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Application of LC–NMR for the study of the volatile metabolite of MK-0869, a substance P receptor antagonist

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Abstract

LC–NMR was applied to identify the polar volatile metabolite of MK-0869. MK-0869, a morpholine-based compound containing a triazolone ring, is a very potent NK_1 receptor antagonist. Currently, it is in development as an anti-emesis agent in chemotherapy treatments. The primary metabolites of MK-0869, M1 and M2, are non-polar and lack the triazolone ring. Incubation of [^{14}C]M1 with liver microsomes from male rats produced a very polar and volatile metabolite, M3. Analysis was not possible by LC–MS or by conventional NMR because of poor ionization, small molecular weight and volatility, leaving chemical derivatization and LC–NMR as alternative methods. Reduction of M3 with $NaBH_4$ resulted in a derivative that had the same retention time as *p*-fluorophenylethylene glycol on HPLC. A small aliquot of the solution containing M3 was passed through the LC of the LC–NMR system, which was connected on-line with a radioactivity detector. The simultaneous UV and radioactivity chromatograms thus identified the chromatographic UV peak that was associated with the metabolite. Analysis was carried out by stop-flow on another portion of this fraction. From the chemical derivatization and the analysis by LC–NMR, M3 is shown to be *p*-fluoro-α-hydroxyacetophenone. Further studies using LC–NMR showed that M3 could be generated from both M1 and M2 in NADPH-dependant reactions catalyzed by microsomes containing recombinant human CYP2C19, CYP1A2 or CYP3A4.

Keywords: LC–NMR; Volatile metabolite of MK-0869; MK-0869; Microsomes containing recombinant human CYP isozymes; *O*-Dealkylation metabolic pathway

1. Introduction

Cancer treatments are associated with distressing side-effects such as severe nausea and vomiting, which deprive the patients of a better quality of life. Substance P, the first member of the tachykinin family of peptides to be discovered [1,2], has been associated with the mediation of the emetic reflex [3] as one of its numerous functions [4], and it has been identified as a preferred agonist for the tachykinin receptor NK_1 [4,5]. Studies suggested that one of the roles of substance P via the NK_1 receptor may be the pathoetiology of

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emesis [3,6–10]. MK-0869 (I) [(2*R*)-(1*R*)-3,5-bis(trifluoromethylphenyl)ethoxy)-(3*S*)-(4-fluoro) phenyl-4-(3-(5-oxo-1*H*,4*H*-1,2,4-triazolo)methyl-morpholine] (Fig. 1), a very potent NK_1 receptor antagonist [11], is therefore a potential drug for use in the treatment of emesis, and is currently in development.

MK-0869 undergoes *N*-dealkylation in vitro, from rat hepatocyte incubations, leading to the formation of two primary non-polar metabolites, M1 (II) and M2 (III) (Fig. 1) [12,13]. Incubation of [^{14}C]M1 with liver microsomes from male rats produced the very polar metabolite designated as M3. Because the radioactivity of the fraction containing this metabolite was lost during evaporation to dryness, this suggested that M3 was possibly volatile and even more under acidic conditions, which made it difficult to determine its identity.

LC–MS, NMR and/or LC–NMR are techniques commonly applied for the structural determination of metabolites [14–16]. There are instances where LC–MS cannot provide definitive characterizations, leaving NMR as the preferred technique [17–21]. One example involves the present case in which parent drug is converted to a very polar small molecular weight species (i.e. less than 200 Da) that is likely to be difficult for MS detection. Conventional NMR is also unsuitable since evaporation of the final solution results in the loss of the metabolite. Hence, LC–NMR was selected as the method of choice.

Here we report the evaluation of the structure of the M3 metabolite using LC–NMR analysis. We also determine which human CYP isozymes are involved in its formation.

