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Investigation of the *in vitro* metabolism of the H_2-antagonist mifentidine by on-line capillary electrophoresis–mass spectrometry using non-aqueous separation conditions

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Abstract

The *in vitro* metabolism of mifentidine, a prototype second-generation histamine H_2-antagonist, is investigated using on-line capillary electrophoresis–mass spectrometry (CE–MS) by analysis of hepatic microsomal incubates. Consideration of the hydrophobicity of this drug and putative metabolites led to the development of a non-aqueous CE separation medium consisting of 5 m*M* NH_4OAc in methanol containing 100 m*M* acetic acid. Benefits of non-aqueous media in CE–MS studies of small hydrophobic molecules are discussed. In addition, we elucidate both chemical transformations and the *in vitro* metabolism of mifentidine using guinea pig hepatic microsomes.

1. Introduction

The utilization of capillary electrophoresis (CE) in the separation of complex mixtures of biopolymers has experienced an explosive growth since the introduction of the first commercial instruments in the late 1980s [1–4]. Furthermore, the technique is also finding increased use in the analysis of small, relatively hydrophobic compounds. Analyses of pharmaceuticals [5,6] and metabolites produced by endogenous physiological processes [7,8] as well as phase I and II biotransformation of xenobiotics and therapeutic drugs [9] have been reported recently. A frequent problem encountered with the analysis of such compounds is their poor solubility in the aqueous separation buffers commonly used with CE. However, it has been noted by us and others [9–14] that the addition of organic modifiers, such as methanol, isopropanol or acetonitrile, to the CE separation buffer improves the resolution of small-molecule mixtures in free solution. These increases in performance can be attributed to a change in the viscosity and dielectric constant of the separation buffer as well as the ζ potential of the capillary wall [1,9]. Typically, organic modifiers constitute as high as *ca.* 30% (v/v) of the CE separation buffer, and we have recently demonstrated the enhanced separation of a mixture of phase I metabolites using this approach [9–11].

In addition to changes brought about in separation characteristics in free solution CE (capillary zone electrophoresis, CZE), we and others

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have made the case that addition of organic modifiers, such as methanol, greatly enhances the solubility of relatively hydrophobic small molecules in aqueous solution [7,8,10,15]. This is noteworthy since a large number of therapeutic drugs and their resulting metabolites are poorly soluble in aqueous solution [16] and has led to major obstacles in the analysis of such compounds by CZE. This observation prompted us to investigate the use of a non-aqueous separation medium in the CZE separation of the major phase I metabolites of the hydrophobic, antitumor drug, pyrazoloacridine [17]. In this study, we demonstrated an increase in detected metabolite levels as well as enhanced separation in the non-aqueous electrolyte solution. We attributed this to a reduction in absorptive losses to the capillary wall and changes in the viscosity and dielectric constant of the separation buffer.

The first reported use of a non-aqueous separation medium in CE involved the separation of quinoline-like compounds dissolved in acetonitrile [18] and the resolution of a series of polynuclear aromatic hydrocarbons [19]. To our knowledge, to date, only one other group have reported the use of non-aqueous electrolyte solutions, namely Sahota and Khaledi [20], who separated a mixture of six small peptides using formamide.

