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Assessment of [^{18}F]fluoroethylflumazenil metabolites using high-performance liquid chromatography and tandem mass spectrometry

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Abstract

A simple procedure using HPLC and tandem mass spectrometry has been developed for the determination of fluoroethylflumazenil metabolites. Samples were precipitated with acetonitrile, evaporated to dryness followed by reconstitution with methanol. As mobile phase, 50 m*M* ammonium formate–methanol (58:42, v/v) was used. The method is valid both for cold and radiolabelled metabolites. Various cold metabolites (hydroxylated and/or dealkylated) were identified in rat and human microsome preparations. Radiolabelled metabolites arise from two or more transformations including hydroxylation. The methodology developed can be applied for further characterisation of metabolites, and for the determination of non metabolised [^{18}F]fluoroethylflumazenil in routine clinical analysis.

Keywords: [^{18}F]Fluoroethylflumazenil; Metabolite; Mass spectrometry

1. Introduction

Flumazenil (Ro-1788, Anexate®) is a well-known inverse agonist of the benzodiazepine receptor. It can be radiolabelled with ^{11}C and it has been widely used for mapping central benzodiazepine receptors in vivo, using positron emission tomography (PET) [1–5]. ^{11}C-Flumazenil has several drawbacks, such as a short physical half life (20 min) and a short biological half life due to a rapid in vivo metabolism, which leads to the formation of various radiolabelled metabolites [6–8].

To overcome these difficulties, the synthesis of a ^{18}F-radiolabelled analogue of flumazenil {[^{18}F]fluoroethylflumazenil, or 5-[2′-^{18}F]fluoroethyl-flumazenil(ethyl-8-fluoro-5,6-dihydro-5-[2′-(^{18}F)-fluoroethyl]-6-oxo-4H-imidazo[1,5*a*]benzodiazepine-3-carboxylate} was first proposed by Moerlein and Perlmutter [9,10], and further investigated by others [11]. It was shown that [^{18}F]fluoroethylflumazenil (^{18}FEF) possesses a very high affinity for the benzodiazepine receptor. These previous studies

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demonstrated the appearance of radiolabelled metabolites after intravenous (i.v.) administration to baboons. However, up to now, no data are available on the structures of the ^{18}FEF metabolites.

In order to validate the usefulness of ^{18}FEF as a radiotracer that allows quantification of brain benzodiazepine receptors in man, one must know the fate of the radiolabelled metabolites. It is necessary to clearly demonstrate that the metabolites will not introduce artefacts in the in vivo measurements. For example, the lipophilicity is an important feature in order to predict if a metabolite can cross the blood–brain barrier and accumulate in the brain.

In this study, we report a method for the identification of cold fluoroethylflumazenil (FEF) metabolites using high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS–MS). We first studied the in vitro transformation of FEF in rat and human liver microsomal preparations, and attempted to elucidate the structure of the separated metabolites. We also studied the metabolisation pattern obtained with the radiolabelled molecule (^{18}FEF) under the same incubation conditions.

2. Experimental

2.1. Chemicals

FEF was synthesised from fluorotosylethane and desmethylflumazenil in anhydrous acetonitrile, according to a previously published scheme [9].

^{18}F-FEF was synthesised according to the same procedure, using ^{18}F-fluorotosylethane. $^{18}F^-$ was obtained from the IBA Cyclone 30 cyclotron of the Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

Methanol and acetonitrile were purchased from Fluka (Bornem, Belgium, HPLC grade).

Isocitrate dehydrogenase, isocitric acid and cofactors (NADP, NADPH) were all from Sigma (Bornem, Belgium).

Others chemicals were of analytical grade from Merck.

2.2. Enzyme preparation

Dexamethasone (10 mg/kg, i.p.) was injected into male Wistar rats (Animalerie Facultaire, UCL Brussels, Belgium) for 5 days. Rats were killed by decapitation on day 6, and their livers were sampled for the preparation of microsomes, according to Amar-Costesec et al. [12].

Human liver was obtained from an organ donor (St. Luc University Hospital, Brussels, Belgium), and microsomes prepared as follows.

