. However, its complete metabolic features in rats and humans have not yet been fully understood. Liver metabolism accompanied by an abundant hydroxylation mechanism has been mentioned as playing an important role in the disposition of the drug. Several metabolites containing the chromophore of unmodified taxol have been detected.[11,12] Recently the major metabolites have been isolated and purified from rat bile and human faeces and then



eak number(Rt)	Compound	T-series-fragment ions	S-series-fragment ions	△ mass vs. taxol
metabolite **3** (Rt 5.44)	T-series-hydroxytaxol	585, 525	286	T-series +16 amu S-series + 0 amu
metabolite **4** (Rt 6.40)	S-series hyroxytaxol (3'- p- hydroxytaxol)	569, 509	302	T-series + 0 amu S-series + 16 amu
metabolite **5** (Rt 7.11)	S-series-hydroxytaxol	569, 509	302	T-series + 0 amu S-series +16 a mu
metabolite **6** (Rt 8.14)	T-series hydroxytaxol (6α-hydroxytaxol)	585, 525	286	T-series +16 amu S-series + 0 amu
peak **10** (Rt 15.45)	taxol	569, 509	286	

Figure 7. Analysis of mono-hydroxylated paclitaxel metabolites — Table of main fragment ions of metabolites 3, 4, 5, 6 and taxol (peak 10) obtained by HPLC/tandem mass spectrometry, using CAD experiments (E_{lab}30 eV; collision gas nitrogen, 8 mTorr).

structurally characterized.[9,21] The introduction of the HPLC/APCI$^-$ MS method[16] allowed the detection of 6α-hydroxytaxol and 'new possible metabolites' in human plasma and urine. To this end a reinvestigation of the taxol derivatives in rat bile and human patients was suggested because it is very likely that they have escaped detection.

In the present report, we studied the metabolism directly in rat bile and achieved the structural characterization of several mono- and dihydroxylated metabolites, using a chromatographic separation combined with mass spectrometry without performing a separation of each component by semi-preparative HPLC column. We investigated the advantages of performing mass separation of taxol derivatives using HPLC/ISP-MS as well as HPLC/ISP-MS/MS, which provides extreme selectivity.

Only the collision-activated decomposition with tandem mass spectrometry permitted us to explore the mass range of interest in order to detect the chemical modifications and to assign them either to the S-side chain or the T-taxane ring of paclitaxel.

We concluded that the simple observation of intense-molecular ions, even if these are well separated from the chemical noise, is not enough to establish the presence of taxol analogs let alone their chemical modifications. Tandem mass spectrometry allowed us to extract from the rat bile information on the presence of nine metabolites and distinguish them from the other endogenous contaminants. These metabolites were recognized as three dihydroxytaxol, four monohydroxytaxol, one deacetyltaxol and one containing the taxane ring. With this methodology, we were able to identify four new metabolites of paclitaxel belonging to the dihydroxy and monohydroxy series, never previously detected.

Among the dihydroxytaxol derivatives, metabolite 1 confirmed the presence in rat bile of the already known 6α-3'-*p*-dihydroxytaxol, metabolite 8 was characterized as its isomer and metabolite 2 was recognized as a new compound called S-series-dihydroxytaxol due to the presence of two hydroxyl groups on the S-side chain of the unmodified taxol.

Among the monohydroxytaxol derivatives, two confirmed the presence of 6α-hydroxytaxol and 3'-*p*-hydroxytaxol (metabolites 4 and 6) and the other two were structurally characterized as two isomers of the above mentioned metabolites with superimposable MS/MS spectra and called S- and T-series-hydroxytaxol respectively. None of the other peaks can be assigned to hydrolytic metabolites or baccatin III. None of the chromatographic peaks which showed molecular ions corresponding to glucuroconjugated or sulfated derivatives showed the characteristic fragment ions of the taxane ring under MS/MS conditions; for this reason these metabolites can be excluded from the metabolic pathway in rat bile.

In conclusion, this study provides a highly sensitive analytical tool based on the selectivity of tandem mass spectrometry combined to a chromatographic separation to detect new taxol derivatives directly in biological fluids in order to study the effect of metabolism on the clinical activity of taxoids.

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