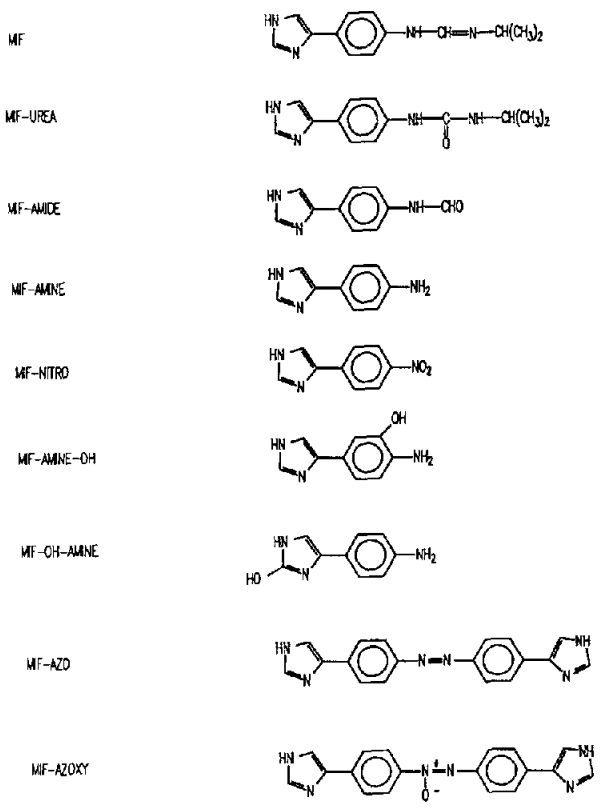

Fig. 1. Structures of the H_2-antagonist mifentidine (MIF) and eight synthetic standards/putative metabolites.

In drug metabolism studies, structure elucidation of biologically transformed parent drugs is vitally important in order to understand the physical and biological effects of the parent drug. A physical method with a proven history for obtaining this type of data is mass spectrometry (MS) [21–26]. However, since most drug metabolites are often components of a complex biological matrix, separation prior to MS detection is often necessary [27,28]. In this regard, the application of on-line CE–MS pioneered by Smith and co-workers [29–33] and Henion and co-workers [34–38] is extremely attractive. We have also recently reported the usefulness of this technique in the study of the phase I metabolites of the neuroleptic drug haloperidol [11].

In the present study, we have investigated the phase I metabolism of the H_2-antagonist mifentidine (MIF), N^1-[(4-imidazole) phenyl]-N^2-isopropyl formamidine (see Fig. 1 for structure). This drug is a representative prototype of the second generation of histamine H_2-antagonists and possesses both antisecretory and antiulcer activity [39]. In preliminary studies of this drug's metabolism [39,40], the metabolites identified were the amine (MIF-amine), formamide (MIF-amide) and urea (MIF-urea). In the present study we report, to our knowledge, the first use of non-aqueous separation solvent in conjunction with on-line CE–MS to investigate the metabolism of a therapeutic drug.

2. Experimental

2.1. Chemicals

Mifentidine and all synthetic standards of putative metabolites (see Fig. 1) were synthesized and supplied by the Institute DeAngeli

(Milan, Italy). Potassium phosphate (monobasic), and zinc sulphate were obtained from Sigma (St. Louis, MO, USA). Gold-grade ammonium acetate, glacial acetic acid, and magnesium chloride were obtained from Aldrich (Milwaukee, WI, USA). Nicotinamide–adenine dinucleotide phosphate (NADP), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were supplied by Boehringer Mannheim (Indianapolis, IN, USA). HPLC-grade methanol and high-purity water were obtained from Baxter (Minneapolis, MN, USA).

2.2. Capillary electrophoresis

CE separations were performed using a modified Beckman P/ACE 2100 Model CE (Fullerton, CA, USA), coupled to a Reason Technology 486 PC with control by System Gold software. An uncoated capillary (50 cm × 75 μm I.D.) purchased from Beckman was used throughout. Prior to its use, the capillary was rinsed with 0.1 *M* sodium hydroxide (10 capillary volumes), water (10 capillary volumes), methanol (10 capillary volumes), and separation medium (10 capillary volumes). Between analyses, the capillary was washed with methanol (2 column volumes) followed by separation medium (1 column volume). Synthetic standards were individually dissolved in methanol (1 mg/ml) and 5 μl were removed from each vial and mixed in a single, clean vial to give the mixture containing MIF and all putative metabolites. The mixture was introduced by pressure injection (1 s), and all experiments were conducted with an applied voltage of 20 kV and a capillary temperature maintained at 40°C, monitored at a wavelength of 214 nm. Separation buffers utilized were: (1) 20 m*M* NH_4OAc with 1% acetic acid in 30% aqueous methanol and (2) 20 m*M* NH_4OAc with 1% acetic acid in methanol.

2.3. Capillary electrophoresis–mass spectrometry

CE conditions were used as described above with the exception that the uncoated capillary had dimensions of 65 cm × 50 μm I.D. and the capillary temperature was maintained at 25°C. Prior to its use, the capillary was rinsed with 0.1 *M* sodium hydroxide (20 capillary volumes), water (20 capillary volumes) and CE separation solvent (10 capillary volumes). Between analyses, the capillary was only washed with separation solvent (5 capillary volumes). The CE separation solvent used to afford optimum separation of metabolites was 5 m*M* ammonium acetate (NH_4AOc) in methanol containing 100 m*M* glacial acetic acid.