2. Experimental

2.1. Chemicals

All chemicals were of analytical purity. MK-0869 and M1 were synthesized by Dr J. Hale of Medicinal Chemistry of Merck Research Laboratories (MRL) at Rahway [22]. [Morpholine-2-^{14}C]M1 (specific activity 16.3 μCi/mg) and [morpholine-2-^{14}C]M2 (specific activity 38.56 μCi/mg) were prepared by Dr M. Braun of the Radiosynthesis Group of MRL at Rahway. Microsomes from Baculovirus-infected Sf21 cells containing co-expressed CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4) and cytochrome P-450 reductase were prepared by Dr M. Shou and Dr T. Rushmore of Drug Metabolism of MRL at West Point. *p*-Fluorophenylglyoxal was purchased from Apollo Scientific, Ltd (Derbyshire, UK). The HPLC solvents were of analytical grade. The solvents for the LC–NMR were CH_3CN (ACN) (EM Science, Gibbstown, NJ) and D_2O (Cambridge Isotopes Laboratories, Inc., Andover, MA).

2.2. Preparation of liver microsomes

Liver microsomal and cytosolic fractions were prepared from Sprague Dawley rats according to

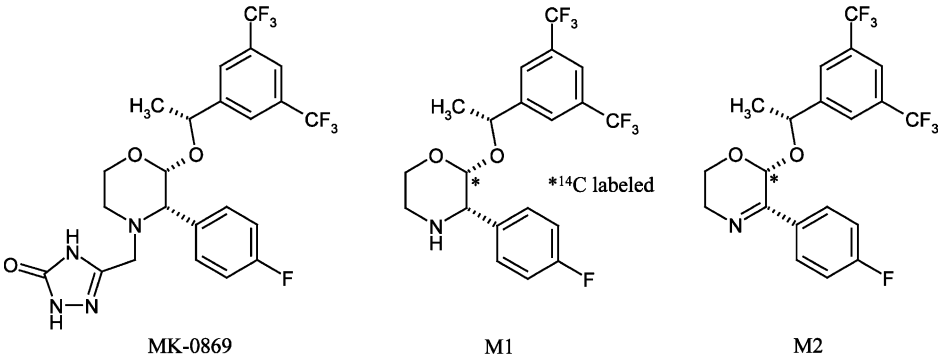


Fig. 1. Structures of compounds MK-0869, M1 and M2.

the published procedure [23]. Briefly, livers were homogenized and subjected to differential centrifugation to obtain cytosolic and microsomal fractions which were subsequently aliquoted into small tubes, and stored at −70 °C. Protein concentrations (42.7 mg/ml) were determined by the BCA assay from Pierce [24]. The specific cytochrome P-450 content (0.40 nmol/mg) in each microsomal preparation was measured as described by Omura and Sato [25].

2.3. Oxidative metabolism in liver microsomes or expressed recombinant CYP isozymes

The incubation mixtures (1 ml) contained 40 μM of the test compound ([^{14}C]M1 or [^{14}C]M2 metabolites), 100 mM phosphate buffer pH 7.4, NADPH-regenerating system (5 mM glucose-6-phosphate, 1 mM NADP and 0.7 IU/ml glucose 6-phosphate dehydrogenase), and 1 mg/ml liver microsomal proteins prepared from rats as final concentrations. Stock solutions of the substrate in methanol were made so that final concentration of methanol in the reaction mixture was 3%. The reaction was initiated by adding 100 μl of 10 mM NADP and proceeded at 37 °C for 90 min. Ten identical incubations were carried out to generate enough material for these studies. After incubation, the reaction was terminated by the addition of four volumes of acetonitrile. The suspension was sonicated for 2 min and centrifuged at 3000*g* for 5 min. The supernatant was collected and concentrated under N_2 stream to 5 ml for pre-purification with a Varian Mega Bond Elut C18 cartridge. The cartridge was washed first with 5 ml of water and then eluted with 10 ml of acetonitrile. The radioactive acetonitrile fraction was concentrated under N_2 stream to 1 ml. This fraction will be referred to as the crude microsomal extract in the text below. A portion of the crude microsomal extract was used for reduction with $NaBH_4$ and another portion was used for the LC–NMR analysis (Sections 2.4 and 2.6).

