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In-vitro metabolism of isotetrandrine, a bisbenzylisoquinoline alkaloid, in rat hepatic S9 fraction by high-performance liquid chromatography–atmospheric pressure ionization mass spectrometry

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Abstract

The objective of this study was to investigate the in-vitro metabolism of isotetrandrine, a bisbenzylisoquinoline alkaloid, using rat hepatic S9 fraction and to profile and identify its metabolites using high-performance liquid chromatography–atmospheric pressure ionization mass spectrometry (HPLC-MS) and tandem mass spectrometry (MS/MS). Isotetrandrine was incubated at a concentration of 100 μg mL^{-1} with male rat hepatic S9 fraction in the presence of an NADPH generating system (Tris buffer, pH 7.4, 37 °C). Samples were removed at 60 min after reaction initiation. Unchanged isotetrandrine (~63% of the sample) and four metabolites were profiled, characterized and tentatively identified using solvent extraction, methyl derivatization, and HPLC-MS and MS/MS techniques. Isotetrandrine metabolites were mainly formed via two main pathways, *N*-demethylation and isoquinoline ring oxidation. The first pathway produced a major metabolite, *N*-desmethyl isotetrandrine (~16% of the sample). The second pathway produced three minor oxidized metabolites, hydroxy-isotetrandrine (~6% of the sample), oxo-isotetrandrine (~7% of the sample), and oxo-hydroxy-isotetrandrine (~7% of the sample). Diazomethane treatment of these metabolites did not produce any methyl derivatives and therefore the hydroxylated sites of the metabolites were tentatively assigned at the heterocyclic moieties of the isoquinoline rings. In conclusion, isotetrandrine is substantially metabolized in this in-vitro rat hepatic system.

Introduction

Natural products have provided an invaluable source of therapeutic agents and are actively pursued in drug discovery screening programmes. The bisbenzylisoquinoline alkaloid series is well known for its therapeutic activity. One of the most well known is d-tubocurarine, a cyclic bisbenzylisoquinoline used as a neuromuscular blocking agent during anaesthesia (Taylor 2001). Other naturally occurring bisbenzylisoquinoline alkaloids are: (i) tetrandrine, which has gained interest for its activity against the Walker carcinosarcoma; (ii) pheanthine, which possesses antitubercular activity; and (iii) isotetrandrine, isolated and identified previously from *Thalictrum sp.* (Ranunculaceae), which is considered an anti-inflammatory, analgesic, antimicrobial and immunosuppressive agent (Mitscher et al 1971; Bhakuni et al 1980; Cordell 1981; Schiff 1983). The structure of isotetrandrine is shown in Figure 1. It consists of two isoquinoline groups each attached to a benzylic group. The two aromatic rings of the isoquinolines are coupled by an ether linkage and the benzylic rings are attached by another ether linkage. A number of reports describing the mass spectral analysis of isoquinoline alkaloids and their related analogues by direct injection mass spectrometry (MS) in electron ionization (EI) mode are available and have shown that distinct MS/MS fragmentation patterns are associated with this class of compound (DeJongh et al 1965; Tomita et al 1966). From the limited information available regarding the characterization of this class of compound by liquid chromatography MS/MS, it is reported that, in general, they exhibit distinct protonated molecular ions ([M+H]$^+$)