All analyses were carried out on a Finnigan MAT 900 mass spectrometer (Bremen, Germany) of EB configuration (where E is an electric sector and B is the magnet) with a PATRIC (position and time resolved ion counter) focal plane detector. A modified Analytica (Banford, CT, USA) electrospray ionization (ESI) source was used in a positive ion mode throughout with the needle assembly at ground potential. The sample needle of the ESI source was replaced by the CE capillary from which 2–3 mm of the polyamide coating had been removed from the MS end with hydrofluoric acid. A liquid sheath electrode of isopropanol–water–acetic acid (60:40:1, v/v) at a flow-rate of 2 μl/min was used to boost the flow through the ESI needle and serve as a ground for the CE capillary as described previously [11]. The nitrogen ESI drying gas was at 140°C with a flow-rate of 3.6 l/min and an ESI voltage of −3400 V was employed. For CZE–MS runs, the scan range was 50–400 u (exponential magnet scan from low to high mass) at a rate of 2 s/decade. An instrument resolution of *ca.* 1000 was employed throughout.

2.4. Microsomal incubations

English short-hair male guinea pigs were obtained from the Charles River Co. (Montreal, Canada). Animals were fasted overnight before sacrifice. Hepatic microsomes were prepared using the centrifugation method described previously [41]. Rat microsomes were obtained as described by Lipsky [42], with the exception that the microsomes were made to a comparable concentration using a buffer consisting of 250 m*M* sucrose plus 80 m*M* KCl and 25 m*M* imidazole, instead of Triton X-100.

Incubation procedures were as follows: an

NADP, reduced (NADPH)-generating system consisting of the sodium salt of NADPH ($NADP^+$) (2 μmol), glucose-6-phosphate disodium salt (10 μmol), glucose-6-phosphate dehydrogenase (1 unit) and $MgCl_2$ (2 mg), all in 2 ml phosphate buffer (0.2 *M*, pH 7.4) was preincubated for 2 min. Enzymatic reactions were initiated by addition of MIF (2 μmol) and microsomal preparations equivalent to 0.5 g original tissue. In control incubates, heat-inactivated microsomes (boiled at 100°C for 30 min) were used instead of fresh microsomal preparations. Incubations were carried out for 30 min at 37°C.

Enzymic reactions were terminated by addition of $ZnSO_4$ (200 mg) to the incubation mixture. The precipitated proteins were removed by centrifuging (IEC Cru-5000) at *ca.* 1200 *g* for 20 min. The supernatant was passed through a preconditioned [methanol (4 ml) followed by distilled water (4 ml)] Sep-Pak C_{18} cartridge. Excess $ZnSO_4$ was removed by washing with distilled water (4 ml). The retained compounds were eluted by methanol (4 ml), which was subsequently evaporated to dryness at 45°C under nitrogen [43]. The residues were reconstituted in methanol (20 μl) and subjected to CZE separation with MS detection.

3. Results and discussion

3.1. Analysis of a mixture of mifentidine and synthetic standards

Initial studies focused on the development of optimal CZE conditions for the separation of MIF and a series of synthetic standards, including MIF-urea, MIF-amide, MIF-amine, MIF-nitro, MIF-amine-OH, MIF-azo, and MIF-azoxy (see Fig. 1 for structures). A variety of CZE aqueous separation buffers containing variable concentrations of NH_4OAc and acetic acid were investigated. Results (not shown) indicated that substantial analyte–wall interactions occurred causing considerable peak tailing. Also, resolution of MIF and the standards was poor and losses of compounds due to their poor solubility in water, and interaction with the capillary wall were also apparent [9].

