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Use of chiral liquid chromatography–tandem mass spectrometry to investigate the metabolism of racemic cholecystokinin-B antagonists

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Abstract

In an attempt to establish the enantiomeric specificity of metabolism for a series of racemic cholecystokinin-B receptor antagonists, chiral LC–MS–MS conditions were established using a Pirkle DNBL chiral stationary phase operating in the reversed-phase mode. Rat liver microsomal incubations of the compounds were analysed using these conditions and it was demonstrated that resolution of oxygenated and demethylated metabolites could be achieved. A single model compound was investigated in detail by obtaining product-ion spectra on all mono-oxygenated species in an attempt to correlate these and identify enantiomeric pairs of metabolites. In this example a lack of differentiation in the product ion spectra did not allow correlation but the results suggest that such an approach may still be viable for the chiral metabolic analysis of racemic material.

Keywords: Enantiomer separation; Pharmaceutical analysis; Cholecystokinins; Peptides; Hormones; Benzodiazepines

1. Introduction

The 33 amino acid polypeptide hormone cholecystokinin (CCK), which is found in both the gastrointestinal tract and the central nervous system, mediates its action through two distinct CCK receptor subtypes designated A (CCK_A) and B (CCK_B). Activity at the peripheral CCK_A receptor has been shown to play a role in satiety [1], gastric secretion [2] and regulation of pancreatic secretion [3], whilst the CCK_B receptor is found primarily in the brain and has been implicated in, amongst other things, spatial recognition memory [4], control of feeding behaviour [5] and neurotransmitter release [6]. As such the therapeutic potential of CCK_B receptor ligands appears promising and extensive.

Previously reported non-peptidic antagonists of the CCK_B receptor have included substituted 1,4-benzodiazepin-2-ones in which the *R* stereochemistry at C3 of the benzodiazepine was crucial for CCK_B receptor selectivity over CCK_A [7]. The chromatographic chiral separation of these racemic compounds has previously been documented using Pirkle type dinitrobenzoyl phenylglycine (DNBPG) or dinitrobenzoyl leucine (DNBL) chiral stationary phases (CSPs) [8,9] and these have been used extensively to prepare milligram quantities of material for initial binding and efficacy studies. However, for a drug discovery programme in which many analogues are prepared this approach can be rate limiting. A pragmatic solution to this is to perform a limited number of studies on the racemate with the belief that this is likely to be only two-fold away from the active enantiomer in terms of potency. Whilst this may be true in vitro, in vivo the situation becomes

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much more complex as enantiomers may have markedly different pharmacokinetic and metabolic properties [10,11], hence we have sought to establish whether, by using chiral high-performance liquid chromatography–mass spectrometry (HPLC–MS) it is possible to assess the metabolic fate of enantiomers as a racemic mixture.

We have investigated the in vitro metabolism of a series of racemic benzodiazepine amidines (for structures see Fig. 1). Given the extremely high enantioselectivities observed for such molecules using a Pirkle DNBL CSP [8], we hypothesised that it may be possible to perform the analysis using such a phase in a reversed-phase mode, albeit with some loss of enantioselectivity as it is known that Pirkle columns may be operated in this mode [12]. This would then be fully compatible with liquid chromatography (LC) coupled to mass spectrometry and aqueous samples. Then, given the multiplicity of peaks one might observe, the use of the additional dimension of tandem mass spectrometry to generate distinct product-ion spectra may allow us to match enantiomeric metabolites. Therefore, it might be possible through assessment of capacity factor and product ion spectra, to assign metabolites to either the *R* or *S* parent molecule. Detailed data for the metabolic fate of a representative compound using rat liver microsomes is presented demonstrating the feasibility of this approach.

2. Experimental

2.1. Materials

All test compounds were synthesised in the laboratory with identity and purity confirmed by nuclear magnetic resonance spectroscopy (NMR), MS, HPLC and elemental analysis. Ammonium formate was analytical-reagent grade and acetonitrile was Chromasolv grade (Riedel-de Haen), supplied by Fisher (Loughborough, UK). Methanol (HPLC grade) and β-nicotinamide adenine dinucleotide phosphate (NADPH) was from Sigma–Aldrich (Poole, UK). Dulbecco's phosphate-buffered saline (DPBS) was from Life Technologies (Paisely, UK).

2.2. Instrumentation

An 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 at 230 nm and 280 nm corresponding to the first and second λ_{max} of the compounds with a bandwidth of 10 nm.