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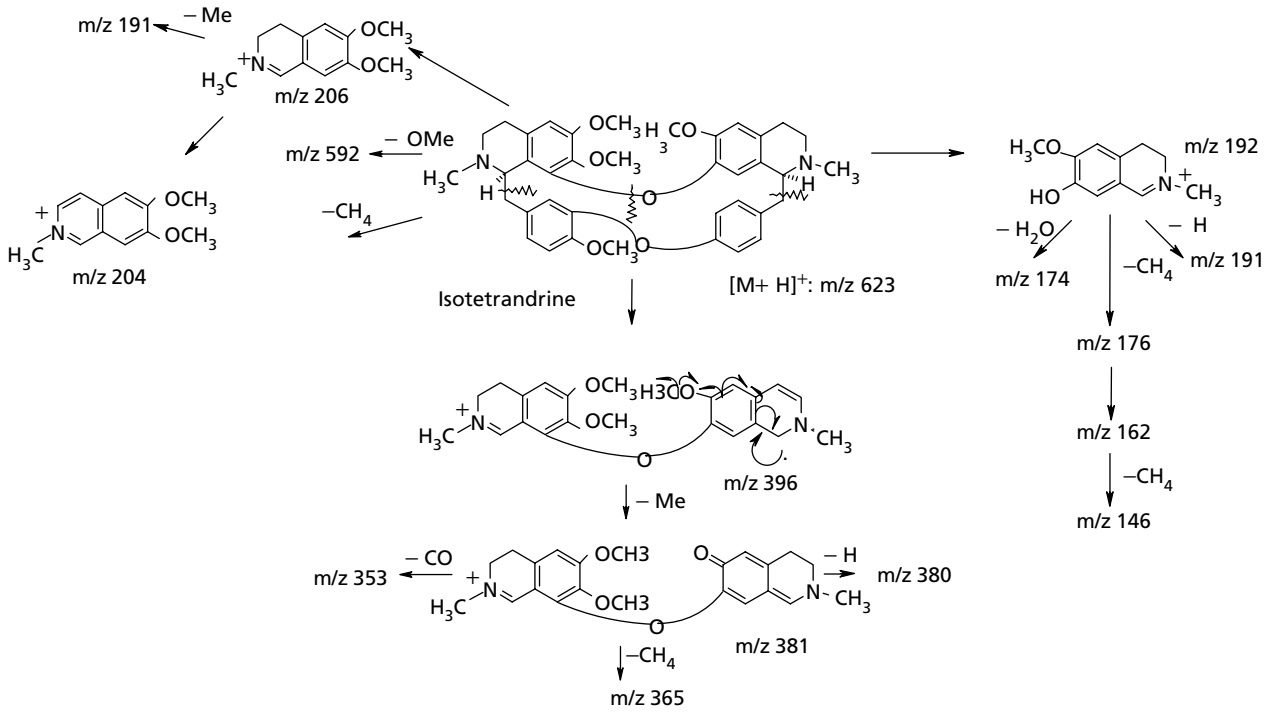

Figure 1 Proposed MS/MS fragmentation pathways of isotetrandrine.

and very intense doubly protonated molecular ions ($[M+2H]^{2+}$) in Q1 scan MS spectra, and prominent as well as diagnostic product ions for the structural information in MS/MS spectra (Wu & Moyer 2004). The objective of this study was to utilize atmospheric pressure ionization (API)-MS and MS/MS techniques to characterize this tertiary isoquinoline and its in-vitro metabolites formed in the rat hepatic S9 fraction. The Sciex API-MS and MS/MS analysis of the alkaloid liver incubate is a unique technique for metabolite profiling and identification because of its higher sensitivity and powerful mass-fragmentation for the quantification and structural elucidation of alkaloid molecules. This is the first reported metabolism study of isotetrandrine, although the metabolism of similar alkaloids such as thalicarpine, d-tubocurarine, atracurium, and mivacurium has been previously investigated. A preliminary account of this study has been presented (Wu et al 2003).

Materials and Methods

Chemicals

Isotetrandrine was isolated from *Thalcitrum sp* (Ranunculaceae) as previously described (Mitscher et al 1971) and had a purity of >97% (API-MS/MS, high-performance liquid chromatography (HPLC)). HPLC-grade solvents were obtained from the Fisher Scientific Co. (Fairlawn, NJ, USA) and glass-distilled solvents were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI, USA). The incubation components for hepatic S9 Tris, potassium chloride, magnesium chloride, $NADP^+$ and glucose-6-phosphate were purchased from Sigma (St Louis, MO, USA).

Hepatic S9 fraction

The hepatic S9 fraction was generated from a male Crl:CD (SD)IGSBR VAF/Plus rat at Johnson & Johnson Pharmaceutical Research & Development (Spring House, PA, USA) following our routine laboratory procedure (Wu & McKown 2002).

Rat hepatic S9 incubation

Chilled, freshly made components were added to each flask (on ice) in the following order: 1.1% KCl in 0.05 M Tris buffer (pH 7.4), 5 mM $MgCl_2$, 5mM glucose-6-phosphate, 0.5 mM $NADP^+$, hepatic S9, and isotetrandrine spike, to obtain a final volume of 5 mL and an isotetrandrine concentration of 100 μg mL^{-1}. After the addition of the last component, each flask was incubated at 37 °C in a Dubnoff metabolic shaker incubator (Precision Scientific, Chicago, IL, USA). Samples were removed at 0 and 60 min. A control flask was incubated without rat subcellular fraction to determine substrate stability under the incubation conditions.

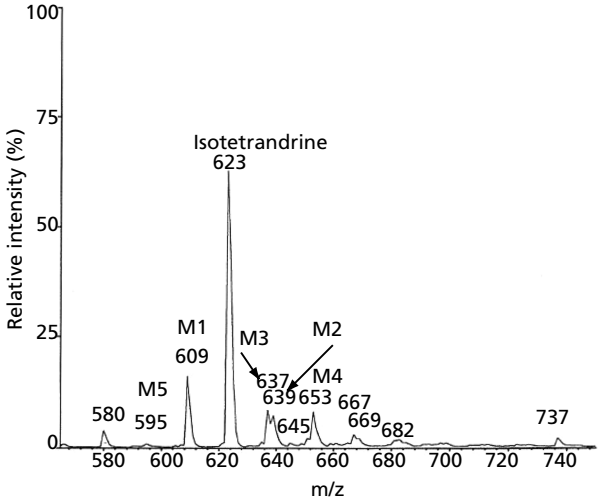

Figure 2 Total ion chromatogram (Q1-MS) of the extract of a 60-min rat hepatic S9 incubation showing protonated unchanged isotetrandrine and its metabolites.