A substantial improvement in resolution, and recovery of the standards in the mixture was observed on increasing the organic solvent content from 0 to 30 to 100% methanol, and this is demonstrated in Fig. 2 (A: 20 m*M* NH_4OAc in 30% aqueous methanol containing 1% acetic acid; B: 20 m*M* NH_4OAc in 100% methanol containing 1% acetic acid). Furthermore, using the non-aqueous separation medium, improved recovery of each component was observed, indicating that analyte–wall interactions and losses of compounds to the capillary wall were minimized. We believe this is due to the increased solvation cage surrounding analyte species when dissolved in organic solvent. We have also noted that another successful strategy to avoid losses of such compounds to the capillary wall is to electrophorese with a 10 μM MIF solution in separation solvent/buffer prior to CZE analysis of the mixture of standards [9]. This pretreatment of the capillary leads to absorption of parent drug on "active sites" present on the silica wall, hence minimizing loses of subsequent analytes injected on to the capillary. A further consequence of using a non-aqueous separation solvent system is that relative migration times (compared to MIF) of standards can undergo some change in comparison to aqueous buffers. Hence, the MIF-azo, MIF-azoxy and MIF-amide standards all migrated slower (relative to MIF) in 100% methanol (Fig. 2B) compared to 30% methanol (Fig. 2A).

We have recently reported [44] that CZE buffers (aqueous or containing organic solvents) that have low electrolyte concentrations, which result in low CZE capillary current values (μA), are particularly compatible with on-line CE–MS analysis since such low current values are comparable to those generated in the ESI interface used to couple CE to MS. We have found from empirical observations, that if both the CE and ESI currents are comparable, sensitivity limits of the mass spectrometer are maximized and the stability of the CE and ESI is optimal. Grounding the CE capillary is also found to be more effective at lower CE currents. This in turn

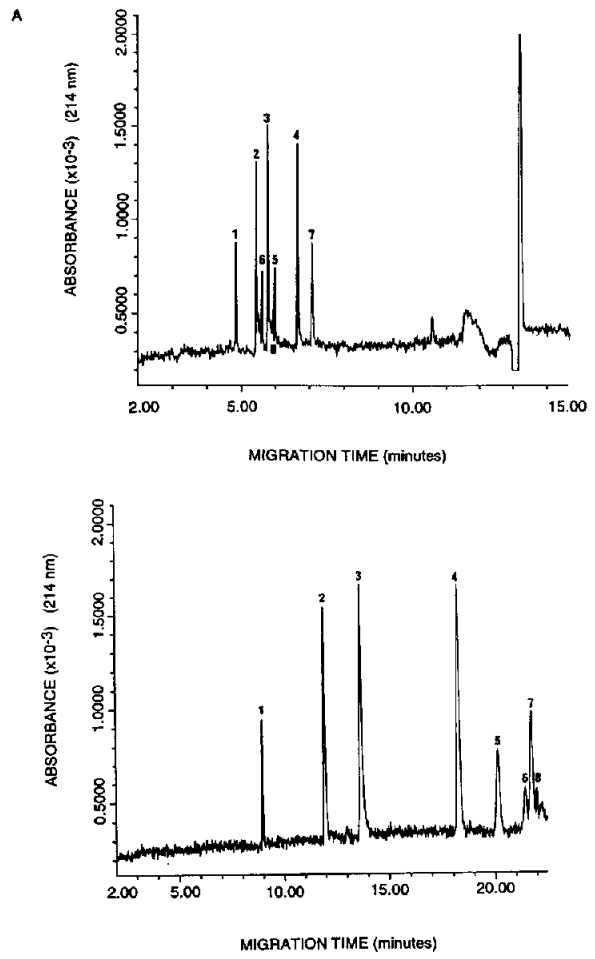

Fig. 2. Separation of MIF and eight synthetic standards by CZE using variable concentrations of organic modifier. Analysis performed on a Beckman P/ACE 2100 CE instrument using an uncoated fused-silica capillary [57 cm (50 cm to the detector) × 75 μm I.D.] maintained at 40°C and monitored at UV of 214 nm. The mixture was by pressure injection (1 s) and electrophoresed at 20 kV. Peaks: 1 = MIF; 2 = MIF-amine; 3 = MIF-amine-OH; 4 = MIF-urea; 5 = MIF-amide; 6 = MIF-azo; 7 = MIF-nitro; 8 = MIF-azoxy. CZE separation buffer/solvent: (A) 20 m*M* NH_4OAc in 30% aqueous methanol containing 1% acetic acid; (B) 20 m*M* NH_4OAc in 100% methanol containing 1% acetic acid.

produces extremely stable ESI conditions. Hence, we systematically reduced the NH_4OAc concentration in order to reduce the CE current and found that 5 m*M* NH_4OAc in 100% methanol containing 100 m*M* acetic acid still afforded separation of MIF and eight synthetic standards but substantially reduced the CE current to approximately 6 μA. The separation and subsequent ion detection of the components of the mixture using a non-aqueous solvent system by CE–MS is shown in Fig. 3.