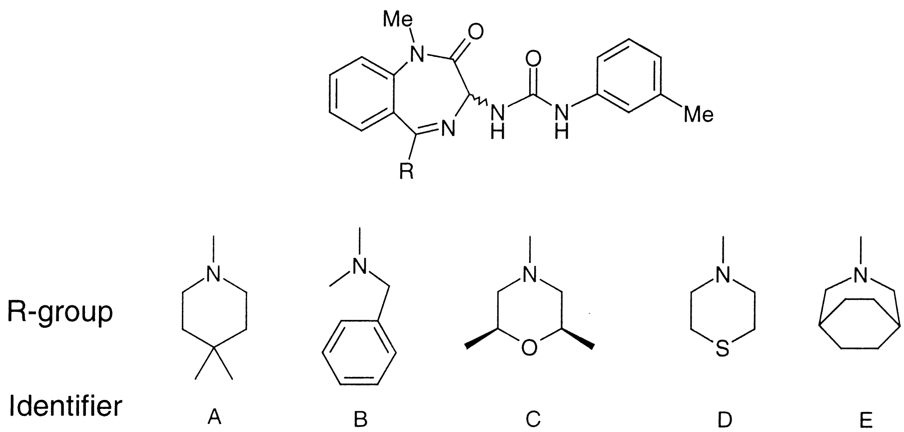

Fig. 1. General structures of 3-substituted racemic benzodiazepines showing variation of amidine groups investigated at C5.

Data were processed using a HP ChemStation (rev. 6.03).

Liquid chromatography–tandem mass spectrometry 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). The following conditions were used; capillary voltage 3.5 kV, cone voltage 30 V, extractor 1 V, source temperature 120°C, drying gas temperature 300°C, collision energy 55 eV. Both Q1 and Q3 were set to unit mass resolution.

2.3. Chromatographic conditions

HPLC analysis was performed using a Pirkle DNBL (250×4.6 mm I.D.) (Hichrom, UK) column with a mobile phase of 25 m*M* NH_4COOH buffer, adjusted to varying pH values with formic acid and modified with acetonitrile or methanol. The majority of analyses were conducted at 40% MeCN in 25 m*M* NH_4COOH, pH 7 buffer with a total run time of 30 min. All separations were performed at ambient (25±1°C) temperature. The flow-rate was 0.8 ml/min, used without splitting, this being a compromise between maximal efficiency at 1 ml/min and the drying capacity of the mass spectrometer source. Injection volumes of 5 μl of pure standards and 50 μl of reconstituted microsomal incubations were used.

2.4. Microsomal incubations

Rat liver microsomes were prepared in the laboratory by differential centrifugation of fresh rat liver homogenate. 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 NADPH concentration of 2 m*M*. A typical incubation consisted of DPBS (550