Small slices of liver were homogenised with a Potter Elvehjem in a volume of iced 0.1 *M* phosphate buffer (pH 7.4, 0.1 *M* KCl, 1 m*M* EDTA) corresponding to three times the mass of liver. Homogenate was then centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was collected and centrifuged a second time at 17 000 *g* for 60 min at 4°C. The supernatant was discarded, and the pellets were resuspended in the iced buffer, and kept at −80°C until used.

Protein content was estimated by the method of Bradford [13], and the P450 concentration by the method of Omura and Sato [14].

2.3. Incubation

The incubation mixture consisted of 8.3 mg (rat) or 6 mg (human) microsomal protein in 100 m*M* sodium phosphate buffer (pH 7.4, final volume 2 ml) in the presence of an NADPH generating system (1 m*M* NADP, 1 m*M* NADPH, 50 m*M* isocitrate, 5 m*M* $MgCl_2$, 1 U of isocitrate dehydrogenase) and was kept at 37°C for 5 min before adding the substrate, a methanolic solution of FEF (final concentration 10 μ*M*) or ^{18}FEF. The final methanol concentration was under 1%.

The reaction was terminated by adding an equal volume of acetonitrile. The denatured proteins were removed by centrifugation (5 min, 1000 *g*). A 300-μl volume of supernatant was evaporated to dryness under vacuum, then redissolved in 100 μl of methanol. A 20-μl volume was injected for HPLC analysis.

2.4. Instrumentation

Separation of metabolites was performed using a TSP (Thermo Separation Products) HPLC system consisting of an autosampler (AS3000), a pump (P1000xr), and a UV detector (UV1000) operating at 240 nm. We used a C_{18} Alltima column (125×4.6 mm; Alltech, Laarne, Belgium) equipped with a

guard column. The mobile phase was a 50 m*M* ammonium formate (pH 7)–methanol (58:42, v/v) solution, pumped at a flow-rate of 1 ml/min.

A TSQ7000 triple quadrupole (Finnigan MAT, Palo Alto, CA, USA) with an atmospheric pressure chemical ionization (APCI) ion source was used for detection and for tandem mass spectrometry to obtain MS–MS spectra. The operating conditions were: capillary temperature 220°C, vaporisation chamber 400°C, sheath gas (N_2) 70 p.s.i., auxiliary gas (N_2) 10 p.s.i., corona discharge 5 μA (1 p.s.i.= 6894.76 Pa). Collision gas for MS–MS was xenon at 0.8 mTorr (1 Torr=133.322 Pa).

For the separation of the radiolabelled metabolite, we used a HPLC system from Gilson (Villiers-le-Bel, France) consisting of a pump (Gilson 302) a manometric module (Gilson 805), a high-pressure mixer (Gilson 811C), a variable-wavelength UV detector operating at 240 nm (Gilson 118), a Rheodyne injection valve, and a fraction collector (Pharmacia ReadyFrac).

Eluent was collected every 30 s, and counted in a Berthold NaI well counter (Berthold Gammazint, Perkin-Elmer Life Science, Turku, Finland).

3. Results and discussion

3.1. Separation conditions

Among the various ionisation modes used in MS, APCI offers the possibility of using conventional 4.6 mm I.D. column at a normal flow-rate (1 ml/min). Using this system, it is necessary to use a volatile mobile phase. The choice of buffer is limited to volatile salts such as ammonium acetate or formate. Consequently we used a high-purity silica-based C_{18} column which allows for a sufficient separation of our metabolites, without excessive tailing.

Typical chromatograms obtained for rat and human preparations are shown in Fig. 1.

The full MS scan gives for each peak its protonated molecular ion (Table 1) and also shows that there were no co-elutions of the peaks of interest.

From the comparison of the chromatograms (Fig. 1), one can conclude that the metabolisation in vitro is less extensive in human microsomes than in induced rat microsomes. Human microsomes give rise to the formation of four metabolites whereas rat microsomes give eight products (Table 1).

3.2. MS–MS analysis of standards

Mass spectra are valuable tools that can help in the elucidation of chemical structures.

As no MS–MS spectra are available in the literature, either for FEF or flumazenil (FMZ), we first recorded the fragmentation spectrum of standards of known structures [i.e., FMZ, desmethylflumazenil (DFZ) and FEF (Fig. 2)].