Table 1 In-vitro metabolism of isotetrandrine in the 60-min rat hepatic S9 fraction.

Parent/metabolite	Percent of sample
Unchanged isotetrandrine	63
N-Desmethyl-isotetrandrine (M1)	16
Hydroxy-isotetrandrine (M2)	6
Oxo-isotetrandrine (M3)	7
Oxo-hydroxy-isotetrandrine (M4)	7
N,N-Didesmethyl-isotetrandrine (M5)	< 1

Data were obtained from the integrated ion chromatograms via Q1-MS.

Sample processing

Following ethyl acetate (3 mL) extraction of each ammonium hydroxide-basified (pH∼9) incubate (1 mL), the extract residue was reconstituted in mobile phase (0.5 mL of acetonitrile–water (50/50, v/v) with 2 mM ammonium acetate buffer, adjusted to pH 4.0) with acetic acid and then analysed via 20-μL flow injection using LC-MS and LC-MS/MS.

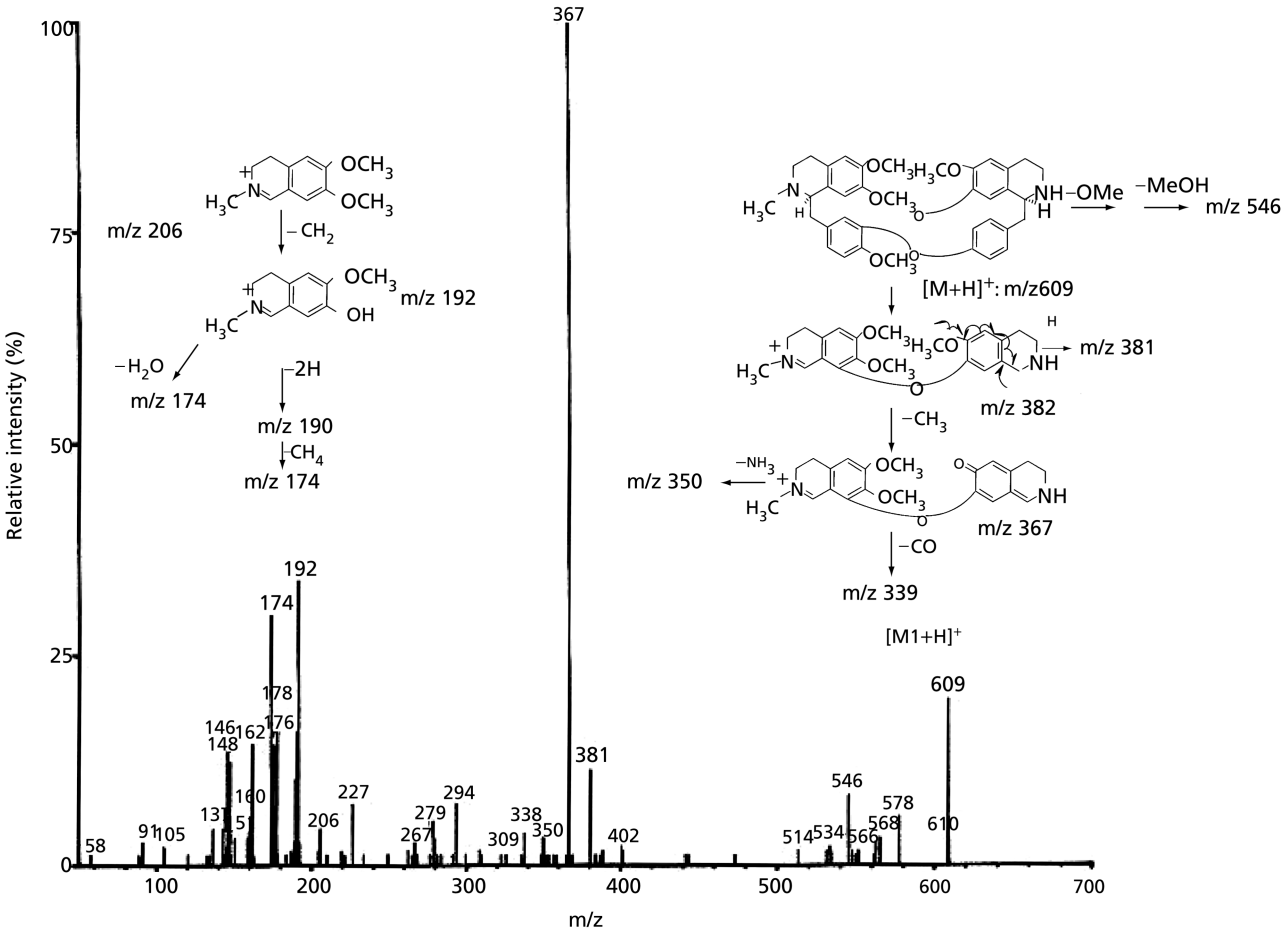

Figure 3 MS/MS spectrum of metabolite 1 (M1) in the extract of a 60-min rat hepatic S9 incubation.

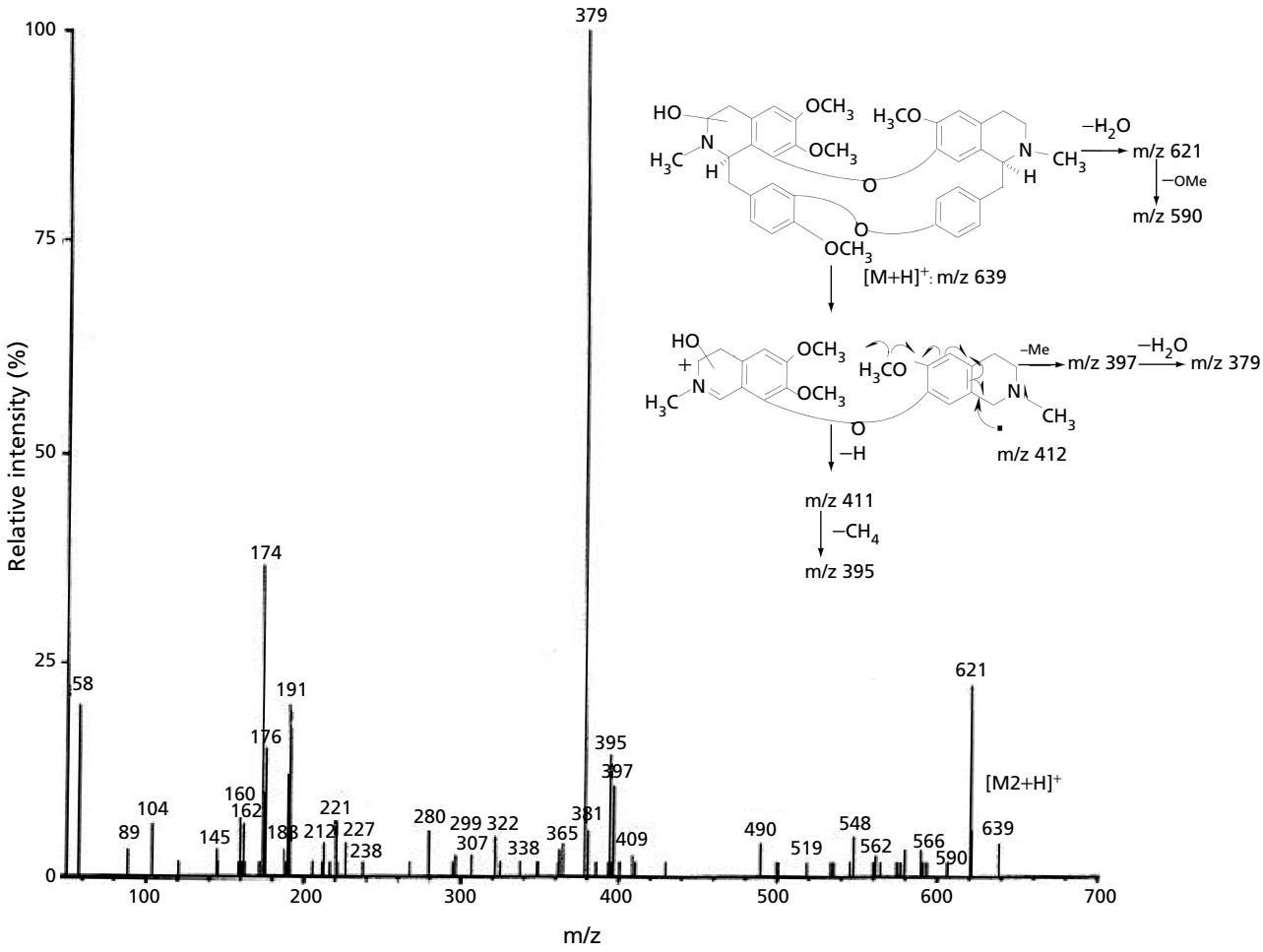

Figure 4 MS/MS spectrum of metabolite 2 (M2) in an extract of a 60-min rat hepatic S9 incubation.