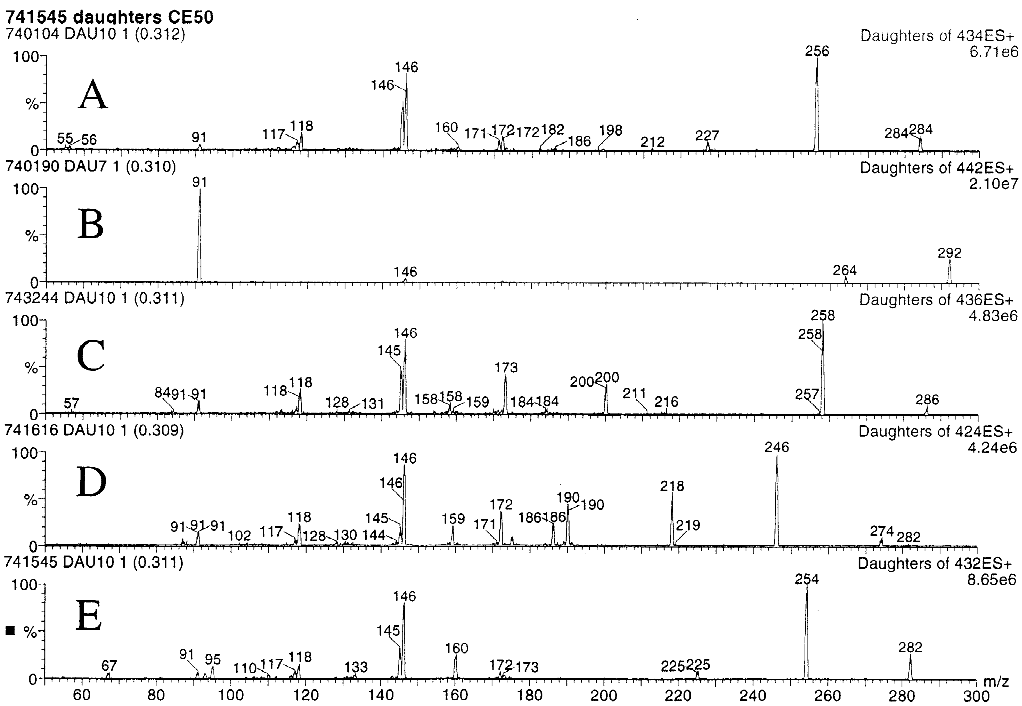

Fig. 2. CID product-ion spectra of compounds A–E obtained at a collision energy of 50 eV (except compound B at 35 eV).

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, incubated for 30 min in a shaking water bath then terminated by the addition of an equal volume of MeCN. The sample was then centrifuged (15 min, 2000 *g*) 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

In order to analyse aqueous samples by LC–MS, it was first investigated whether the DNBL stationary phase employed previously could be employed in the reversed-phase mode to give adequate chiral discrimination. This was additionally important as it was likely that the products of metabolism under investigation would be more polar than the parent compounds hence it would be preferred if they were to elute before rather than after parent. Secondly, should this prove successful, in order to achieve adequate sensitivity using electrospray ionisation, low pH buffers would be preferred hence an investigation of the pH dependence on separation was performed. The separation for five analogues was conducted at pH 7 using electrospray LC–MS to select the m/z for the molecular ion of the parent compound [i.e., single-ion recording (SIR) mode] and product ion spectra for all compounds were obtained (Fig. 2). These were all shown to be well resolved (Fig. 3) although a decrease in separation selectivity and resolution were observed compared to normal-phase conditions (Table 1). Additionally, both MeOH and MeCN were assessed as organic modifiers and showed no difference in resolution

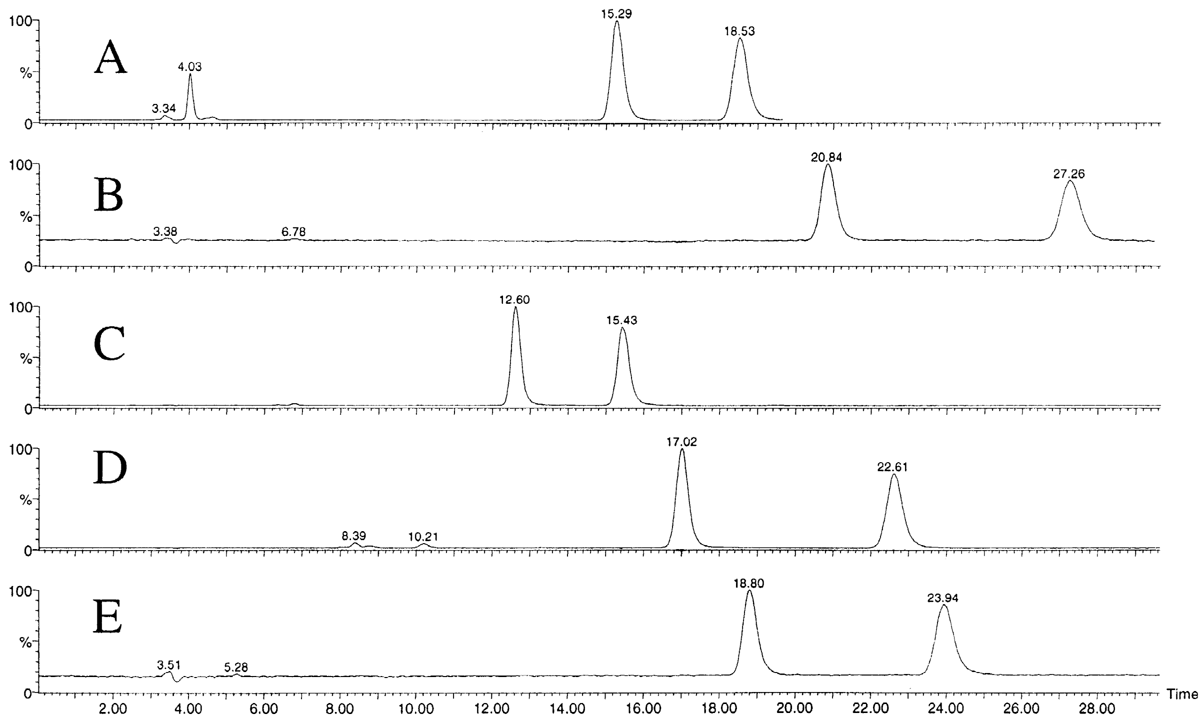

Fig. 3. Single-ion chromatograms for racemic compounds A–E using reversed-phase chiral HPLC with a Pirkle DNBL CSP. Conditions as described in text. Time scale in min.