Looking at the main fragments for desmethylflumazenil ($[MH^+]=290$) (Fig. 2), we note a first fragment at m/z 261.8, resulting from a loss of 28 Da from the pseudomolecular ion. A further loss of water gives rise to the fragment at m/z 244. This sequence most probably affects the ester lateral chain: the 28 Da loss can be the ethyl moiety of the ester lateral chain. Then, a loss of water gives the fragment at m/z 244 which is thought to be the acylium ion. Another loss of 28 Da (probably CO^+) leads to the m/z 217 ion.

This scheme is also found with flumazenil (Fig. 2): pseudomolecular ion ($[MH^+]=304$) gives a fragment at m/z 276 (28 Da loss), a further loss of water giving the acylium ion at m/z 258. Then a loss of 28 Da (CO^+) is found again.

The same pattern is observed for fluoroethylflumazenil (Fig. 2) (fragmentation of the ester lateral chain giving the acylium ion at m/z 290). It shows two other interesting fragments: a first product ion at m/z 270 that may be a loss of 20 Da from the acylium ion. This is probably due to the loss of HF. As this is not seen with the other two standards, which lack a fluorine on the N-alkylated chain of the diazo ring, HF probably comes from that very chain.

A fragment at m/z 246 is also seen and matches with a loss of 44 from the acylium ion. It can result from a retro Diels–Alder reaction in the phenyl ring.

3.3. HPLC–MS–MS analysis of metabolites

In order to elucidate the structure of the isolated metabolites, we first deduced a probable molecular mass from the protonated molecular ion measured, and proposed a structural modification based on a theoretical scheme (e.g., hydrolysis, hydroxylation, etc.).