Diazomethane derivatization

An excess of ethereal diazomethane was added to each extract residue, which was dissolved in 1 mL of methanol, and allowed to react at room temperature overnight. The organic layer was subsequently evaporated under nitrogen to yield a residue that was dissolved in mobile phase and analysed as described below.

Sample analysis

MS and MS/MS analyses were performed on a PE Sciex API-III-Plus (Applied Biosystems MDS Sciex, Thornhill, ON, Canada) triple quadrupole mass spectrometer, interfaced to an Hitachi HPLC solvent delivery system (L-6200 A Intelligent pump) via an ion-sprayer using nitrogen as curtain gas and nebulizing gas, and argon as collision gas. The mobile phase was the same as described above for the residue reconstitution, and pumped at the same flow rate of 0.1 mL min^{-1}. For each sample, the relative percentage of unchanged isotetrandrine and metabolites was estimated using the integrated total ion chromatogram generated by Q1 MS scanning. Unchanged isotetrandrine, its metabolites, as well as the presence or absence of methyl derivatives were elucidated on the basis of MS and MS/MS data.

Results and Discussion

Mass spectral analysis of isotetrandrine has been reported and is consistent with our results (Wu & Moyer 2004). The MS/MS spectrum of the $[M+H]^+$ of isotetrandrine included intense product ions at m/z 607 (10%), 592 (22%), 580 (22%), 381 (80%), 365 (10%), 353 (10%), 191 (75%), 174 (100%), 162 (70%) and 146 (60%).

The prominent product ion at m/z 381 is likely the result of the preferred cleavage of the carbon–carbon bond both β to nitrogen and β to the two aromatic systems followed by *O*-demethylation of an aromatic methoxy group. The product ion at m/z 191 is most likely a single isoquinoline moiety that gives rise to the base peak at m/z 174 following the loss of H_2O. The structure of