Table 1
Comparison of separation selectivity and resolution using a DNBL CSP with normal- and reversed-phase conditions[a]

	Reversed-phase		Normal-phase	
	α	R_s	α	R_s
A	1.3	4.6	2.1	9.6
B	1.4	7.3		
C	1.3	5.1	2.3	9.6
D	1.4	7.2	2.8	13.0
E	1.3	6.2	2.3	10.9

[a] Separation conditions: normal-phase 10% MeOH in 1-chlorobutane, flow 1 ml/min, detection 280 nm; reversed-phase 40% MeCN in 25 m*M* NH_4COOH, pH 7, flow 0.8 ml/min, detection 280 nm.

hence MeCN was preferred as this allowed operation at reduced back pressure compared to MeOH. Compound A was further investigated by performing the separation at decreasing pH values down to pH 3, the pH normally used for LC–MS as at this pH sufficient protonation of the analytes should occur using atmospheric pressure ionisation techniques. It was

Table 2
Comparison of capacity factor, separation selectivity and resolution as a function of pH for compound A using a DNBL CSP under reversed-phase conditions

pH	k'_1	k'_2	α	R_s
4.0	6.3	8.2	1.29	4.1
4.5	9.6	12.4	1.29	5.4
5.0	10.1	13.0	1.29	5.1
5.5	7.3	9.5	1.30	4.8
6.5	8.4	10.9	1.29	5.2
7.0	9.4	12.1	1.29	5.0

found (Table 2) that decreasing pH values had no effect on separation selectivity and resolution until pH 4, at which point a decrease in the capacity factors was observed. Below this, capacity factors decreased markedly with no separation observed at pH 3 and the molecules eluting at the solvent front. It may have been possible to adjust this through re-optimisation of organic modifier concentration but it was decided to adhere to neutral pH buffers on LC–MS.

