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Determination of the in vitro metabolism of (+)- and (−)-epibatidine

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Abstract

The oxidative in vitro metabolism of epibatidine was investigated using liver microsomes from rat, dog, rhesus monkey and human. Analysis was performed using liquid chromatography–mass spectrometry (LC–MS) using both achiral and chiral stationary phases. Comparison of the metabolism of the (+)- and (−)-enantiomers revealed species differences in the extent of metabolism, with rhesus monkey>dog>rat=human. Furthermore, differences in the routes of metabolism for epibatidine enantiomers were also observed, with mass spectra consistent with hydroxylation of the azabicycle for (−)-epibatidine and with the formation of diastereomeric *N*-oxides for (+)-epibatidine being obtained. For chiral LC–MS, a volatile ion-pair reagent of heptafluorobutyric acid was used in place of pentanesulphonic acid with no deterioration in chiral selectivity. Analysis of the same samples by chiral LC–MS revealed no evidence for metabolic chiral interconversion and chiral analysis from a metabolic time course of racemic material revealed enantiomers to be metabolised to approximately the same extent. Such findings may be important particularly should epibatidine be investigated in non-rodent species.
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1. Introduction

Epibatidine, *exo*-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane (Fig. 1) is a natural product isolated from the poison arrow frog *Epipedobates tricolor* first described in 1992 [1]. This compound has been shown to possess potent activity at nicotinic acetylcholine receptors and has been utilised as a novel pharmacological tool to probe such receptor function [2]. Many total syntheses of epibatidine as both a racemate [3] and as the separate enantiomers [4,5] have been reported and the identity of the natural enantiomer determined to be the (+)-isomer (as the HCl salt) [6] with the absolute configuration 1*R*,2*R*,4*S* [7]. Even with the availability of single enantiomers many in vivo studies are still conducted with racemic material [8–10]. Although reports suggest there is little difference in the pharmacological actions of the enantiomers [11] there are no reports regarding the enantioselectivity of epibatidine metabolism. Since it is well documented that metabolism, and in particular oxidative phase I processes mediated through the cytochrome P450 enzyme

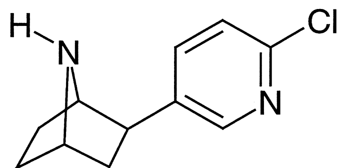

Fig. 1. Structure of *exo*-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane (epibatidine).

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system, shows enantioselectivity [12,13] investigation of the potential metabolic fate of epibatidine enantiomers using in vitro microsomal systems would be of value. Furthermore, since metabolic chiral inversion is a known process this could also be investigated as this would clearly negate the benefit of dealing with single enantiomers.

In order to investigate whether differences in the oxidative metabolism of the enantiomers of epibatidine existed and indeed whether metabolic activation leading to interconversion between the isomers was possible both chiral and achiral liquid chromatography–mass spectrometry (LC–MS) was employed. Although epibatidine possesses three chiral centres, at both bridgeheads of the azabicyclo ring and at the carbon alpha to the pyridyl ring, one does not observe the predicted 2^3 (=8) isomers. This is because the relative stereochemistries around the ring are fixed with the two bridgehead protons always *cis* and the pyridyl ring *exo* thus constraining the molecule to exist as two enantiomeric forms only. Consequently, metabolic hydroxylation of one of the prochiral methylene groups of the bicyclic ring (for a single epibatidine enantiomer) would produces a mixture of diastereoisomers insofar as the resulting hydroxyl group could be *exo* or *endo*. Consequently, in working with single enantiomers of epibatidine it is appropriate to use achiral chromatography to separate potential metabolites. Should each enantiomer form a metabolic product with the same m/z and retention characteristics, it is possible that these are enantiomeric; otherwise they are distinct diastereomeric products. Chiral high-performance liquid chromatography (HPLC) is therefore relevant in dealing with the fate of the racemic mixture or in the racemisation of single enantiomers.

Chiral HPLC conditions had been established previously for (±)-epibatidine [7]. This separation employed a chiral α_1-acid glycoprotein (AGP) stationary phase with a mobile phase containing the ion-pair reagent pentanesulphonic acid (PSA). Due to the hydrophilicity of the compound it had been found necessary to include the ion-pair to allow sufficient retention and to augment chiral selectivity. To overcome the anticipated difficulties of contamination of the mass spectrometer using a mobile phase containing an involatile ion-pair component, a volatile ion-pair reagent was desired. Perfluorinated carboxylic acids have previously been reported as ion-pair reagents for reversed-phase chromatography [14] and for coupling ion-pair HPLC to MS [15] but not for chiral HPLC. We demonstrate the application of heptafluorobutyric acid (HFBA) as an ion-pair reagent for chiral AGP and its utilisation in the separation of epibatidine enantiomers.