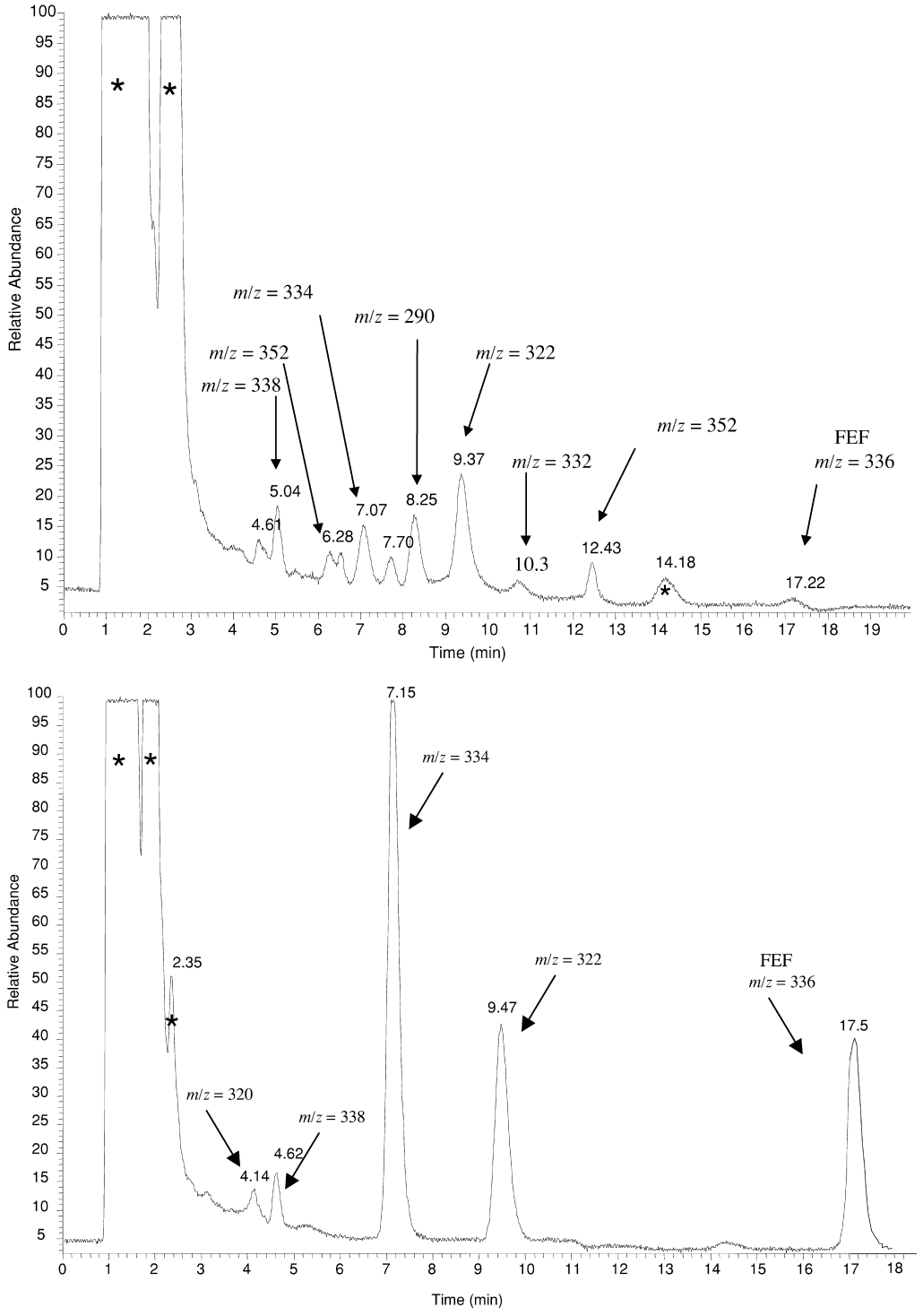

Fig. 1. UV chromatogram obtained from rat microsomal preparation (top) and human microsomal preparation (bottom). * Labelled peaks are matrix peaks.

Table 1
Detected peaks by HPLC and their m/z [MH^+]

Peak	Retention time	m/z [MH^+]	Rat microsomes[a]	Human microsomes[a]
1	17 min 50 s	336 (FEF)	+	+
2	12 min 30 s	352	+	−
3	10 min 30 s	332	+	−
4	9 min 30 s	322	+	+
5	8 min 30 s	290	+	−
6	7 min	334	+	+
7	6 min 30s	352	+	−
8	5 min 4 s	338	+	+
9	4 min 30 s	320	+	+

[a] "+" Stands for present and "−" for not present in the preparation.

Then we compared the individual fragmentation patterns of the metabolites with the spectra of our standards (FEF, FMZ and DFZ). This allows us to verify if our theoretical hypothesis is realistic or not, and to establish which part of the molecule is affected by the biotransformation.

Table 1 shows two HPLC peaks (peaks 2 and 7) with a protonated molecular ion at m/z 352, but eluting at different times. These can be hydroxylated metabolites, since a hydroxylation is consistent with the measured 16 Da increase of mass compared to FEF.

HPLC peak 2 displays the fragmentation already noted with the standards (Fig. 3A): loss of the ethyl chain (28 Da to yield m/z 324) followed by a dehydration leading to the acylium ion (m/z 306). A further loss of HF gives the fragment at m/z 286. This brings us to the conclusion that the ester lateral chain is probably not modified, nor is the N-alkylated chain of the diazo ring. Hydroxylation is the most probable transformation, possibly in position No. 3 of the diazo ring, a classical position for hydroxylation in benzodiazepine.

The fragmentation scheme for the other HPLC peak with a m/z of 352 is different. Fig. 3B presents a fragment (m/z 334) resulting from a direct dehydration of the protonated molecular ion. A fragment is observed at m/z 308 which results from a loss of 46 Da, possibly ethanol, followed by a dehydration giving the acylium ion (m/z 290). The latter can lose HF (20 Da) giving the m/z 270. A retro Diels–Alder from the acylium ion is also possible, and results in the loss of 44 Da to yield the m/z 247 ion. This is consistent with a hydroxylation on the ester lateral chain.

For peak 3 (m/z=332, Fig. 3C), it is difficult to propose a structure, since the variation of mass from the parent compound (FEF, m/z=336) is only 4 Da, and does not correspond to a single transformation, but more likely to two. Nevertheless, one can see from the fragmentation scheme that the ester lateral chain is not modified since we found the usual pattern due to loss of fragment from this lateral chain (Fig. 3C) leading to the formation of the acylium ion (m/z 286). A loss of 44 Da from retro Diels–Alder is noted (m/z 241) but there is no loss of HF (20 Da) indicating that the N-alkyl chain may be modified. This compound is present in the preparation of rat microsomes, but not in human ones.