The detection limit for each of these standards was determined to be *ca.* 80 fmol applied onto the CE capillary. This was achieved using a PATRIC focal plane scanning array detector on the MAT 900 mass spectrometer, which is an ion counting device that allows the simultaneous detection of up to an 8% (in the case of the

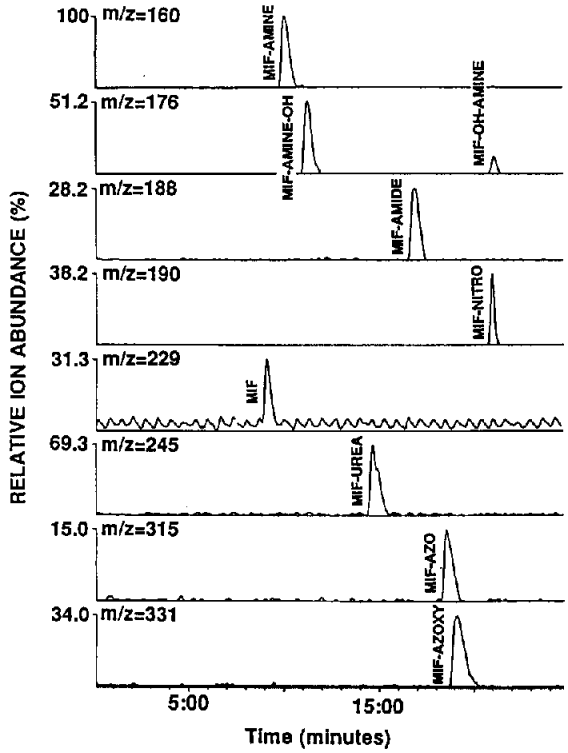

Fig. 3. Non-aqueous CE–MS analysis of a mixture of synthetic standards containing MIF-amine ($MH^+ = 160$), MIF-amine-OH ($MH^+ = 176$), MIF-OH-amine ($MH^+ = 176$), MIF-amide ($MH^+ = 188$), MIF-nitro ($MH^+ = 190$), parent drug MIF ($MH^+ = 229$), MIF-urea ($MH^+ = 245$), MIF-azo ($MH^+ = 315$), and MIF-azoxy ($MH^+ = 331$). Separation was carried out on an uncoated fused-silica capillary (65 cm × 50 μm I.D.) at 20 kV and 25°C. Detection was by a MAT 900 mass spectrometer equipped with a PATRIC focal plane detector, and the CE–MS separation solvent system was 5 m*M* NH_4OAc with 100 m*M* acetic acid in 100% methanol.

PATRIC detector) mass window. Hence the effective time counting ions of each specific m/z value focused by the mass spectrometer is increased, resulting in enhanced signal for each ion species detected. Array detectors can be up to *ca.* 100-fold more sensitive than point detectors such as secondary electron multipliers. A further advantage of the PATRIC detector is that high sensitivity measurements can be maintained while scanning the mass spectrometer. This is particularly useful in the arena of drug metabolism studies, since minor metabolites are difficult to detect with static array detection devices due to dynamic range problems. This can be overcome by using a scanning array detector in conjunction with on-line chromatography, *e.g.* CE–MS to determine the molecular mass of an unidentified minor metabolite that has been temporally separated from excess, unmetabolized parent drug.