2. Experimental

2.1. Materials

(+)-, (−)- and (±)-Epibatidine were synthesised in the laboratory as previously described [4] with identity and purity confirmed by nuclear magnetic resonance (NMR), MS, HPLC and elemental analysis. Ammonium formate was analytical-reagent grade and acetonitrile Chromasolv grade (Riedel-de Haen) from Fisher (Loughborough, UK). HFBA was from Sigma–Aldrich (Poole, UK). Kromasil and chiral AGP columns were obtained through Hichrom (Reading, UK). Dulbecco's phosphate-buffered saline (DPBS) from was from Life Technologies (Paisley, UK). β-Nicotinamide adenine dinucleotide phosphate (NADPH) was from Sigma (Poole, UK).

2.2. Instrumentation

A HP1090M series high-performance liquid chromatograph was used for the HPLC–UV separations (Hewlett-Packard, Avondale, PA, USA). The system comprises an autoinjector, consisting of a Rheodyne 7010 injection valve fitted with a 250-μl loop, an autosampler and a binary DR-5 solvent delivery system. Detection was by UV using a built-in diode-array detection (DAD) system and data were processed using a HP ChemStation (rev. 6.03).

LC–MS was performed using a Micromass Quattro LC system (Micromass, Manchester, UK) running MassLynx software (rev. 3.02) coupled to a HP1050 quaternary pump (Hewlett-Packard) and a CTC PAL autosampler (Presearch, Hitchin, UK).

2.3. Chromatographic conditions

HPLC analysis was performed using either achiral or chiral chromatography. For the achiral separa-

tions, the system consisted of a Kromasil KR100 (150×3.2 mm I.D., 5 μm) $5C_8$ column with a typical mobile phase of 25 m*M* ammonium formate (NH_4COOH), adjusted to pH 3.0 with formic acid and a flow of 0.5 ml/min modified under gradient elution conditions as follows: t=0 min, MeCN=5%; t=2 min, MeCN=5%; t=15 min, MeCN=30%; t=15.1 min, MeCN=5%; total runtime 20 min. For chiral separations, a chiral AGP (150×4.6 mm I.D., 5 μm) column was employed with a mobile phase of 10 m*M* NH_4COOH adjusted to pH 7.4 with aqueous NH_3 and a flow-rate of 0.7 ml/min. HFBA concentration was optimised by adding increasing concentrations (100 μ*M* to 1 m*M*) to formate buffer and readjusting the pH with aqueous NH_3 to pH 7.4. Injection volumes of 5 μl of pure standards and 50 μl of reconstituted microsomal incubations were used. For LC–UV work the DAD system was set to 270 nm with a bandwidth of 10 nm as this was the second λ_{max} and away from background interference due to the formate buffer. For LC–MS the flow was taken directly into the source without splitting.

2.4. Mass spectrometer conditions

The mass spectrometer was operated using the following conditions; electrospray ionisation, capillary voltage 3.5 kV, RF lens 0.2 V, cone voltage 31 V, extractor lens 1 V, source temperature 100°C, drying gas temperature 300°C and collision energy 25 eV. Both Q1 and Q2 were set to unit mass resolution.

2.5. Microsomal incubations

Liver microsomes were prepared in the laboratory by differential centrifugation of fresh liver homogenate as described in the literature [16]. These were diluted to a protein concentration of 4 mg/ml and stored at −80°C prior to use. Incubations were conducted at a final substrate concentration of 10 μ*M*, microsomal protein concentration of 1 mg/ml and a NADPH concentration of 2 m*M*. A typical incubation consisted of DPBS (550 μl), microsomes (250 μl), substrate (100 μl of a 100 μ*M* solution in water) and NADPH (100 μl of a 20 m*M* solution in DPBS) to give a total incubation volume of 1 ml. DPBS, microsomes and substrate were mixed and pre-incubated to 37°C in a shaking water bath for 5 min. NADPH solution was separately pre-incubated at 37°C. The reaction was initiated by the addition of NADPH solution, and the samples incubated in a shaking water bath for 30 min for a single time–point study or at varying time points up to 1 h for a time–course study, then terminated by the addition of an equal volume of MeCN. The sample was then centrifuged to remove the protein pellet, the supernatant dried under a stream of nitrogen at 50°C then redissolved in 250 μl water for HPLC analysis.

3. Results and discussion

LC–MS has become the methodology of choice for the analysis of xenobiotics in biological media due to its enhanced sensitivity and selectivity over traditional LC–UV technology [17]. This allows assays to be conducted at more physiologically relevant concentrations and less stringent sample preparation methods are required. Epibatidine enantiomers were incubated at 37°C with liver microsomes from rat, dog, rhesus monkey and human at 10 μ*M* for 30 min and prepared for analysis as described. In incubating individual enantiomers, formation of hydroxylated or oxidised products on the azabicyclo ring will give rise to individual products of fixed relative stereochemistry due to the constrained nature of the ring (i.e., *exo* or *endo* hydroxylations or *N*-oxidation). Therefore, achiral chromatography was initially employed as, should (+)- and (−)-epibatidine be hydroxylated differentially then these compounds would be separable under achiral conditions as they are diastereomeric. Only if both enantiomers produce the same product (e.g., a specific *exo* hydroxylation) would the products be enantiomeric and therefore indistinguishable by achiral HPLC.

