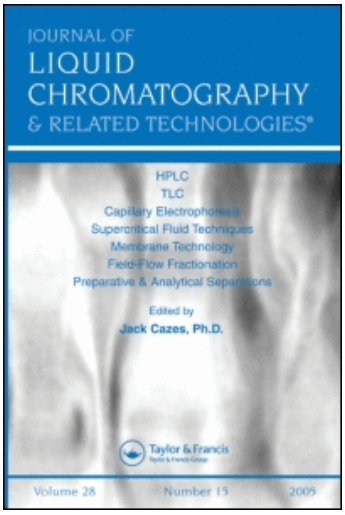

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In Vitro Studies of the Oxidative Metabolism of L-737,415, A C5-Cycloalkylamine-1,4-Benzodiazepin-2-One CCK_B Receptor Antagonist

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IN VITRO STUDIES OF THE OXIDATIVE METABOLISM OF L-737,415, A C5-CYCLOALKYLAMINE-1,4-BENZODIAZEPIN-2-ONE CCK_B RECEPTOR ANTAGONIST

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ABSTRACT

In an effort to investigate and characterise the metabolism of the C5-cycloalkylamine-1,4-benzodiazepin-2-one CCK_B receptor antagonist L-737,415 a study was undertaken using rat liver microsomes. A procedure is described in which the metabolism of the unlabelled compound is firstly investigated analytically under a controlled set of conditions, then scaled up to allow further metabolite elucidation. We also report the use of HPLC with diode array detection, and thermospray LC-MS and LC-MS-MS, to detect and characterise the observed metabolites. From the UV spectra obtained with diode array detection and fragmentation analysis from LC-MS-MS it is demonstrated that, contrary to other known benzodiazepinones such as diazepam or the CCK_B antagonist L-365,260, metabolism seems to occur predominantly at the C5 substituent. Further work in which urine from rats dosed with L-737,415 was analysed indicated that the *in vitro* microsomal assay provides a good model for *in vivo* metabolism for this class of compounds.

INTRODUCTION

Cholecystokinin (CCK) is a 33 amino acid polypetide hormone which occurs in numerous molecular forms throughout both the central and peripheral nervous systems. CCK exerts a variety of actions on peripheral organs, such as regulating pancreatic secretion and gut motility, and may also function as a neurotransmitter or neuromodulator in the CNS [1,2]. The actions of CCK are mediated by two receptor subtypes designated CCK_A and CCK_B [3], with the majority of the central receptors being of the CCK_B subtype.

A number of non-peptidic CCK_B receptor antagonists have been reported. Structures based upon the natural product asperlicin [4] have given rise to a series of 1,4-benzodiazepin-2-ones, including MK-329 and L-365,260 (Figure 1)[5-7]. Further modifications of this latter structure to increase affinity, selectivity and solubility have led to a series of modifications at the C5 position of the benzodiazepine in which the phenyl ring of L-365,260 has been replaced by a cycloalkylamine to generate an amidine. The homopiperidine L-737,415 (Figure 2) is one example from this series showing an improved profile [8].

Typical benzodiazepines, such as diazepam, are known to undergo a number of biotransformations of which N1 demethylation, C3 oxidations and C5-phenyl oxidations are characteristic [9]. Additionally, investigation of the metabolic fate of L-365,260 has demonstrated that in the dog two hydroxylated metabolites were present, namely the 5-(3-hydroxyphenyl) and the N'-(3-methyl-4-hydroxy-phenyl) analogues identified by NMR [10].

EXPERIMENTAL

Materials

L-737,415 was synthesised in-house as previously described [8] with identity and purity confirmed by NMR, MS, HPLC and elemental analysis. Acetonitrile, ammonium acetate and methyl t-butyl ether were of HPLC grade, obtained from Fisons (Loughborough, UK). NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (Poole, UK). TRIS, EDTA, potassium chloride and potassium phosphate were all of Analar

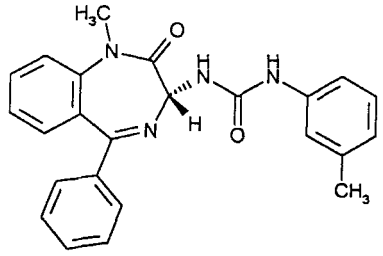

Figure 1. Structure of L-365,260.