3.2. *In vitro microsomal modifications of mifentidine*

A guinea pig hepatic microsomal incubate of MIF was subsequently subjected to CE–MS analysis (after preliminary C_{18} Sep-Pak clean-up [43]) using the non-aqueous separation conditions described above for the synthetic standards. Ions at m/z 160, 188, 229, 245, 315 and 331 were clearly discernible above background in the CE–MS ion electropherogram (Fig. 4). Comparison of relative migration times (compared to MIF) with standards (Fig. 3) confirmed the presence of MIF-amine ($MH^+ = 160$), MIF-amide ($MH^+ = 188$), unmetabolized parent drug MIF ($MH^+ = 229$), MIF-azo ($MH^+ = 315$) and MIF-azoxy ($MH^+ = 331$). The relative CE–MS migration times of MIF-azo and MIF-azoxy (Fig. 4) compared to standards (Fig. 3) was somewhat variable. This can be explained by the fact that small differences in the methanol concentration of the CE separation medium can affect the migration times of MIF-azo and MIF-azoxy standards as seen in Fig. 2. However, the relative migration time of the molecular ion at m/z 245 (Fig. 4) was *ca.* 6 min later than observed for the MIF-urea standard (Fig. 3). This indicates

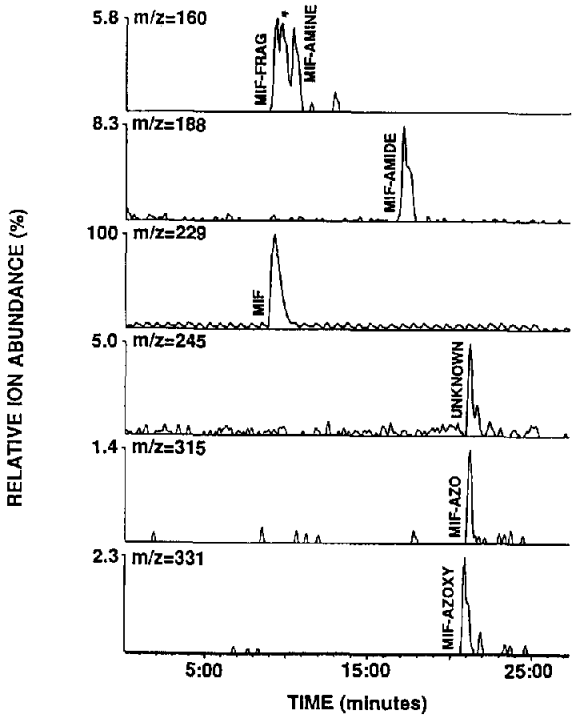

Fig. 4. Non-aqueous CE–MS analysis of a metabolite mixture derived from a guinea pig hepatic microsomal incubation of MIF. Conditions as for Fig. 3. Metabolites corresponding to MIF-amine ($MH^+ = 160$), MIF-amide ($MH^+ = 188$), unmetabolized MIF ($MH^+ = 229$), MIF-azo ($MH^+ = 315$), MIF-azoxy ($MH^+ = 331$), and an unidentified metabolite "unknown" ($MH^+ = 245$) were detected. Other ions at m/z 160 (MIF-FRAG) correspond to a fragment ion of MIF (*ca.* 9.25 min) and an unidentified component (*) of the microsomal mixture (*ca.* 9.5 min).

that our original suggestion that MIF-urea may be formed in the *in vitro* microsomal metabolism of MIF [40] is open to question. Further work is in progress, but evidence to date suggests the tentative presence of the N^2-hydroxylamine metabolite [45].

In the CE–MS ion electropherogram of the microsomal incubate (Fig. 4), there are three distinct time-resolved ions at m/z 160. The m/z 160 ion at *ca.* 10.5 min is assignable to the protonated molecular ion (MH^+) of the metabolite MIF-amine (*cf.* with MIF-amine standard, Fig. 3). The ion detected at *ca.* 9.25 min is derived from the fragmentation of the large amount of unmetabolized MIF present in the mixture. Fragmentation of the parent compound

($MH^+ = 229$) occurs in the ESI source to afford the ion at m/z 160. It has previously been noted that fragmentation of MIF to afford an ion at m/z 160 is facile under collision-induced dissociation conditions [40]. The origin of this ion is also confirmed by inspection of a CE–MS analysis of a control microsomal incubate containing only heat-inactivated microsomes plus parent drug MIF. An ion at m/z 160 with a migration time identical to MIF (*ca.* 9.5 min) is observed in the CE–MS ion electropherogram (Fig. 5). The third ion at m/z 160 (*ca.* 10.00 min) is probably derived from fragmentation of an unidentified component in the mixture (Fig. 4), although only a small amount of this component is observed in the CE–MS analysis of the control incubate (Fig. 5).