Similar incubations (1 ml each) with test compound ([^{14}C]M1 or [^{14}C]M2) were carried out using 1 mg/ml microsomes from Baculovirus-infected Sf21 cells containing 150–200 pmol of CYP isoform (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4) co-expressed with cytochrome P-450 reductase. Another incubation was carried out with *p*-fluorophenylglyoxal and control microsomes prepared from Sf21 cells without transfection. After incubation, the reactions mixtures were precipitated with four volumes of acetonitrile and centrifuged at 3000*g* for 5 min. The supernatants were collected and concentrated to 1.5 ml for HPLC semi-purification (25–80% B in 40 min; A, ammonium acetate (10 mM) in H_2O; B, ammonium acetate (7.3 mM) in MeOH:ACN (7.3:92.7, v/v), the flow rate was 1 ml/min, and Zorbax RX C8 (4.6 × 250 mm) column was used). The eluents were collected in 1 ml fractions and an aliquot of each fraction was monitored for radioactivity. The radioactive fractions were concentrated under N_2 stream to ca. 500 μl or less for the LC–NMR analysis. The incubation with *p*-fluorophenylglyoxal was analyzed by HPLC (conditions indicated above) and LC–NMR (Section 2.5) monitored by UV.

2.4. Reduction with $NaBH_4$

A volume of 400 μl of the crude microsomal extract (Section 2.3) was divided into two equal portions. A pinch of $NaBH_4$ was added to one of the portions. Both aliquots were stirred and kept overnight at room temperature. Analysis of both portions was carried out via HPLC and monitored by radioactivity using the conditions in Section 2.3.

2.5. LC–NMR

The HPLC conditions for LC–NMR used ACN and ammonium acetate in D_2O in the gradient solvent system (25–80%B in 40 min; A, ammonium acetate (10 mM) in D_2O; B, ACN, 1ml/min, 210 nm and using the column Zorbax RX C8 4.6 × 250 mm).

A Varian Unity Inova 600 MHz NMR instrument (Palo Alto, CA) equipped with a ^{1}H{^{13}C/^{15}N} pulse field gradient triple resonance microflow NMR probe (flow cell 60 μl; 3 mm OD) was used. Reverse-phase HPLC of the samples was carried out on a Varian modular HPLC system (a

9012 pump and a 9065 photodiode array UV detector). The Varian HPLC software was also equipped with the capability for programmable stop-flow experiments based on UV peak detection. This software was designed to work with a radioactivity detector equipped with a liquid cell (Radiomatic C150TR, Packard, Meriden, CT) connected on-line to the LC–UV system of the LC–NMR. The ^{2}H resonance of the D_2O was used for field-frequency lock, and the spectra were centered on the ACN methyl resonance. Suppression of resonances from HOD, methyl of the ammonium acetate, and methyl of ACN and its two ^{13}C satellites was accomplished using a train of four selective WET pulses, each followed by a B_o gradient pulse and a composite 90° read pulse [26]. ^{1}H NMR spectra were acquired in stop-flow mode using the UV maximum to trigger peak detection. After peak detection and a time delay of about 28 s, the HPLC pump was stopped, trapping the peak of interest in the LC–NMR microprobe. ^{1}H NMR stop-flow spectra were acquired using an acquisition time of 1.5 s, a delay between the successive pulses of 0.5 s, a spectral width of 9000 Hz, and 32 K time-domain data points. The methyl resonance of ACN was referenced to 1.94 ppm.

2.6. Analysis of microsomal incubations by LC–NMR

Two hundred μl of the crude microsomal extract (Section 2.3) was analyzed by LC–UV, LC–UV–Radioactivity and LC–NMR, respectively, in the LC–NMR system. An LC–UV chromatogram on a 20 μl injection (ca. 0.5 μg based on radioactivity) displayed three UV peaks with retention times of 5.5, 8.0 and 13.1 min in the region where the metabolite M3 was expected to be present. LC–UV–Radioactivity analysis on a second 20 μl injection indicated that only the 8.0 min peak was radioactive. LC–NMR stop-flow analysis was carried out injecting the rest of the sample (160 μl, ca. 4 μg based on radioactivity). Conditions for the LC–NMR are indicated in Section 2.5. ^{1}H NMR spectra were acquired for 37 min (1024 scans) and one day (40 000 scans).