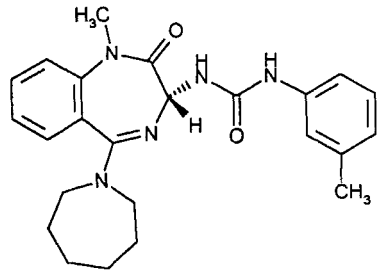

Figure 2. Structure of L-737,415.

grade from BDH (Poole, UK). (+)-Sucrose was from Janssen Chimica sourced through Cambio (Cambridge, UK). Water was of Millipore MilliQ grade and all solvents were filtered using a glass Millipore system with a 0.45μm filter. Male Sprague-Dawley rats (200-250g) were purchased from Bantin and Kingman (Hull, UK).

Instrumentation

An HP1090M series high performance liquid chromatograph (Hewlett Packard, Avondale, USA) was used for HPLC. The system comprised an autoinjector, consisting of a Rheodyne 7010 injection valve fitted with a 250 μl loop, an

autosampler and a DR-5 solvent delivery system. Detection was by UV at 230nm with a bandwidth of 10nm using a built-in linear photodiode array detector saving peak-controlled spectra and data was processed using a 79994A PASCAL workstation. The column was a Spherisorb S5P (250 x 4.6mm i.d.) from Phase Separations Ltd (Deeside, UK) with a mobile phase of 32% MeCN in 100mM NH_4OAc at pH 6.8 and a flow rate of 1.0ml min^{-1}. Analyses were performed at ambient temperature.

Mass spectrometry was performed by interfacing an HP1090L high performance liquid chromatograph (Hewlett Packard, Avondale, USA) to a VG Quattro triple stage mass spectrometer (VG Biotech, Manchester, UK) through a thermospray interface. HPLC mobile phase conditions were modified to 50% MeCN in 100mM NH_4OAc at pH 6.8 and a flow rate of 0.8ml min^{-1}. The source temperature was 250°C and the ion repeller 120V. The capillary was maintained at 240°C with a collision energy of 200V and air as the collision gas in the collision hexapole.

L-737,415 administration

Three male Sprague-Dawley rats (200-250g) were dosed i.v. at 1mg/kg with L-737,415 dissolved in 10mM HCl at 3mg/ml. Animals were allowed water *ad libitum* in a metabowl and urine was collected from t = 0 to 4 hours.

Preparation of microsomes

Whole livers, each weighing approximately 10g, were obtained from freshly euthanised male Sprague-Dawley rats. The livers were minced with scissors and homogenized in 25ml of cold 50mM TRIS containing 1.15% potassium chloride, pH 7.4, using a Potter-Elvehjem teflon pestle homogeniser. The homogenate was centrifuged at 40,000g at 4°C for 20 min and the supernatant portion filtered through cheesecloth and then re-centrifuged at 150,000g at 4°C for 60 min. The supernatant was discarded and the microsomal pellet suspended in 25ml of cold 10mM EDTA containing 1.15% potassium chloride, pH 7.4, and then re-centrifuged at 150,000g at 4°C for 60 min. The supernatant was discarded and the final microsomal pellet resuspended in 10ml of cold 10mM potassium phosphate buffer containing 250mM sucrose, pH 7.4.

Microsomal Incubations

Microsomal incubations were conducted at pH 7.4 under aerobic conditions at 37°C. Initially 100µl of a 250µM dimethylsulphoxide solution of L-737,415 (i.e. 10µg) was incubated with 100µl of microsomes (ca. 2mg protein) in the presence of a NADPH generating system consisting of 100µl of 50mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 100µl of 10mM NADP and 100µl of 50mM magnesium chloride. The mixture was made up to a final volume of 1ml with 100mM potassium phosphate buffer, pH 7.4, giving a final concentration for L-737,415 of 25µM.

To produce sufficient quantities of metabolites for structural identification the incubation was scaled up with 13mg of L-737,415 being incubated with 8ml of microsomes in a total volume of 100ml. The a final concentration for L-737,415 was 310µM and the concentrations of the other reagents the same as in the initial incubation.