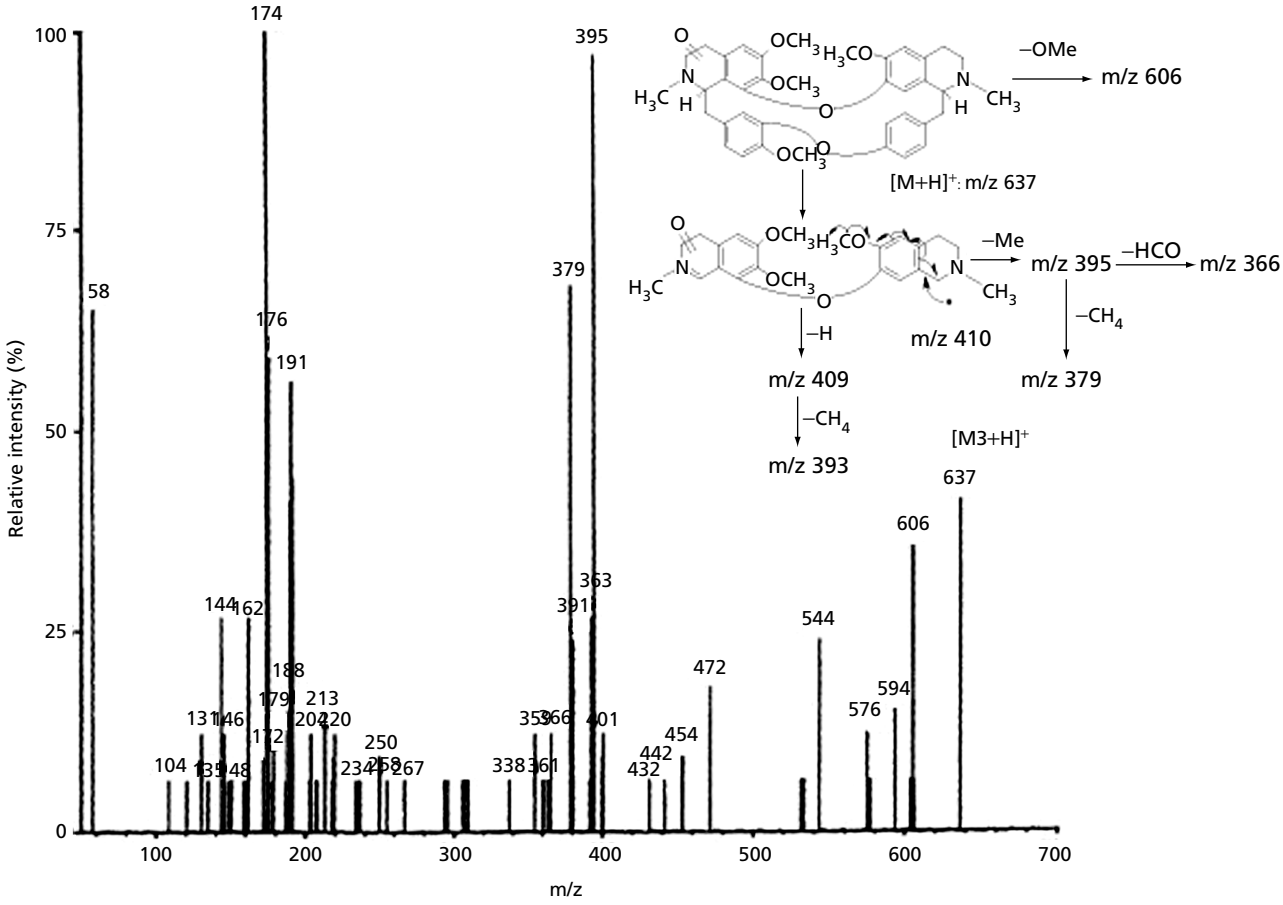

Figure 5 MS/MS spectrum of metabolite 3 (M3) in an extract of a 60-min rat hepatic S9 incubation.

isotetrandrine and a proposed MS/MS fragmentation pattern of its $[M+H]^+$ is shown in Figure 1, which, interestingly, bears similarity to the EI-MS spectrum of isotetrandrine when analysed by direct injection (DeJongh et al 1965; Tomita et al 1966).

The in-vitro metabolism of isotetrandrine was conducted in the rat hepatic S9 fraction. The partial (m/z 500–800) API-ionspray Q1-MS spectrum of the 60-min incubate tentatively identified the protonated molecular ions ($[M+H]^+$) of unchanged isotetrandrine at m/z 623 and those of five metabolites (M1, M2, M3, M4 and M5) at m/z 609, 637, 639, 653 and 595, respectively (Figure 2). The corresponding doubly protonated molecular ions for isotetrandrine, M1, M2, M3 and M4 at m/z 312, 305, 319, 320 and 327, respectively, were also observed in the full (m/z 100–1100) Q1-MS spectrum collected. Isotetrandrine, M1, M2, M3, M4 and M5 accounted for 63, 16, 6, 7, 7 and $< 1\%$ of the sample, respectively (Table 1).

In general, the MS/MS spectra of M1–M4 were very similar to that observed for isotetrandrine with fragmentation patterns consistent with bond cleavage between the isoquinoline and the benzyl portion of the parent molecule as well as cleavage of the ether bond linking the isoquinoline moieties. M1, *N*-desmethyl-isotetrandrine, was characterized by an $[M+H]^+$ at m/z 609, which indicates the loss of 14 amu, consistent with the demethylation of the parent compound (MH^+-14). As observed for the parent compound, the MS/MS spectrum of m/z 609 (Figure 3) shows a fragmentation pattern consistent with a characteristic cleavage occurring between the isoquinolines and the benzyl portion of the molecules to produce the ion m/z 381 initially, followed by the loss of a methyl group to form the ion m/z 367 as the most abundant peak of the spectrum. As proposed for isotetrandrine, the ions m/z 192 and 174 are likely products of a single isoquinoline moiety following cleavage of the ether linkage and followed by the loss of H_2O.

M2, hydroxy-isotetrandrine, is a minor metabolite accounting for 6% of the total sample analysed by LC-MS. The $[M+H]^+$ of this metabolite at m/z 639 is consistent with the addition of an OH group to the parent compound (MH^++16). The loss of water from its $[M+H]^+$ yielded the fragment ion m/z 621 (MH^+-18), typical of hydroxylated compounds. The structure of the compound was determined from the MS/MS spectrum of m/z 639, which was characterized by the most abundant ion at m/z