HPLC peak 4 elutes at 9 min 30 s and possesses an m/z of 322. The usual pattern corresponding to the progressive loss of the lateral chain is not found in the MS spectrum (Fig. 3D). A first loss of 14 Da (m/z 308), followed by a loss of water giving the acylium ion at m/z 290. It can be summarised as a global loss of methanol. The rest of the spectrum is homologous to standard FEF.

The HPLC peak 5 eluting at 8 min 30 s and giving a measured [MH^+] at m/z 290, was identified as the desmethylflumazenil, resulting from N-defluoroethylation of the diazo ring, as the fragmentation pattern is identical to the MS–MS spectrum of a the standard reference (Fig. 3E). It is absent from the human microsome preparation.

Peak 6 eluting at 7 min gives a m/z at 334.

A direct loss of water from the protonated molecu-

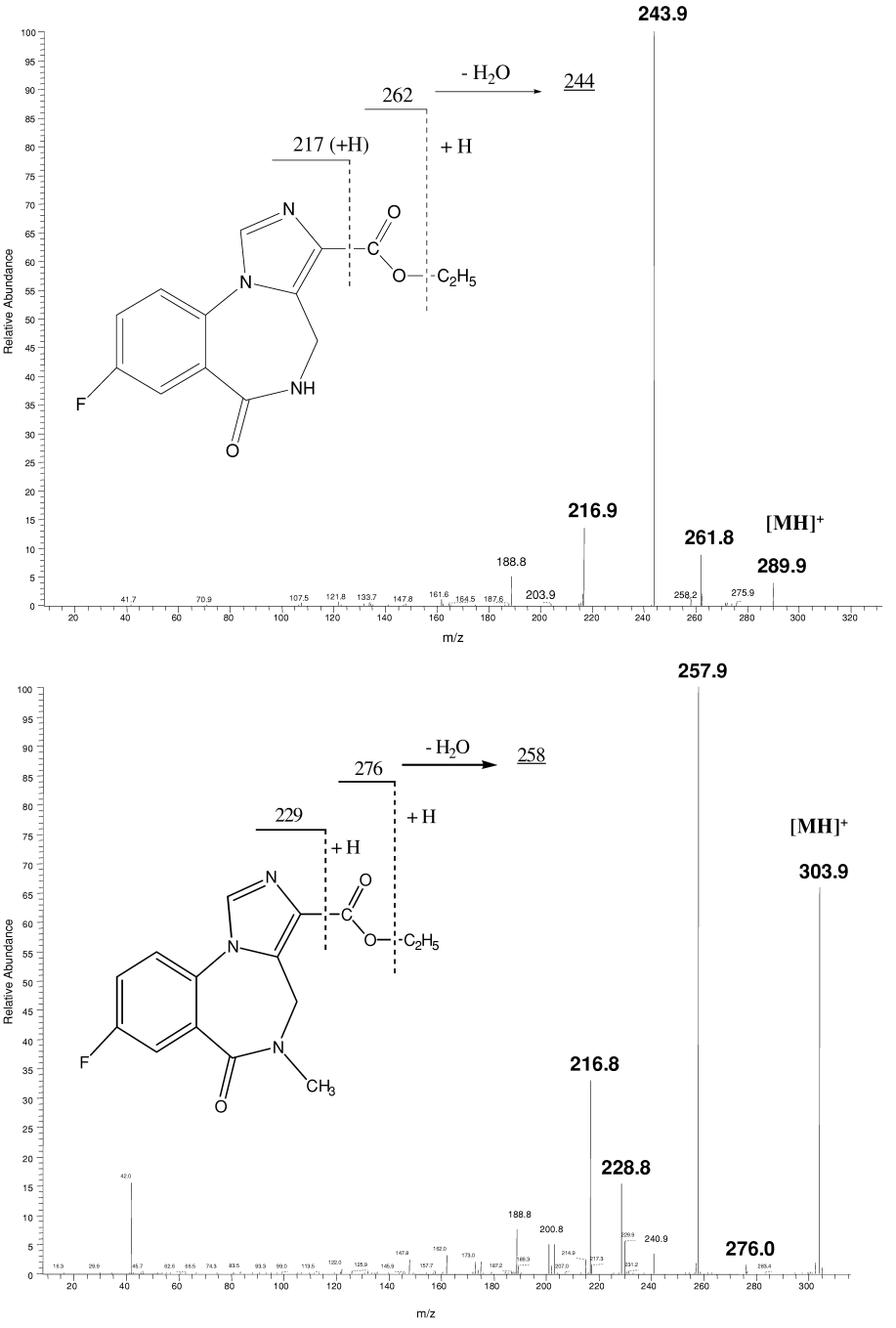

Fig. 2. MS–MS spectra of standards: desmethylflumazenil (top), flumazenil (centre), fluoroethylflumazenil (bottom).