Sample preparation

Samples from the initial incubation were prepared for HPLC analysis by taking a 100µl aliquot at the desired time and quenching metabolism by the addition of an equal volume of acetonitrile. Samples taken at t=0, 30, 60 and 240 mins and samples were stored at -20°C prior to analysis by HPLC. Vortex mixing and centrifugation of this sample separated the denatured protein from the supernatant. The supernatant could then be directly injected onto the HPLC system. Parent compound and metabolites were extracted from both the scaled-up microsome incubation and the urine using liquid-liquid extraction. Typically, 5ml of sample was taken and basified by addition of 100µl 0.1M NaOH, then extracted by vortex mixing with 10ml MTBE. After centrifugation the organic layer was removed and evaporated under a stream of dry nitrogen. Samples were reconstituted in mobile phase and injected directly.

RESULTS

HPLC analysis of parent compound in the initial incubation showed L-737,415 to be rapidly metabolised under oxidative microsomal conditions with 25µM

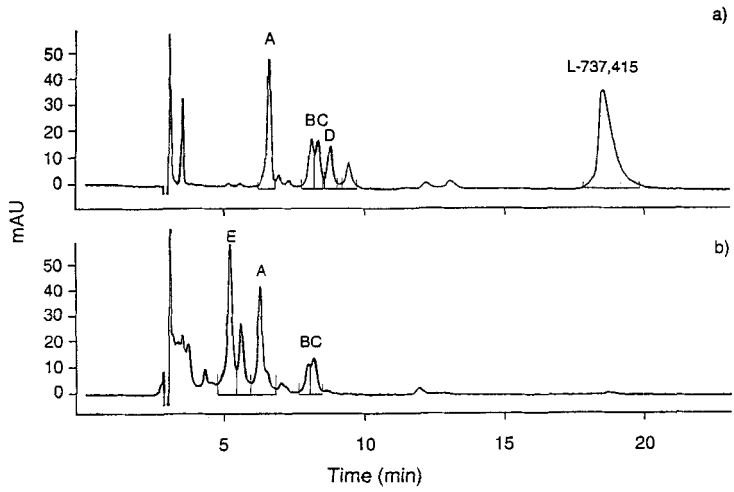

Figure 3. HPLC analysis of L-737,415 metabolites: (a) microsomal metabolites; (b) urinary metabolites. For conditions see text.

substrate. After 60 min, only 10% of the parent compound remained unmetabolised and at 240 min no parent compound was detected.

In order to obtain sufficient material for LC-MS and LC-MS-MS the experiment was scaled-up as described above. At this higher concentration the proportion of parent compound metabolised was lower, but significant concentrations of metabolites were present after 4 hours. Samples were initially analysed using HPLC with diode array detection. Four main groups of peaks were identified from the chromatograms as being potential metabolites and were designated A,B,C and D (Figure 3a). By saving peak controlled spectra using the diode-array detector, we were able to obtain some spectral information on these peaks. The UV spectrum of L-737,415 is characterised by having a λ_{max} at 238nm and an extended UV absorption to above 310nm (Figure 4a). The UV spectra of metabolites A, B, C and D (Figure 4b) are essentially identical to that of L-737,415. This indicates that metabolism is not affecting the chromophore of the molecule, hence aromatic oxidation is unlikely to be a significant metabolic pathway.