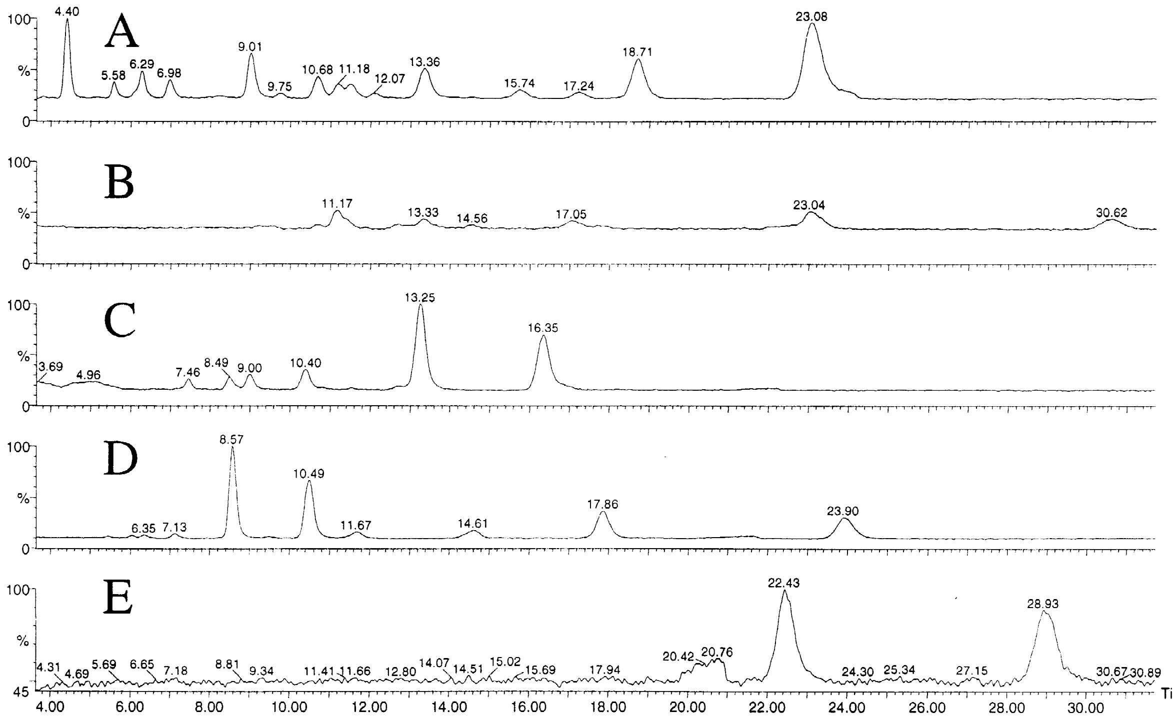

Fig. 4. Chromatograms for racemic compounds A–E following incubation with rat liver microsomes. Traces are total ion chromatograms with ions saved for the respective parent compound plus [M−14], [M+14], [M+16], [M+18] and [M+32] ions. Time scale in min.

The in vitro metabolism of the five analogues was investigated using the chiral HPLC–MS conditions described, from which it was hoped that a single compound could be used for further investigations. Using LC–MS operating in full scan mode from 200 to 500 u, it was shown that the metabolism consisted of [M−14], corresponding to N1-demethylation, [M+16], corresponding to oxidation, [M+18], corresponding to oxidation with concomitant ring opening and [M+32], corresponding to a double oxidation. Since the background signal was high and sensitivity not optimal operating at neutral pH, these were reanalysed operating the mass spectrometer in the SIR mode and monitoring *m/z* ratios corresponding to the ions previously observed (Fig. 4). The sensitivity of mass spectrometric response was poor for B and E and the metabolic turnover of C even after an incubation period of 1 h was low. Reconstruction of the individual ion chromatograms revealed that the predominant metabolism of D corresponded to [M−14] which was of little interest as it was clear that this corresponded simply to an N-demethylation. In contrast A demonstrated a good metabolic profile with a multitude of oxidised products and was selected for further investigation.

The product ion spectrum for compound A was determined at a series of collision energies (Fig. 5). The highest mass ion observed, at *m/z* 301, may be rationalised as being due to the cleavage of the C3 side chain with retention of charge on the benzodiazepine moiety (Fig. 6). The additional fragments are also assigned tentative structures which are consistent with their molecular masses derived from the initial benzodiazepine fragment (Fig. 6) resulting