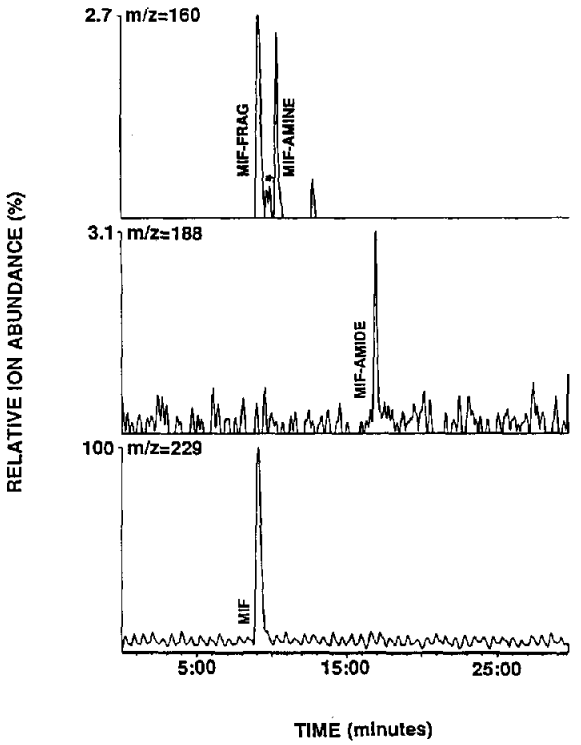

Fig. 5. Non-aqueous CE–MS analysis of a control incubate containing only MIF and heat-deactivated microsomes. Conditions as for Fig. 3. MIF-amine ($MH^+ = 160$, *ca.* 10.5 min) and MIF-amide ($MH^+ = 188$) are formed by chemical hydrolysis of the amidine side chain of MIF. The other ions at m/z 160 (*ca.* 9.35 min) corresponds to a fragment ion derived from MIF (MIF-FRAG) and an unidentified compound (*).

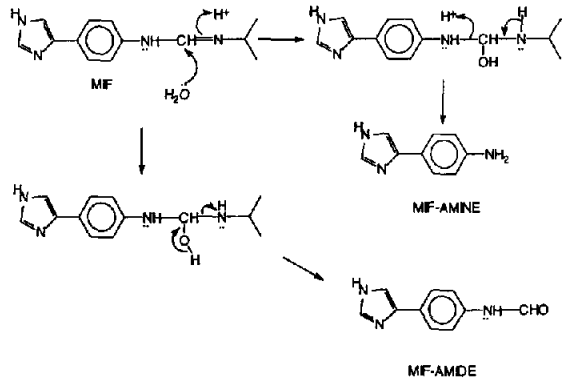

Fig. 6. Postulated mechanism for the chemical formation of MIF-amine and MIF-amide from the hydrolysis of the amidine side chain of the parent drug MIF.

Further inspection of the CE–MS analysis of the control incubation also revealed ions at m/z 160 and 188 with both protonated molecular ion and migration time values identical to MIF-amine and MIF-amide respectively (Fig. 5). This observation suggests that these two compounds are also produced chemically during the incubation process. Hydrolysis of the amidine side chain of the parent drug MIF would result in the formation of these two compounds and a postulated mechanism is shown in Fig. 6 [46].

4. Conclusions

It is possible using on-line CE–MS to analyze mixtures of small hydrophobic compounds such as drug metabolites. In particular by using a non-aqueous separation solvent containing a low concentration of an electrolyte (in this case NH_4OAc) and acid (100 m*M* acetic acid), it was possible to separate a standard mixture by CE and obtain a stable ESI beam in order to analyze the components by MS. Furthermore, by using such an approach, it was determined that at least five metabolites are formed enzymatically, namely MIF-amine, MIF-amide, MIF-azo, MIF-azoxy, and "unknown" (possibly a N^2-hydroxylamine derivative). Furthermore, MIF-amine and MIF-amide are also formed by chemical hydrolysis of the amidine side chain.

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