lar ion is observed (m/z 316, Fig. 3F). This was not seen with standards, suggesting the presence of a hydroxylated compound. The ester lateral chain fragmentation pattern is observed so that this part of the molecule is not modified. The acylium ion (m/z 288) loses an additional molecule of water (m/z

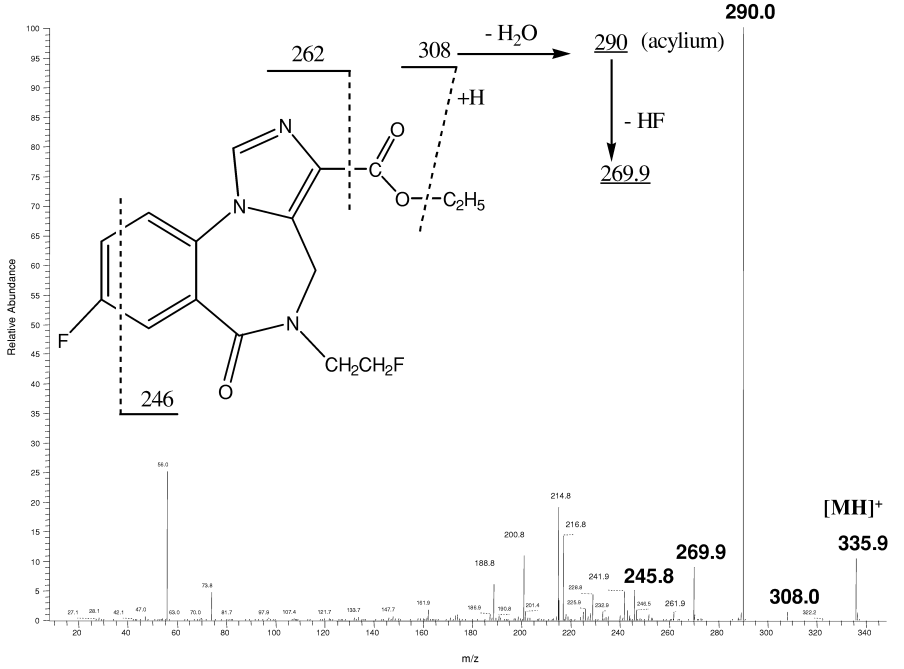

Fig. 2. (*continued*)

270), and a loss of 44 Da due to a retro Diels–Alder reaction is noted (m/z 244). On the other hand, the loss of HF (20 Da) is not observed, suggesting that the N-alkylated chain does not contain the fluorine atom anymore. One can conclude that this molecule is hydroxylated, and that the fluorine on the N-alkyl chain is removed. Such a molecule would have a protonated molecular ion at m/z 334, which matches with the actual measured [MH^+]. Furthermore it is likely that this hydroxylation takes place on the N-alkyl chain.

For peaks 8 and 9, we did not observe the gradual fragmentation of the ester lateral chain (Fig. 3G and H), or a loss of HF (20 Da). The mass of the molecular ion for these two peaks is not consistent with a single chemical modification. It is more likely that two modifications have occurred, affecting both the ester lateral chain and the N-alkyl chain of the diazo ring.

3.4. Metabolisation of [^{18}F]fluoroethylflumazenil

Comparing the HPLC chromatograms (Fig. 1) with the radiochromatogram obtained using radiolabelled substrate (^{18}FEF) in the human microsome preparation (Fig. 4), only two radiolabelled metabolites were detected, possibly three. Based on respective retention times, the first radiolabelled peak could correspond to peak 9 (m/z=320), or peak 8 (m/z=338), or both, since the separation between these two is not optimal. The second radiolabelled peak is peak 4 (m/z=322). Interestingly, there is no peak between peak 4 and peak 8, where the compound with m/z=334 was expected. Therefore, we conclude that a transformation on the fluoroethyl chain has occurred, as this metabolite is not radiolabelled. It confirms the hypothesis made on the basis of the MS–MS analysis.