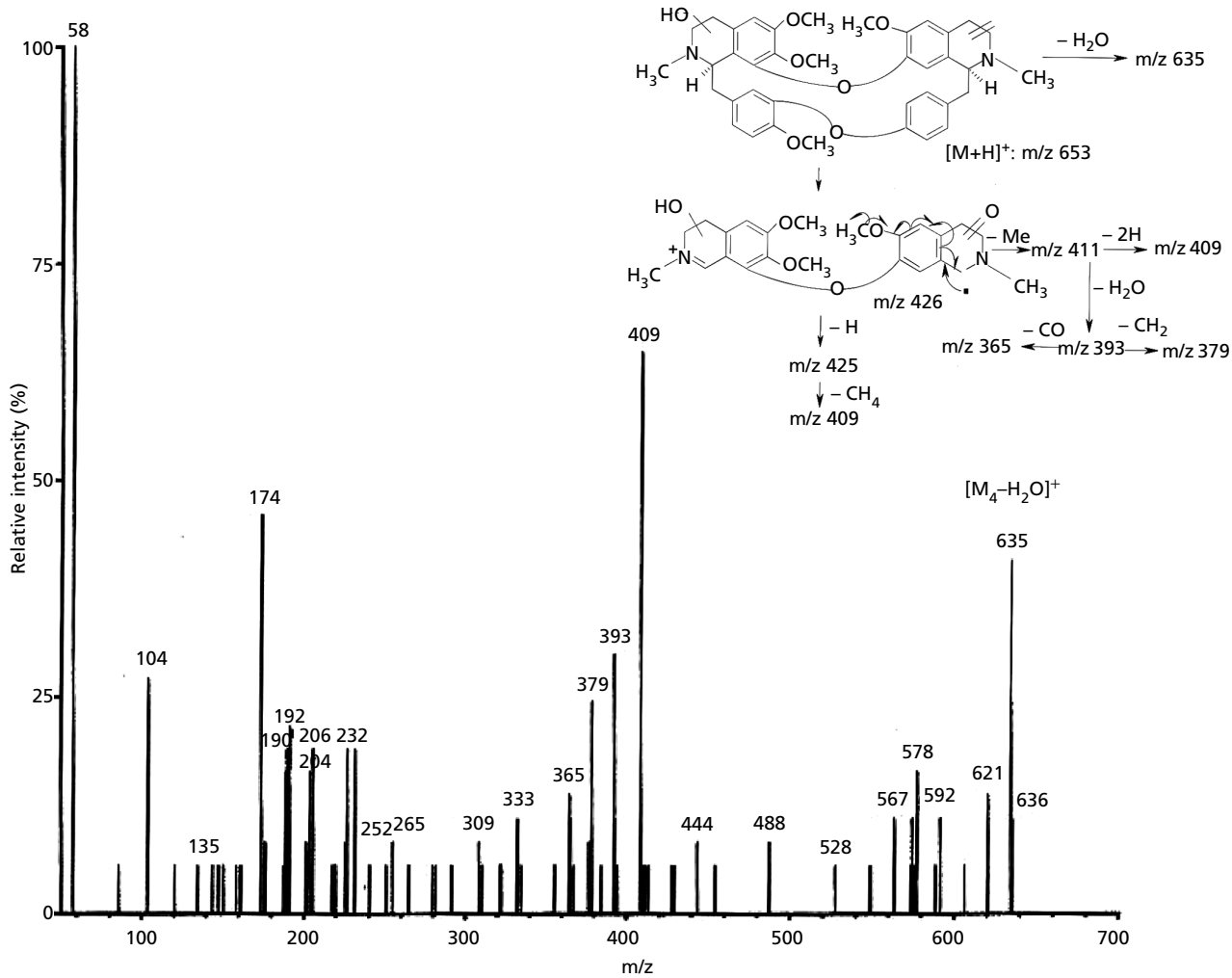

Figure 6 MS/MS spectrum of metabolite 4 (M4) in an extract of a 60-min rat hepatic S9 incubation.

379, the result of bond cleavage between the isoquinoline groups and the benzyl groups, followed by the loss of a methyl group and H_2O (Figure 4). M2 did not react with diazomethane, indicating that the OH group was not phenolic but cyclic as shown in Figure 4. The spectrum is also characterized by less prominent ions at m/z 191 and 174, both arising from the cleavage of the ether bond from the isoquinoline fragment as described previously.

The oxidation of M2 yielded metabolite 3 (M3), oxo-isotetrandrine, which was detected as a minor metabolite of isotetrandrine and accounted for 7% of the sample by LC-MS. The $[M+H]^+$ of this molecule at m/z 637 indicates a gain of 14 amu by oxidation of alcohol to ketone. As expected, the MS/MS spectrum of m/z 637 was characterized by one base peak at m/z 395, yielding the fragment ion m/z 379 following the loss of methane (16 amu). The fragment ion m/z 174 (m/z 191-H-CH_4) was observed as a base peak ion and m/z 191 (isoquinoline fragment) was observed at 60% abundance (Figure 5).