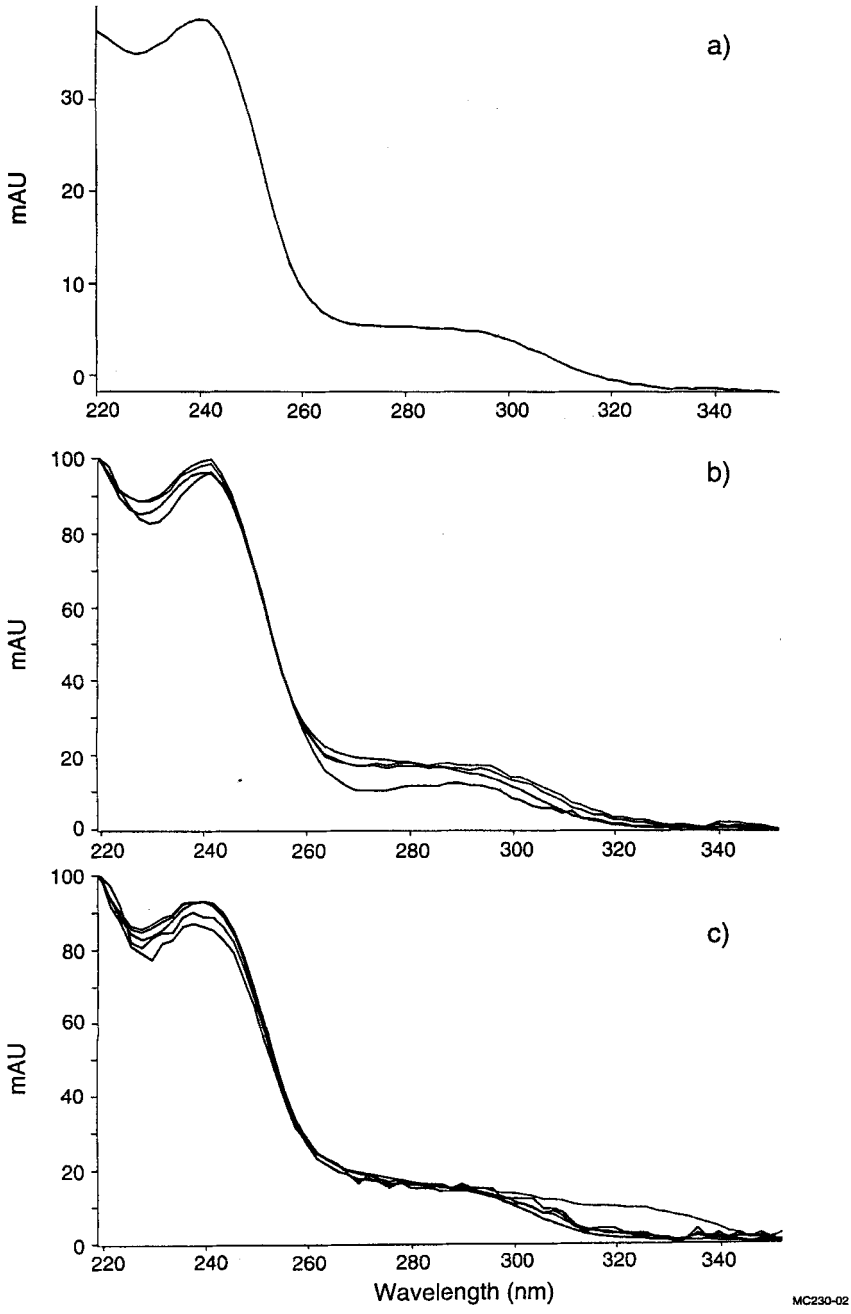

Figure 4. UV spectra of L-737,415 and its metabolites: (a) L-737,415; (b) microsomal metabolites; (c) urinary metabolites.

No parent compound was detected in the extract of combined urine. A number of metabolites were identifiable by HPLC-diode-array of which A, B and C correspond to those produced *in vitro* (Figure 3b). UV spectra of these metabolites again indicate their close relation to parent compound (Figure 4c). A further additional metabolite was seen only in the urine and was designated E. The presence of the metabolites A, B and C in both samples is good evidence that the major metabolic pathways are mediated through hepatic oxidation.

The fragmentation pathway for L-737,415 was elucidated using LC-MS-MS daughter ion scans of the molecular ion m/z 420. It was found that with 50V collision energy only a small amount of fragmentation was observed (Figure 5a) but by increasing the collision energy to 200V further fragments were obtained (Figure 5b) allowing the complete assignment of the pathway (Figure 6). Essentially the fragmentation of L-737,415 involved loss of the tolyl-urea moiety [M - 150] followed by loss of CO with concomitant ring contraction [M - 178]. Finally, the homopiperidine ring was lost [M-275] to produce a fragment of m/z 145.

Full scan LC-MS was performed in order to determine molecular ions for the metabolite peaks. Metabolite A was found to be [M + 14], metabolites B and C [M + 16], metabolite D [M + 18] and metabolite E [M + 30]. The fragmentation pathways of these metabolites were elucidated using LC-MS-MS daughter ion scans of the molecular ion and major fragments. The results from these studies are summarised (Table 1). Crucially the mass differences between the parent and metabolite fragments were maintained until the final fragmentation, leading to a fragment of m/z 145 for A, B, D and E and m/z 160 for metabolite C. This clearly demonstrated the position of metabolism to be on the cycloalkylamine ring.