We can also note that the second radiolabelled peak elutes rather late, and that it could have a high lipophilicity. This is an important parameter since it could potentially cross the blood–brain barrier, and possibly interact with brain receptors, as this compound still possess a benzodiazepine structure.

In radiochromatograms obtained from rat microsomes preparation, more radiolabelled metabolites were observed (Fig. 4). The two most intense peaks show retention time close to those found in human

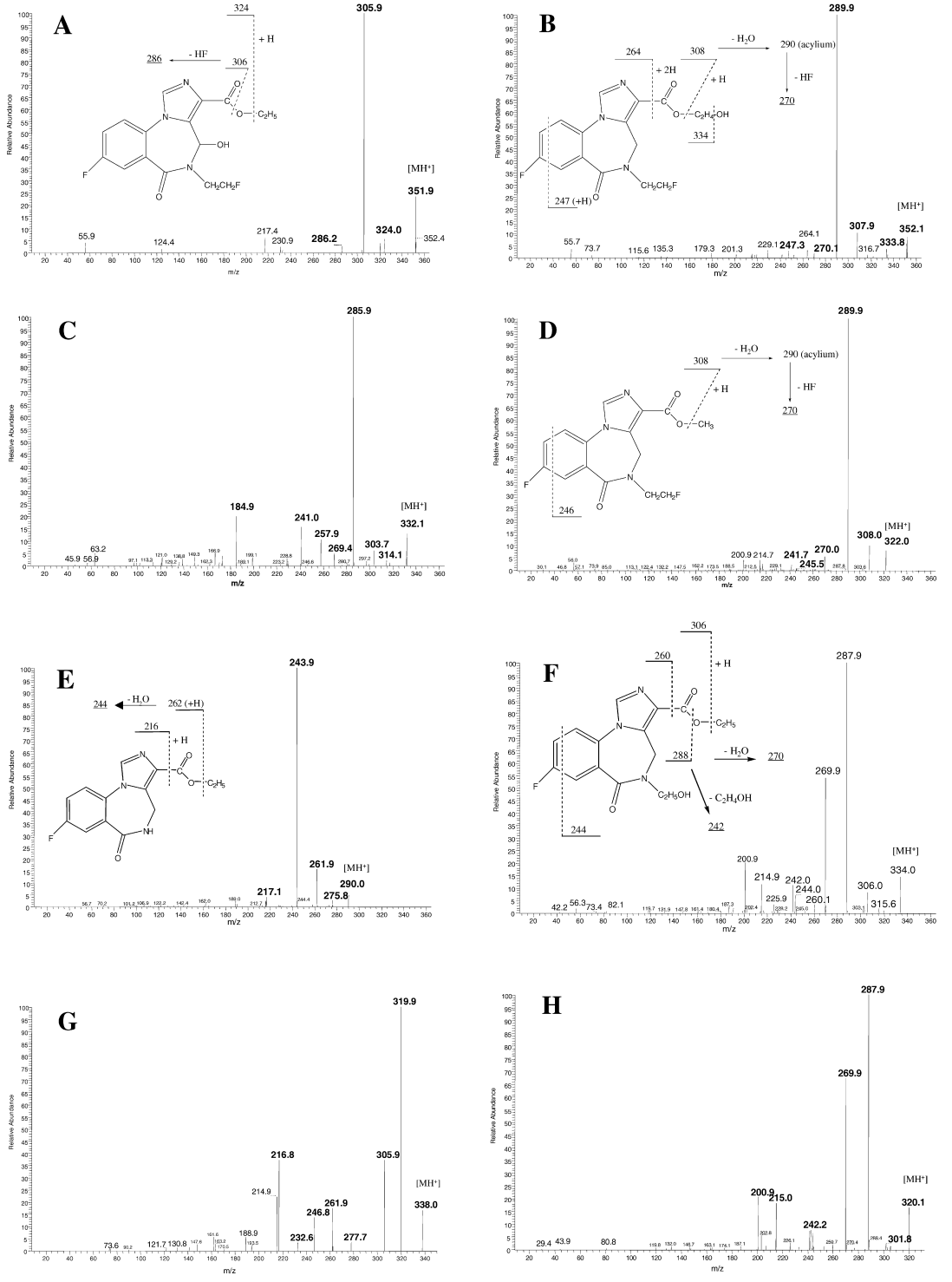

Fig. 3. MS–MS spectra of metabolites, and proposed structures. (A) Peak 2 (m/z=352); (B) peak 7 (m/z=352); (C) peak 3 (m/z=332); (D) peak 4 (m/z=322); (E) peak 5 (m/z=290); (F) peak 6 (m/z=334); (G) peak 8 (m/z=338); (H) peak 9 (m/z=320).

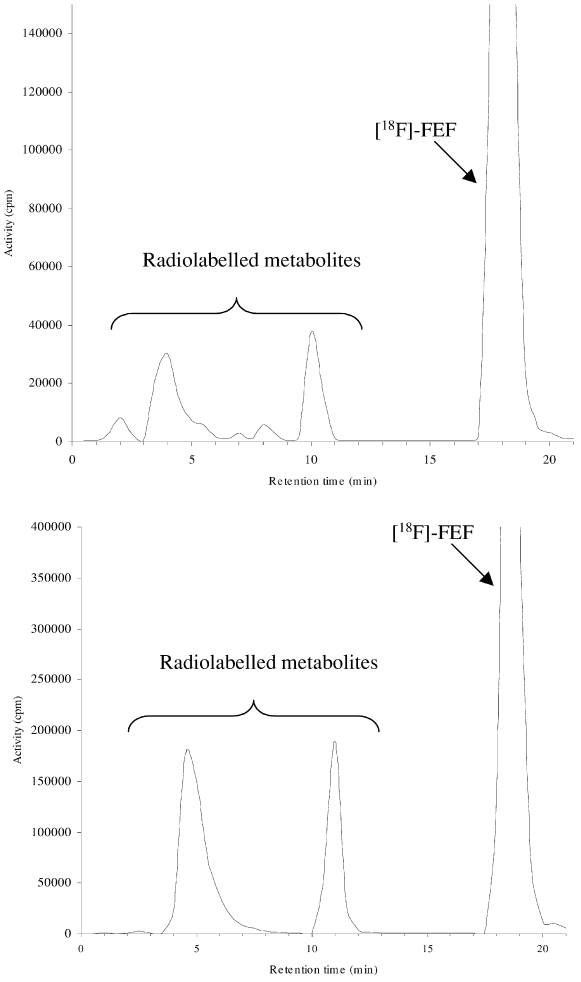

Fig. 4. Radiochromatogram of ^{18}FEF incubated in human microsomes (top), and in rat microsomes (bottom). * Labelled peaks are matrix peaks.

microsomes. Based on the order of elution, we think that they may share the same structure.

4. Conclusion

The in vitro metabolism of FEF is more extensive in rat microsomes than in human microsomes. In human preparations as well as in rat ones, two, possibly three, major radiolabelled metabolites were observed. Their structures are definitely derived from a benzodiazepine motif, with m/z of 320 (or 338) and 322, respectively. They arise from two or more transformations, possibly including a hydroxylation, most probably affecting the lateral chain of the ester group.

Flumazenil, the parent molecule of fluoroethylflumazenil, was mainly metabolised in vivo into its acidic derivative, and to a lesser extent into an hydroxylated compound [6–8]. An N-demethylation is also reported [15] but does not seem to be quantitatively important. Surprisingly, fluoroethylflumazenil shows a rather different pattern; the metabolite resulting from the ester bond cleavage is definitely not found, and the mono hydroxylated compound, which is absent in man, accounts for a small fraction of the total radioactivity, in vitro in rat.

Because of the structure and the lipophilicity of those metabolites, more pharmacological characterisation, such as binding to the benzodiazepine receptor and study of their ability to cross the blood–brain barrier, would be necessary to validate ^{18}FEF as a radiolabelled PET tracer of the GABA receptors.

Finally, a simple HPLC method is presented that allows a good separation of ^{18}FEF from its metabolite. This methodology could be applied for further analysis of metabolites structures, or for the determination of unchanged ^{18}FEF in routine clinical analysis.

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