Samples were analysed by achiral LC–MS operating initially in scan mode (m/z 50 to 250). Although the background signal was found to be high, it was determined that metabolism was solely due to a gain of 16 u over parent, i.e., oxidation or hydroxylation. Since no other metabolism (e.g., dechlorination) could be determined, the samples were re-analysed using single ion recording (SIR), monitoring m/z ions corresponding to parent, and parent +16 u as well as parent +14 u, and parent

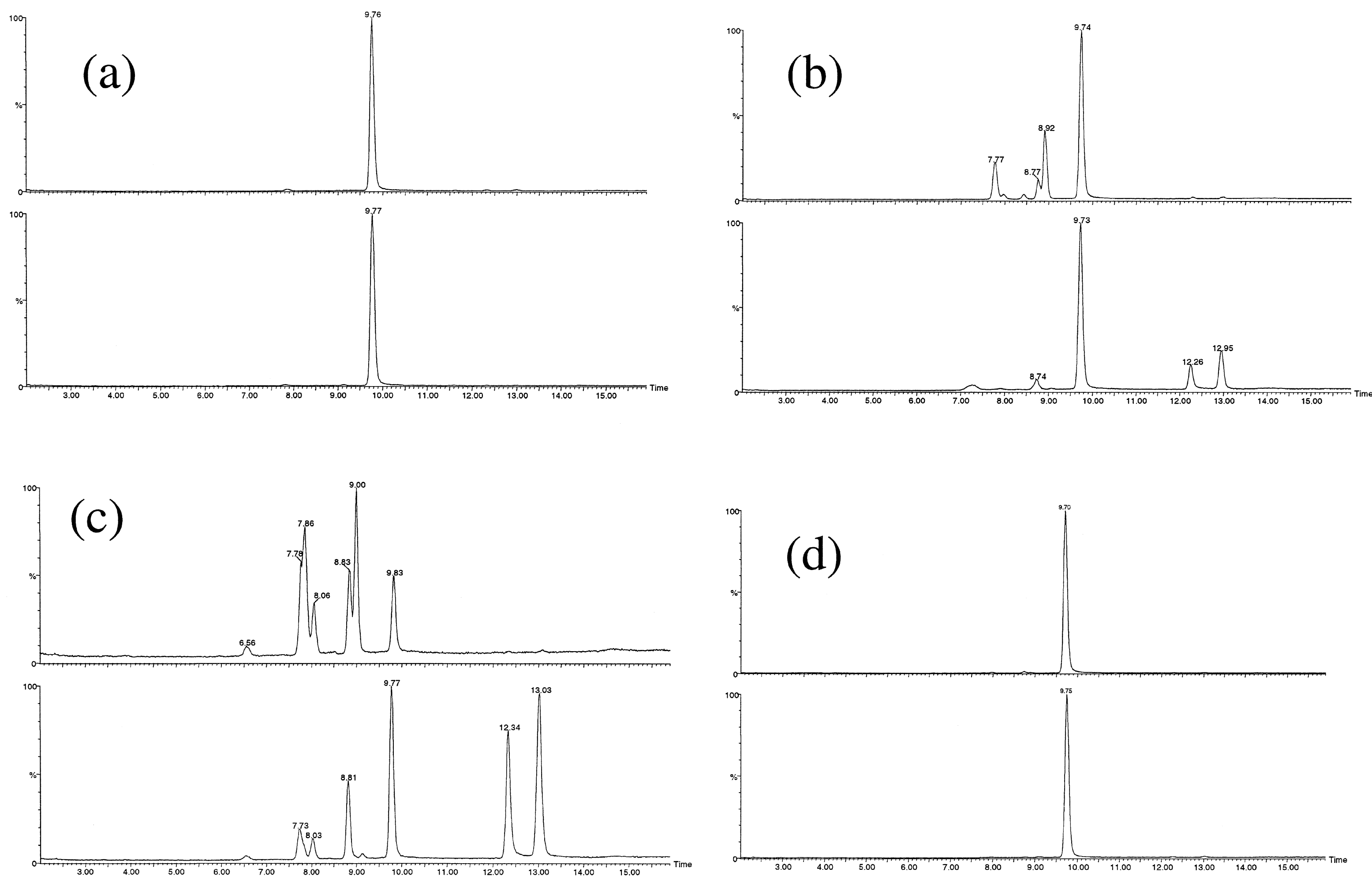

Fig. 2. Ion chromatogram comparison of in vitro metabolism of epibatidine using an achiral LC–MS system for (a) rat, (b) dog, (c) rhesus monkey and (d) human. In all cases the top chromatogram is (+)- and the bottom is (−)-epibatidine. Conditions: Kromasil KR100 (150×3.2 mm I.D., 5 μm) $5C_8$; mobile phase 25 m*M* ammonium formate (NH_4COOH), pH 3.0, flow 0.5 ml/min using gradient elution conditions as described in the text. t_R=9.8 min is epibatidine; all other peaks correspond to [M+16] metabolites.