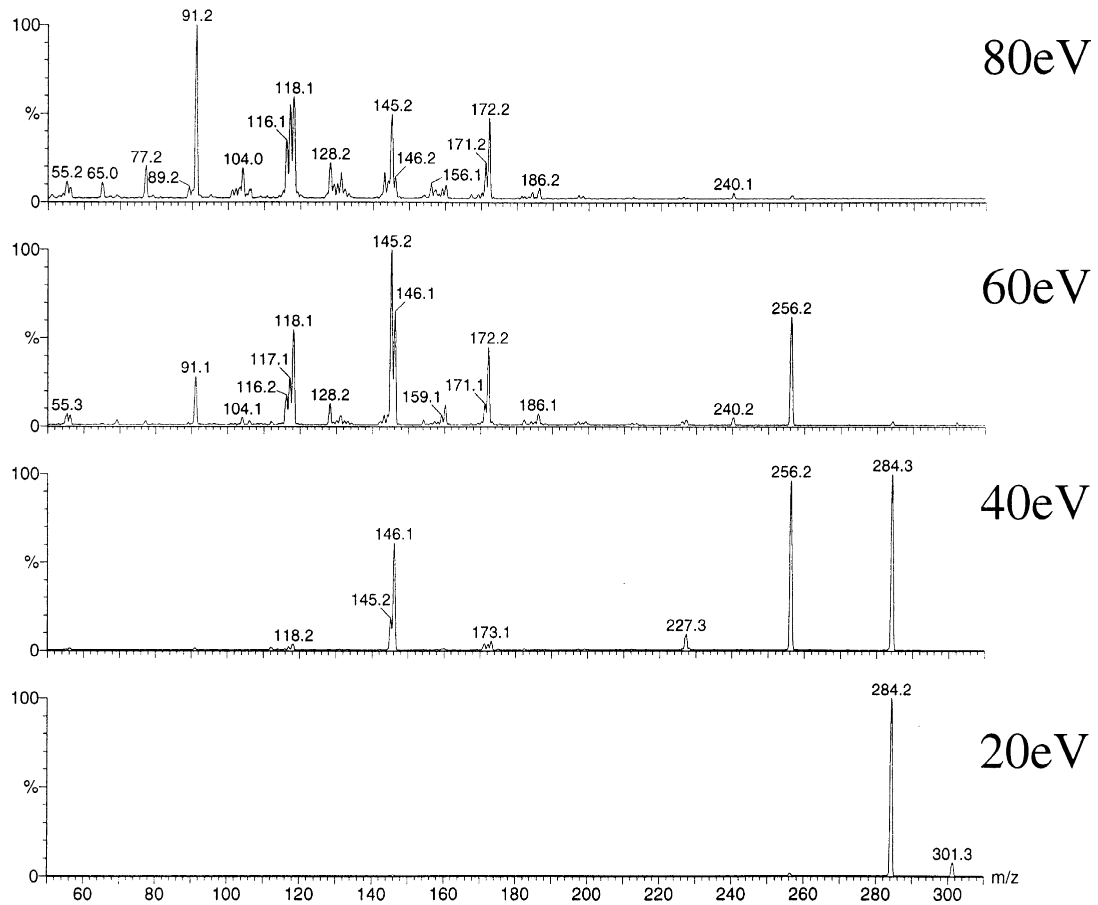

Fig. 5. Product ion spectra for compound A (products of *m/z* 434) conducted at collision energies of 20, 40, 60 and 80 eV.

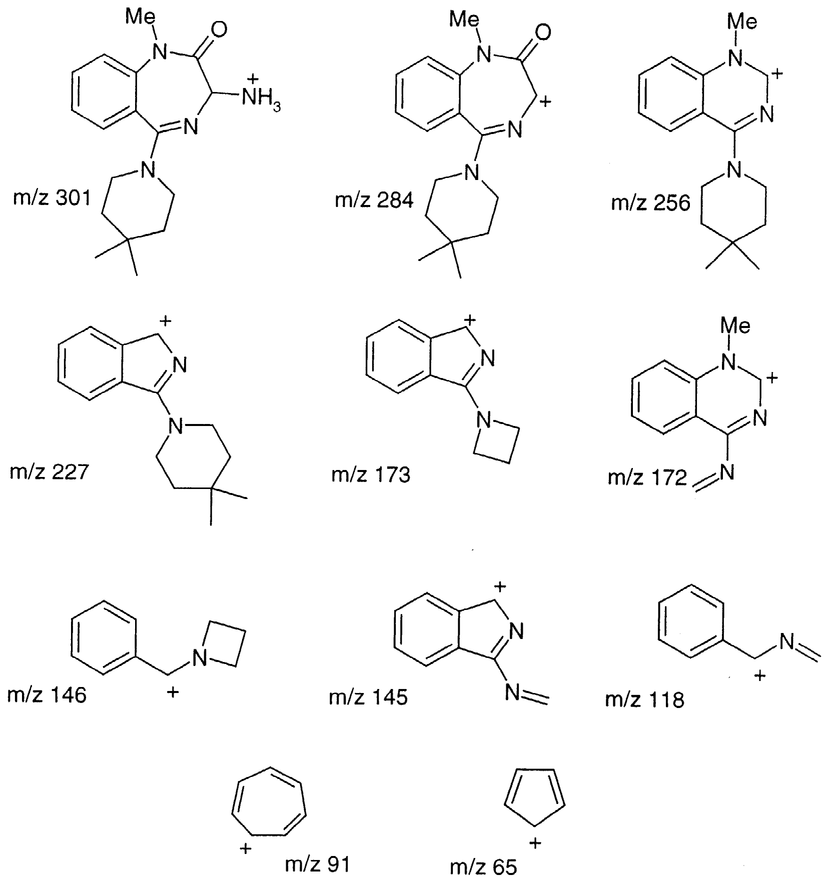

Fig. 6. Proposed CID fragment structures for compound A rationalising the observed product ions.

ultimately in the formation of a tropylium ion. It should therefore be possible to distinguish oxidation on the urea substituent, the benzodiazepine core and the C5 substituent. As a compromise between higher energy collision leading to a large number of low m/z fragment ions and lower collision energy giving higher m/z, more highly abundant but fewer ions, 55 eV was chosen to give the largest range of representative product ions for a single collision energy. The product ion spectra were then recorded for m/z corresponding to [M+16], [M+18] and [M+32] as the identity of the [M−14] ions were already elucidated. It was found, even with sample preconcentration that it was impossible to obtain decent quality product ion spectra for the biotransformed products other than the [M+16] ions presumably due to the well-known sensitivity limitations of the triple quadrupole mass spectrometer in scan mode. It is hoped that this investigation may be carried out in future using a quadrupole time-of-flight mass analysers with a high duty cycle leading to enhanced sensitivity in product ion mode.