DISCUSSION

Structures for each of the metabolites are postulated based on the observation that metabolism occurs at the C5 cycloalkylamine ring (Figure 7). Metabolite A, with [M + H] of m/z 434, is 14 amu heavier than parent compound and is likely to correspond to a lactam. Metabolites B and C, with [M + H] of m/z 436, are 16 amu heavier than parent compound indicating ring hydroxylations. Metabolite D, with [M + H] of m/z 438, is 18 amu heavier than parent compound indicating formation of a ring opened amino alcohol. This conclusion is supported by the benzylic cleavage of the secondary amine to form the m/z 160 rather than the usual

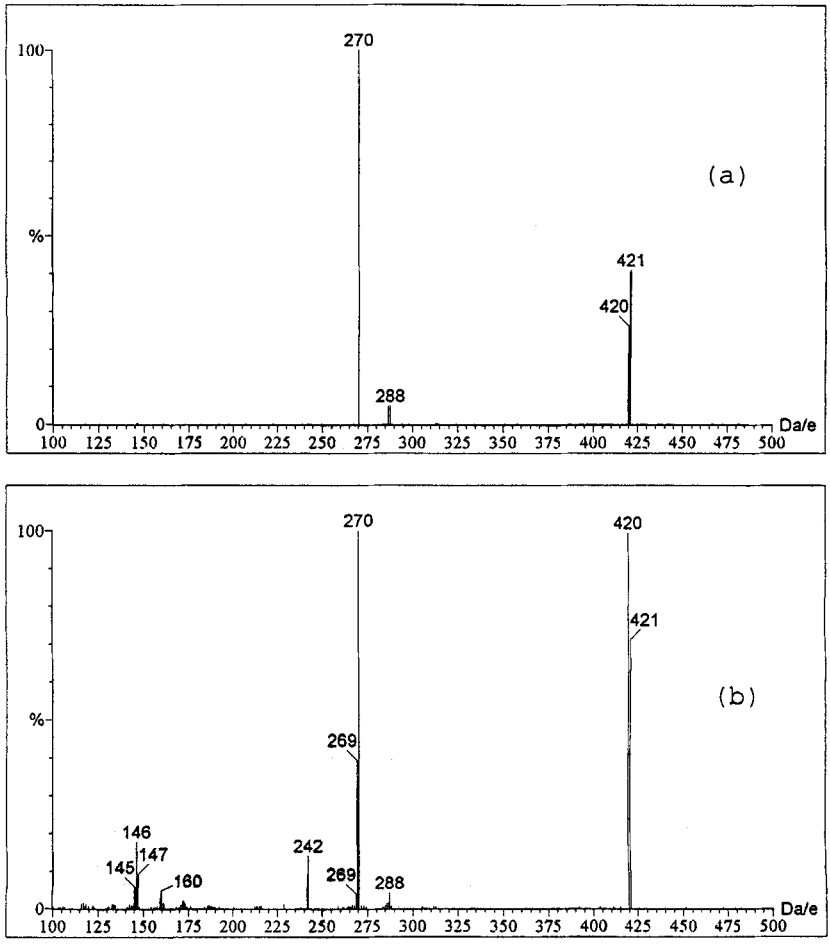

Figure 5. Daughter ion mass spectra of L-737,415 of m/z 420 at (a) 50V and (b) 200V collision energy