+32 u to allow for additional minor ions not seen in the full-scan. The species comparison using gradient elution achiral HPLC for the two enantiomers incubated in rat, dog, rhesus monkey and human liver microsomes is shown (Fig. 2). In each case the incubation resulting from the (−)-enantiomer is the top chromatogram and the (+)-enantiomer the one below. Under these conditions the parent compound elutes at 9.8 min. All other peaks in the chromatogram were found to correspond to [M+16] ions. The first thing of note is that major differences in the extent of metabolism between the four species exist with little metabolism detectable in either the rat or human liver microsomes but extensive oxidation appears to occur with dog and rhesus monkey liver microsomes. Furthermore, the metabolism in dog and rhesus monkey appears to be qualitatively similar hence one need only investigate one system in detail. Secondly, in considering the metabolites that have been formed, it is clear that some are, as expected more polar than the parent compound as evidenced by their shorter retention times whilst others are more lipophilic as they elute after parent compound. This will be discussed further below. Finally, while some metabolites are common to both (+)- and (−)-epibatidine, indicating that these may be enantiomeric products, substantial differences do exist between the nature of the (diastereomeric) metabolites observed for (+)- and (−)-epibatidine. This indicates that the enzyme(s) responsible for the metabolism are able to differentiate the enantiomers of epibatidine and hence form distinct diastereomeric products. For example, if one considers Fig. 2c, the metabolism in rhesus monkey liver microsomes, it might be asserted that the first eluting metabolite peaks (t_R=6.5, 7.8, 8.0 and 8.8 min) are common to both (+)- and (−)-isomers, therefore it is possible that these are hydroxylated at the same position on the molecule and are simply different enantiomeric forms. Crucially though, a major route of metabolism for the (−)-isomer appears to be to yield a metabolite peak at 9.0 min, which is minor for the (+)-isomer. Similarly, the (+)-isomer yields metabolite peaks at 12.3 and 13.0 min which are totally absent from the (−)-isomer.

In order to further explore the metabolic pathways the product ion spectra for the parent compound was obtained (Fig. 3) and the major fragments rationalised (Fig. 4). Given that the molecule contains a chlorine with characteristic 35/37 isotopes product

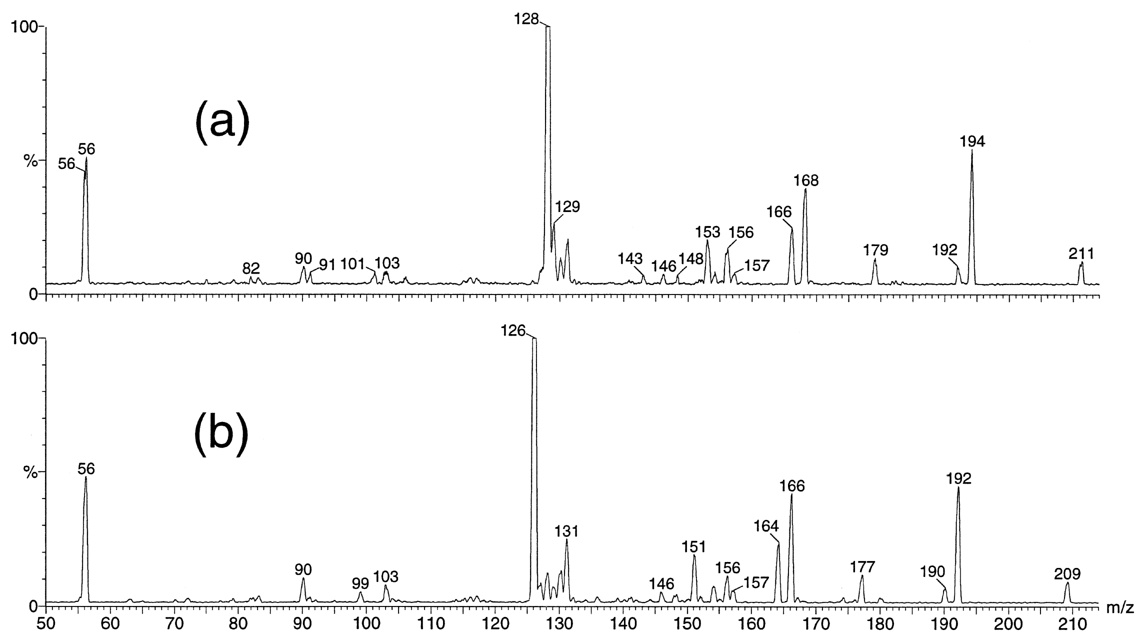

Fig. 3. Product ion spectra of epibatidine acquired at 25 eV for (a) products of 211 and (b) products of 209.