The product ions for the [M+16] metabolite peaks of compound A are summarised (Table 3) and the chromatographic peaks giving rise to them (Fig. 7) labelled in elution order M1 to M7. All peaks demonstrate one of only two different product ion spectra with dominant ions of either m/z 256, which is identical to parent, or m/z 272 corresponding to a gain of 16 u on to the benzodiazepine ring (Fig. 8). Furthermore as m/z 145 and m/z 146 ions are seen, this suggests that oxidation has occurred on the C5 cycloalkyl substituent. For the purposes of a model investigation this was disappointing as it was hoped

Table 3
Comparison of presence of absence of ions for metabolites of compound A (M1–M7)

Metabolite	m/z			
	145	146	256	272
M1	✓	✓	×	✓
M2	✓	✓	✓	×
M3	✓	✓	×	✓
M4	✓	✓	✓	×
M5	✓	✓	✓	×
M6	✓	✓	×	✓
M7	✓	✓	✓	×

that a greater variation in product ion spectra would be observed. However, this does mimic a real-life situation in which low-energy collision-induced dissociation (CID) often fail to give the multiplicity of ions from high-energy CID or even from more classical mass spectrometric ionisation techniques such as electron impact. All that can be surmised is that the two dominant peaks M1 and M7 are not enantiomeric as they have different product ion spectra. Only the groups M1, M3 and M6 or M2, M4, M5 and M7 could be enantiomeric as they have the same product ion spectra, but in this case it is impossible to assign any further. Should the CSP be available in racemic form then enantiomeric pairs should collapse to single peaks. Likewise if the CSP was available with the opposite stereochemistry it should be possible to reverse the elution order; however this was not possible as the phases were not commercially available.

In order to definitively assign which products were produced by biotransformation of which enantiomer, metabolic data for the individual enantiomers was required. To attain this approximately 1 mg of each enantiomer was isolated by preparative HPLC using 10% MeOH in 1-chlorobutane on a Pirkle DNBL column (250×20 mm I.D.; flow 20 ml/min). These were incubated in rat liver microsomes as described for the racemic material and analysed in an analogous fashion. The chromatograms for these incubations are shown (Fig. 9) and the peaks labelled according to the nomenclature devised for the