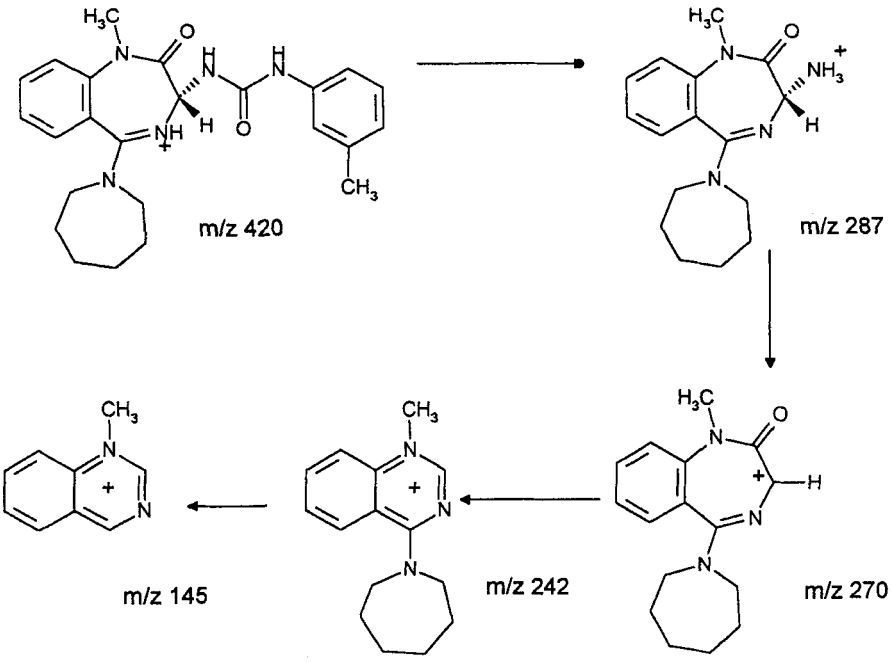

Figure 6. Fragmentation pathway for L-737,415

TABLE 1

Summary of daughter ions observed for L-737,415 and its metabolites

Identity	Molecular ion $[M+H]^+$	Pre-urea fragment	Urea cleavage	Benzo-diazepine	Loss of carbonyl	Loss of amidine
Parent	420	313	287	270	242	145
A	434	327	301	284	256	145
B	436	329	303	286	258	145
C	436	329	303	286	258	145
D	438	331	305	288	260	160
E	450	343	317	300	N.O.	145

N.O. = not observed

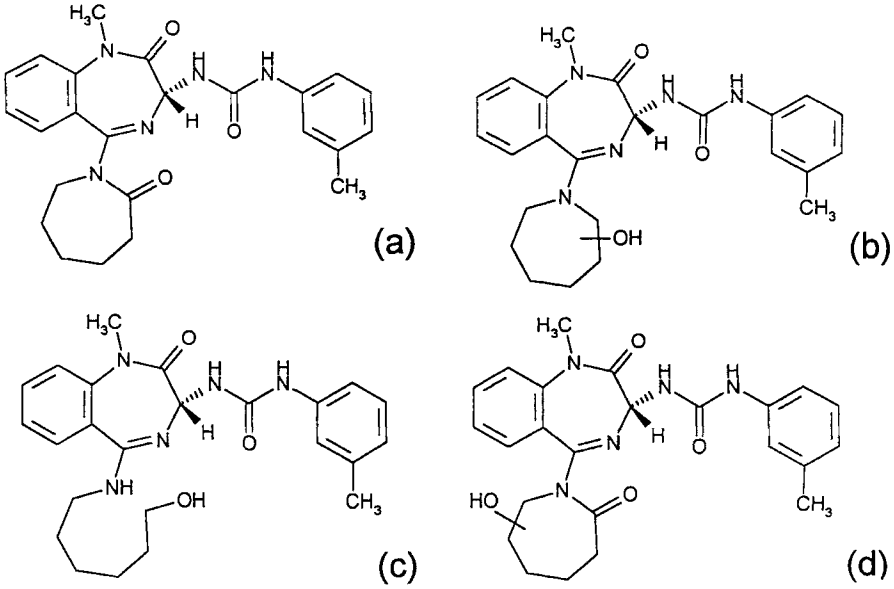

Figure 7. Proposed metabolites of L-737,415; (a) metabolite A, (b) metabolites B and C, (c) metabolite D and (d) metabolite E

m/z 145 ion. Metabolite E, found only in the urine, has [M + H] of m/z 450 and is 30 amu heavier than parent compound. By analogy with the other metabolites, and by consideration of the greater polarity of this compound by HPLC, this is consistent with a hydroxylated lactam.

The evidence consistently suggests that the metabolism of L-737,415 proceeds through oxidative modification of the C5 cycloalkylamine ring, to yield the metabolites A, B, C, D and E (Figure 7). The lack of an exact match of *in vitro* and *in vivo* profiles is perhaps not surprising considering the restricted number of metabolic reactions available *in vitro* compared to the *in vivo* situation. In particular the absence of metabolite D, and the presence of E, in the urine is indicative of metabolism occurring to a greater extent *in vivo* than *in vitro*.

In conclusion, this study has demonstrated the use of scaled-up liver microsomal metabolism assays to produce sufficient quantities of metabolites for structural identification, and the application of LC-MS-MS to obtain rapid information on

the metabolic fate of non-radiolabelled compounds in a pre-development research environment. This provides us with a useful tool for guiding the production of alternative analogues.

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