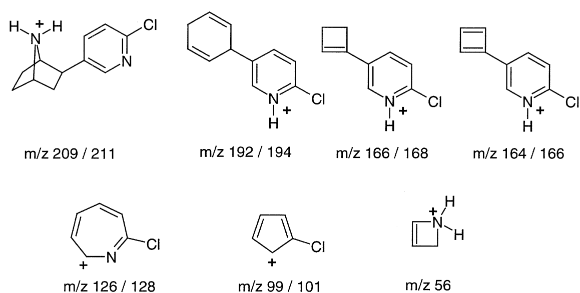

Fig. 4. Proposed structures rationalising the major mass spectrometric fragments of epibatidine.

ion spectra at both m/z 209 and 211 were acquired. The spectrum is dominated by the tropylium-type ion formed from the pyridyl ring at m/z 126/128 but other less abundant ions are also observed. Although the proposed fragment structures are consistent with the observed mass these may exist as forms other than those drawn and would need high resolution data to fully support them. Nevertheless, structural information on the metabolism may be gained.

The product ion chromatograms (Fig. 5) and spectra (Fig. 6) for the major metabolites (m/z 225) resulting from (+)- and (−)-epibatidine were obtained from the rhesus monkey liver microsomes incubations. Due to the poor duty cycle of tandem MS instruments data could only be reliably obtained on three metabolites from the (+)-epibatidine and three from the (−)-epibatidine incubations. The three metabolites from (−)-epibatidine are designated as M1–M3 and those from (+)-epibatidine as M4–M6. Unexpectedly, the mass spectrum for M1 shows no anticipated ions but instead a dominant ion at m/z 68 and is unassigned. By contrast, M2 and M3 both show neutral losses of 17 (ammonia) and 18 (elimi-

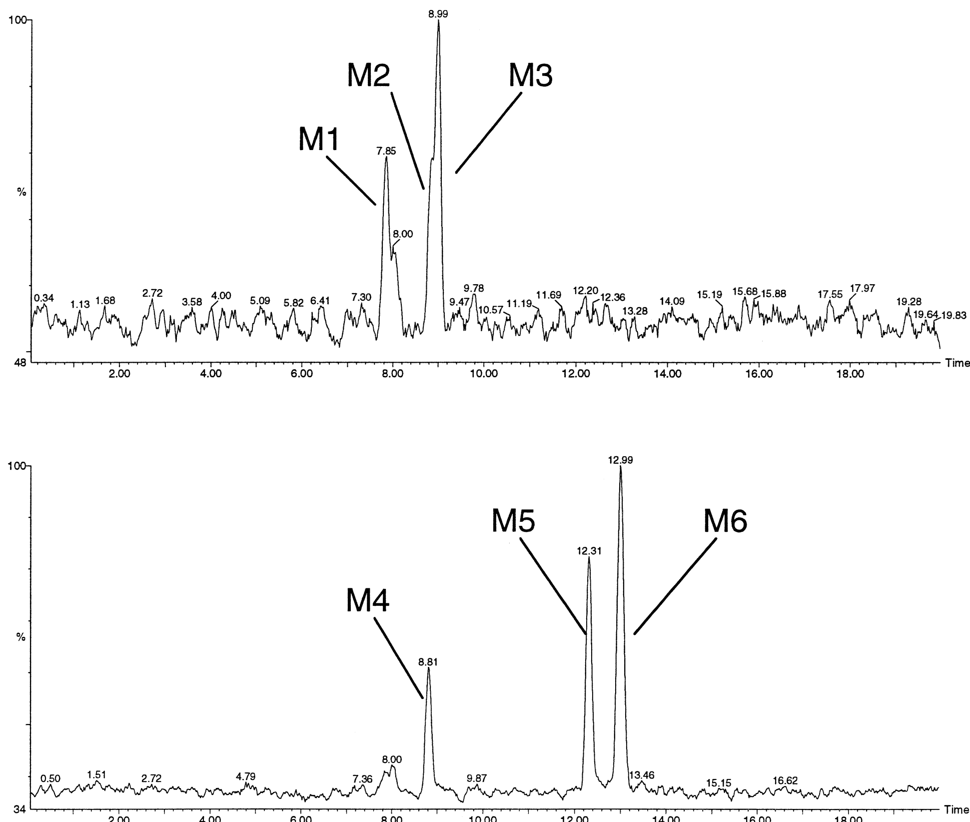

Fig. 5. Total ion chromatogram of products of m/z 225 [M+16] acquired at 25 eV for (a) (−)-epibatidine and (b) (+)-epibatidine. HPLC conditions as in Fig. 2. Time scale in min.