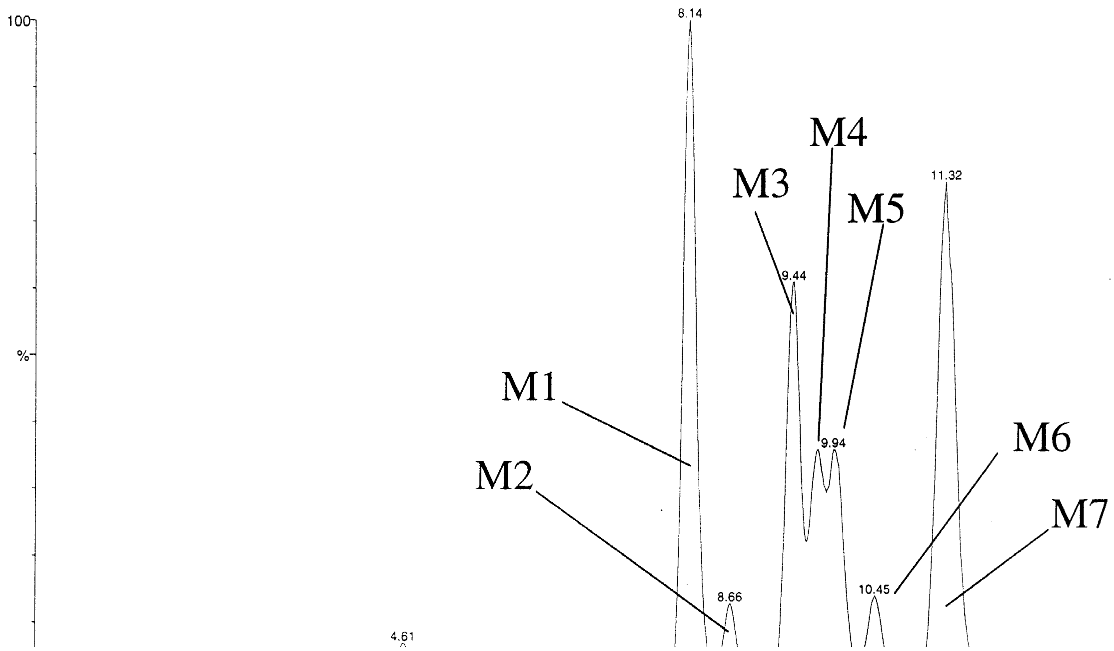

Fig. 7. Chiral LC trace of the single ion chromatogram corresponding to [M+16] for compound A showing of the products of oxidative metabolism produced by incubation of racemic material in rat liver microsomes. Time scale in min.

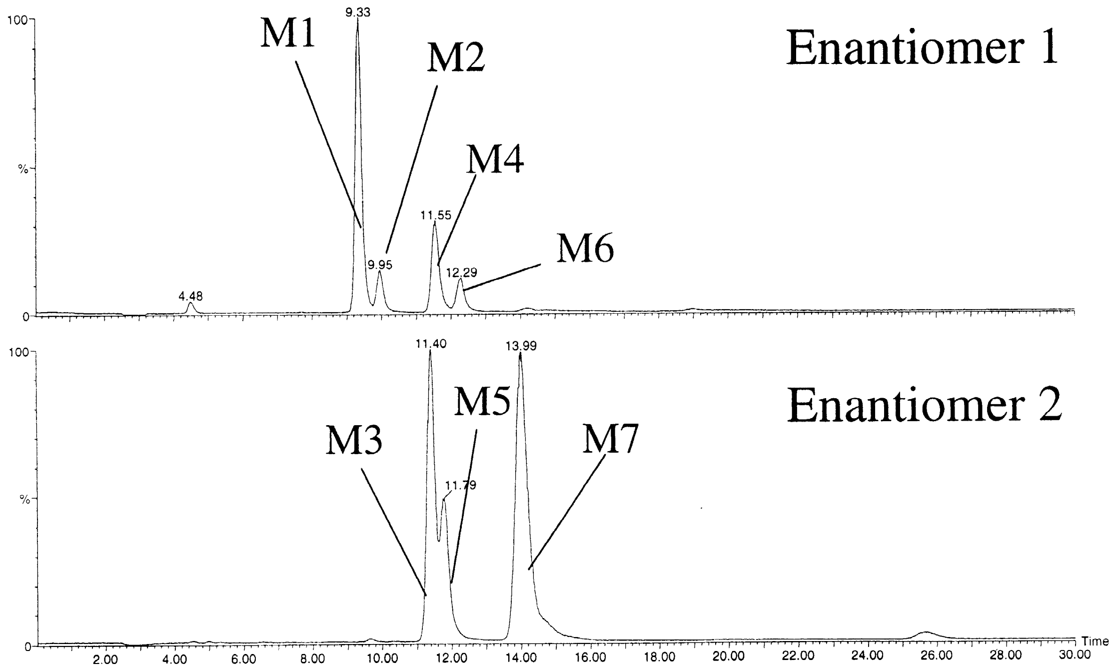

Fig. 9. Chiral LC traces of the single ion chromatograms corresponding to [M+16] for compound A showing of the products of oxidative metabolism produced by incubation of the individual enantiomers of A in rat liver microsomes. Time scale in min.

given the relative simplicity of low-energy CID spectra where the wealth of information from, for example, an electron impact ionisation (EI) spectrum is lost, this is likely to be more of an exceptional rather than a general case.

4. Conclusions

The investigation into the chirality of metabolism of compound A indicates that the technology of converting Pirkle-type CSPs to reversed-phase for compounds of this class to allow mass spectrometric analysis of metabolites is feasible, albeit with a loss of selectivity and resolution over operating in the normal-phase mode. The hope of being able to use product ion spectra as an extra dimension in matching enantiomeric pairs of metabolites to delineate their enantiomeric origin has, in this example, not been realised due partly to the simplicity of the product ion spectra obtained and to the multiplicity of oxidative metabolites formed. Working with racemic material and chiral LC–MS analysis demonstrated that both enantiomers of compound A were metabolised to similar extents hence it should be possible at least to determine whether one enantiomer has a higher metabolic liability than another but the identity of the resulting metabolites may be difficult to gauge without the single isomers or more extensive chromatographic investigations employing alternate CSPs.

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