M4 was characterized as keto-hydroxy-isotetrandrine and accounted for 7% of the sample. MS/MS of m/z 653, its $[M+H]^+$, produced a spectrum characterized by m/z 635, the $[MH^+$-$H_2O]$ fragment ion, and those related to the isoquinolines portion of the molecule at m/z 409, 393 and 379. The product ions m/z 174 and 192 were also observed (Figure 6). M5, *N,N*-didesmethyl-isotetrandrine, was tentatively identified by its $[M+H]^+$ at m/z 595 by Q1-MS, but could not be confirmed by MS/MS due to the small amount detected ($< 1\%$ of sample). The percent composition of unchanged isotetrandrine and five metabolites in the S9 fraction of the rat are summarized in Table 1. The proposed metabolic pathways for the in-vitro metabolism of isotetrandrine in the rat hepatic S9 fraction are depicted in Figure 7.

In conclusion, isotetrandrine was moderately metabolized in the rat hepatic S9 fraction primarily via the *N*-demethylation pathway, while hydroxylation and oxidation of isotetrandrine moieties were less prominent.

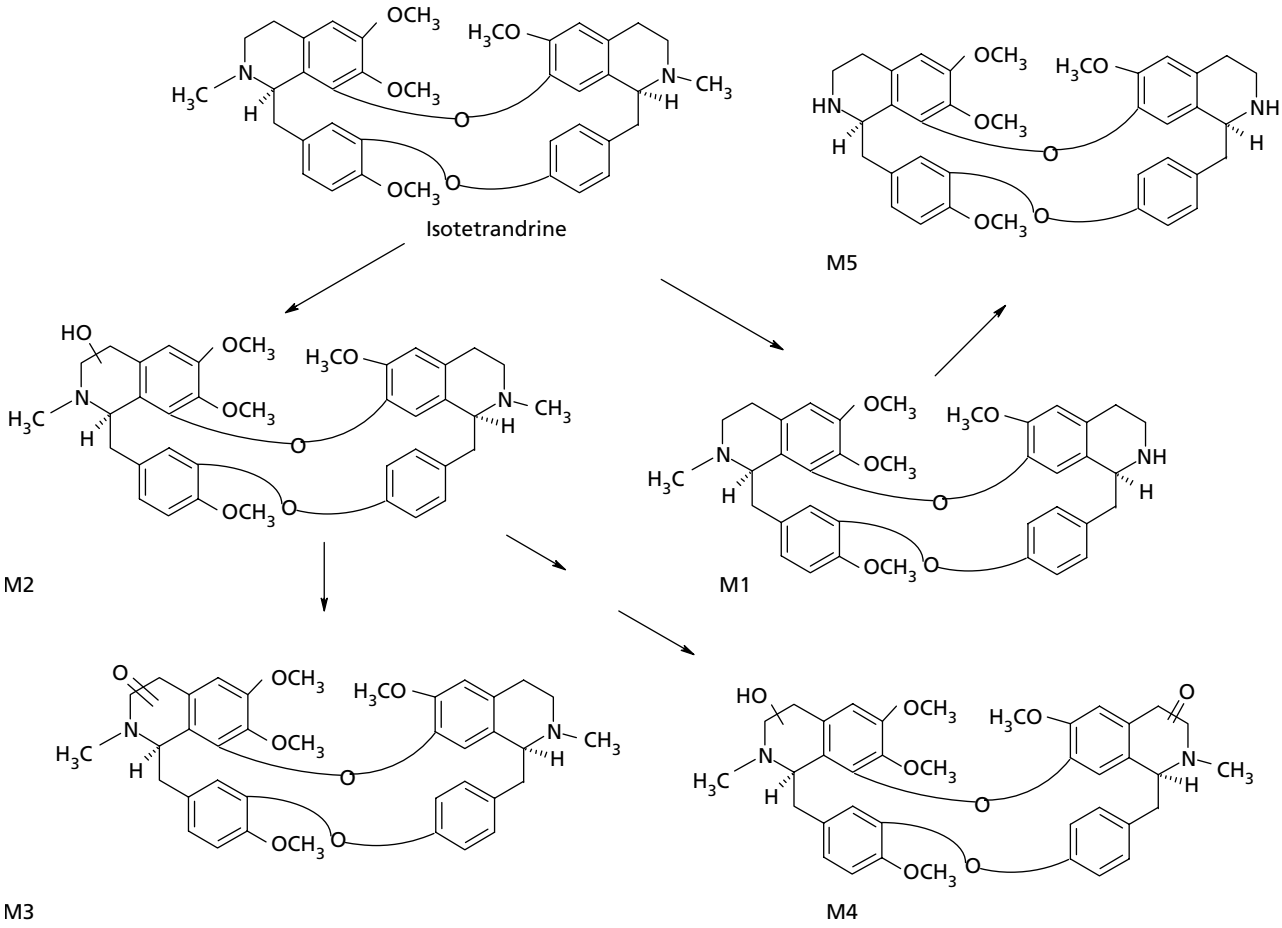

Figure 7 Proposed in-vitro metabolic pathways for isotetrandrine in the rat hepatic S9 fraction.

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