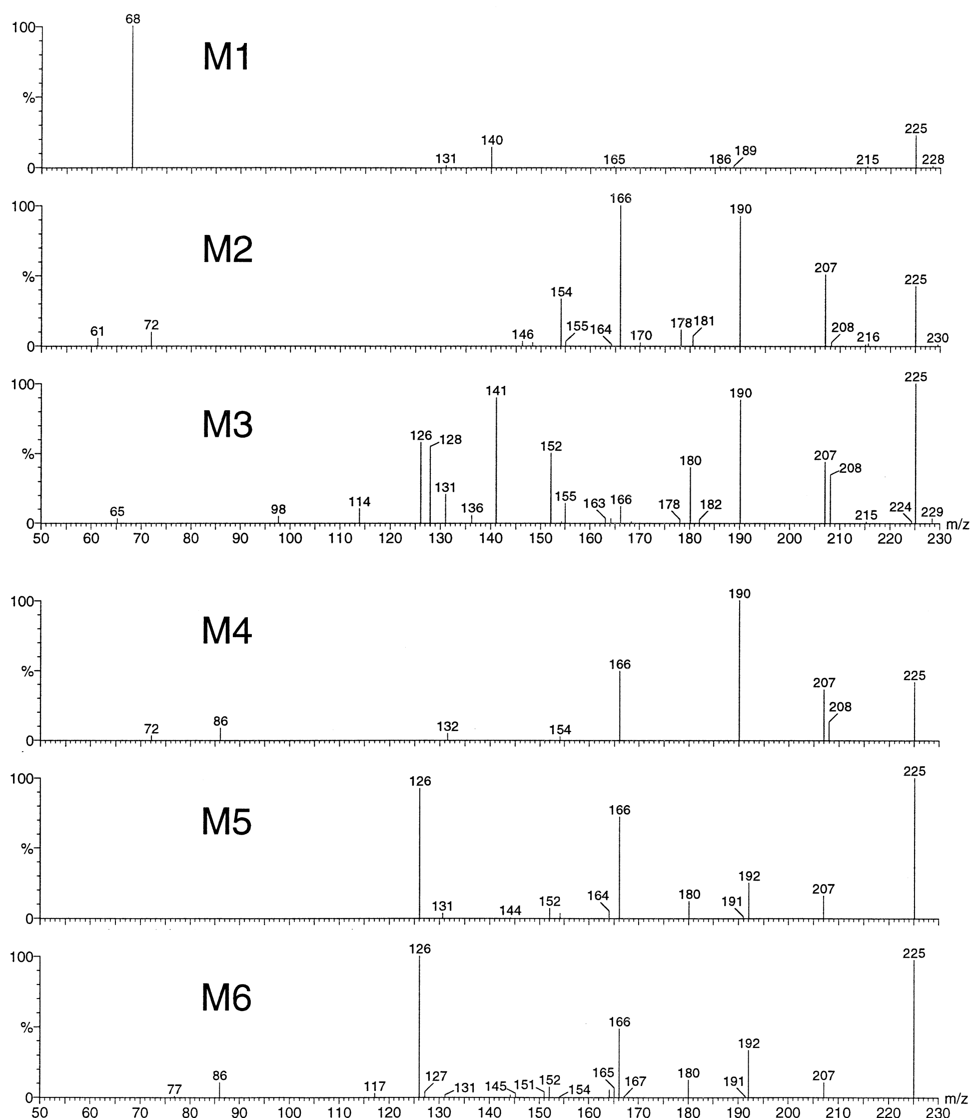

Fig. 6. Product ion spectra for metabolites M1–M6 acquired at 25 eV.

nation of water) and both give a dominant ion at *m/z* 190. This would be consistent with the *m/z* 192 ion seen in the parent but with the loss of two additional protons (resulting from the H_2O loss). This therefore locates the position of hydroxylation to a methylene group on the azabicyclo ring as aromatic hydroxylation would not be able to eliminate water.

Chromatographically, M4 and M6 have the same retention characteristics which, coupled with their near identical product ion spectra suggests that these are enantiomeric products. The products M5 and M6, while chromatographically distinct, have very similar product ion spectra suggesting that these are related metabolites. Of note are the ion at *m/z* 192 suggesting that cycloalkyl ring protons are not lost through the elimination of water and the absence of an ion at *m/z* 208 suggests also that ammonia cannot be eliminated. Coupled with the evidence that these products are more non-polar than the parent compound, this suggests that these may be diastereo-

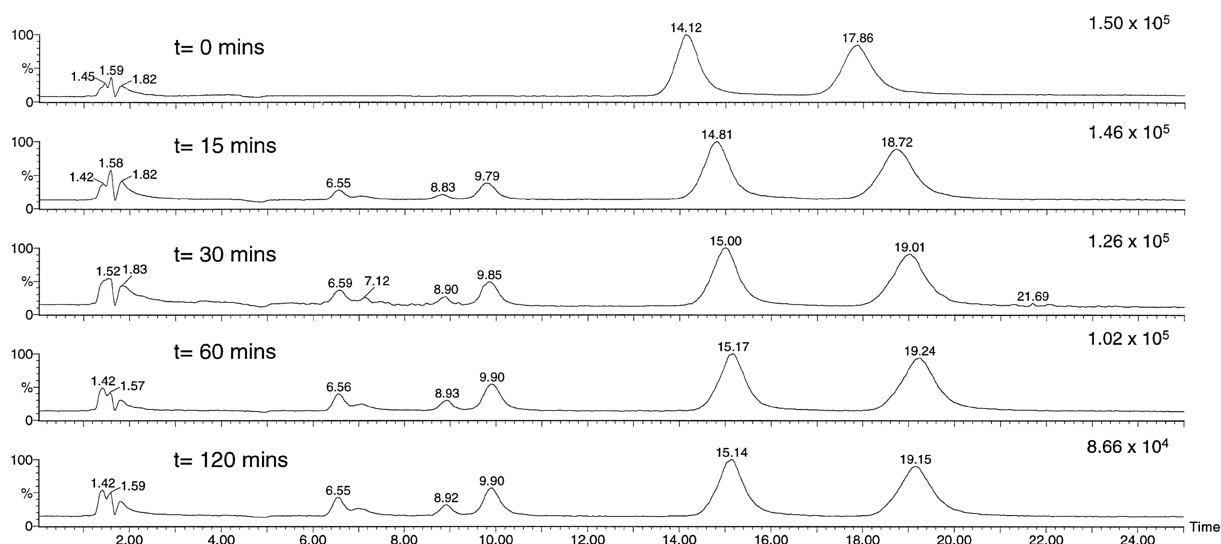

Fig. 7. Time course comparison of the metabolism of racemic epibatidine by chiral LC–MS showing reconstructed ion chromatogram of m/z 209 and 225. Conditions: 5% MeCN in 10 mM NH_4COOH, 1 mM HFBA, pH 7.4 flow 0.7 ml/min. Time scale in min. Parent compound is t_R=15, 19 min; all other peaks correspond to [M+16] metabolites.

meric N-oxides pointing either towards or away from the aromatic ring, the increase in lipophilicity coming from the consequent reduction in basicity of the nitrogen. Consequently, while (−)-epibatidine is metabolised through azabicylic hydroxylation, no N-oxidation is observed and although some azabicylic hydroxylation is observed for (+)-epibatidine, the major pathways appear to be through N-oxidation.

In order to establish chiral HPLC conditions that would be compatible with LC–MS, we first attempted to show that PSA in phosphate buffer, the mobile phase required for enantioseparation of epibatidine using a chiral AGP stationary phase could be replaced by HFBA using a more volatile ammonium formate buffer system. With a mobile phase of 10% MeCN in 10 mM K_2HPO_4 and 5 mM PSA, pH 7.4, baseline separation of the enantiomers using LC–UV analysis was achieved (α=1.20, R_s=2.06). This was replicated using a mobile phase of 10% MeCN in 10 mM NH_4COOH, 1 mM HFBA, pH 7.4 (α=1.18, R_s=2.13) providing a suitable system for investigation by chiral LC–MS. These conditions were used with mass spectrometric detection although it was found that even monitoring in SIR mode for the [M+H]+ ion of parent compound (m/z 209), sensitivity was somewhat lower than anticipated. This can be attributed to the pH of the mobile phase being above the optimum pH for effective electrospray ionisation due to reduced protonation of the analyte. Analysis of a time course incubation of racemic epibatidine in dog liver microsomes (Fig. 7) indicates that both enantiomers are metabolised at approximately equivalent rates. Furthermore, analysis of the same liver microsomes samples as described above using chiral LC–MS conditions are shown (Fig. 8) indicating as expected that no racemisation of the single enantiomers occurs under these incubation conditions.

4. Conclusions

It has been demonstrated that the (+)- and (−)-enantiomers of epibatidine are good substrates for dog and rhesus monkey microsomal enzymes but poor for rat and human. Metabolism across species did, however, appear qualitatively similar. Moreover, in vitro metabolism is stereoselective in that (−)-epibatidine appears to be metabolised through hydroxylation of the azabicyclo ring whereas, although this is a route for (+)-epibatidine, this is appears to be oxidised in vitro to two diastereomeric N-oxides.

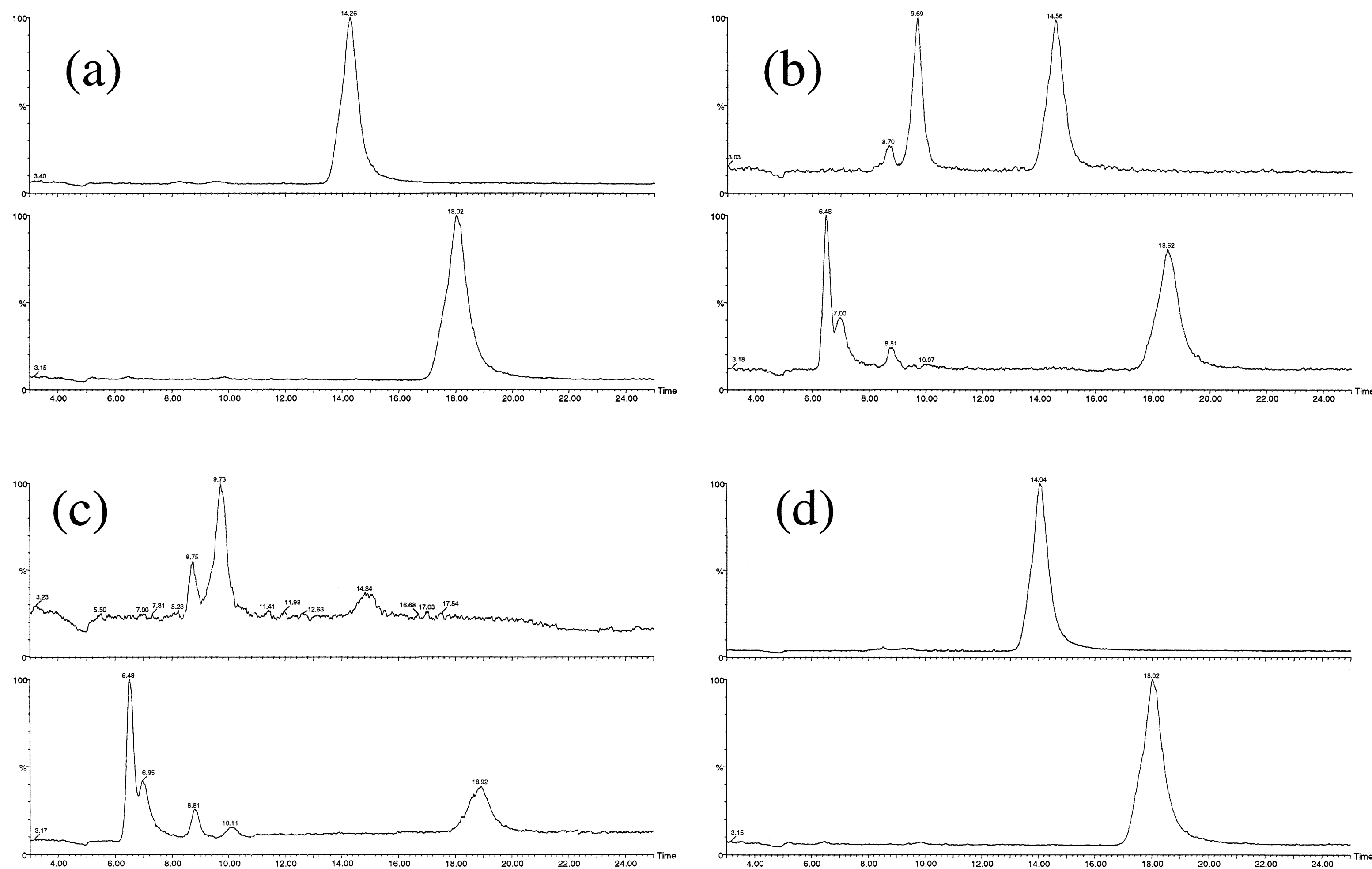

Fig. 8. Reconstructed ion chromatogram (m/z 209 and 225) comparison of in vitro metabolism of epibatidine using a chiral LC–MS system for (a) rat, (b) dog, (c) rhesus monkey and (d) human. In all cases the top chromatogram is (+)- and the bottom is (−)-epibatidine. Conditions as in Fig. 7. Time scale in min. $t_R=14$ min is (+)-epibatidine, $t_R=18$ min is (−)-epibatidine; all other peaks correspond to [M+16] metabolites.

We have additionally demonstrated that it is possible to exchange PSA for HFBA as a suitably volatile ion-pair reagent for the chiral LC–MS analysis of epibatidine enantiomers using a chiral AGP stationary phase. Therefore, in summary, given such species and enantiomeric differences in the metabolism of epibatidine, interpretation of in vivo data for this compound may be confounded.

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