The polar radioactive fractions from the incubations of [^{14}C]M1 with microsomes containing CYP2C19, CYP1A2 or CYP3A4 were estimated to have a total of ca. 5.7, 2, and 3 μg based on radioactivity (^{14}C) for each incubation, respectively. These samples were independently concentrated to smaller volumes (570, 300 and 270 μl for each incubation, respectively) for injection into the LC–NMR system. Only a small portion of the fraction from the incubation with CYP2C19 (130 μl, ca. 1.3 μg based on radioactivity) was used to obtain simultaneous UV and radioactivity (^{14}C) chromatograms in the LC–UV–Radioactivity detector system of the LC–NMR to identify the chromatographic UV peak associated with the radioactive polar metabolite. The peak of interest eluted at ca. 8.13 min in the UV chromatogram and at ca. 8.72 min in the radioactivity (^{14}C) chromatogram, which showed similar retention times as the previously identified M3 metabolite. The stop-flow experiment was carried out with the rest of the sample (ca. 4.4 μg based on radioactivity), stopping on the chromatographic UV peak at ca. 8.11 min. The stop-flow NMR analysis for the radioactive fractions from the incubations with CYP1A2 and CYP3A4 were carried out on the peaks at ca. 8.18 min and 8.22 min, respectively. ^{1}H NMR spectra of all of these three stop-flow experiments were obtained by acquiring for ca. 37 min (1024 scans), 3 h (5120 scans) and 9 h (15 000 scans).

The polar radioactive fractions from the incubations of [^{14}C]M2 with microsomes containing CYP2C19, CYP1A2 or CYP3A4 were estimated to have a total of ca. 2 μg for CYP2C19 and CYP1A2 and ca. 3–4 μg for CYP3A4 based on radioactivity (^{14}C). These fractions were independently concentrated to smaller volumes (300, 210 and 480 μl for each incubation, respectively) for injection into the LC–NMR system. The stop-flow NMR analyses were carried out on the peaks at ca. 8.20, 7.90 and 8.40 min for each incubation, respectively. ^{1}H NMR spectra of all of these three stop-flow experiments were obtained by acquiring for ca. 37 min (1024 scans), 3 h (5120 scans) and 9 h (15 000 scans).

The stop-flow experiment on the incubation with *p*-fluorophenylglyoxal was carried out after

injecting only 180 μl of the reaction mixture, stopping on the chromatographic UV peak at ca. 8.26 min, and acquiring for ca. 37 min (1024 scans).

3. Results and discussion

3.1. Characterization of the M3 metabolite

Previous studies on the metabolism of the very potent antagonist NK_1 receptor MK-0869 indicated that M1 and M2 were the primary metabolites in vitro [12,13]. Incubations of [^{14}C]M1 with liver microsomes from male rats gave rise to a very polar metabolite M3. The radioactivity in the sample was lost when the final solution was evaporated to dryness, suggesting that M3 was volatile. Thus, conventional NMR analysis was contraindicated. The molecular weight could not be determined by LC–MS, suggesting that it was probably less than 200 Da. Two alternative methods, such as chemical derivatization and LC–NMR analysis, were considered.

A small volume of the crude microsomal extract (Section 2.3) was treated with $NaBH_4$ and kept overnight at room temperature. HPLC analysis indicated that the M3 chromatographic peak was absent and revealed the presence of a new more polar peak. Comparison of the retention times of *p*-fluorophenylethylene glycol and the new peak indicated that both had the same retention time (data not shown), suggesting to be the reduction product. This observation suggested three possibilities for the structure of the M3 metabolite, *p*-fluorophenylglyoxal, *p*-fluorophenylglycolaldehyde and *p*-fluoro-α-hydroxyacetophenone.

Two hundred μl of the crude microsomal extract (Section 2.3) was used for the LC–NMR studies. When non-deuterated solvents were used in the HPLC analyses (see conditions in Section 2.3), the retention time of the radioactive peak designated as M3 was 10 min. A 20 μl injection of the fraction containing M3 using the LC–NMR solvent system revealed three chromatographic UV peaks (5.5, 8.0 and 13.1 min) in the area of interest (data not shown). Based only on the UV chromatogram, it was not possible to identify the radioactive metabolite peak.

To carry out a stop-flow experiment on the peak of interest, a radioactivity detector was hooked up on-line to the LC–NMR system after the UV detector (LC–UV–Radioactivity system). The delay from the detection of the UV peak to the radioactivity detector was ca. 34 s. Because of the transfer delay from the UV to the NMR was ca. 28 s, the radioactivity detector could be used only for comparison between the two detectors and not for triggering the peak for NMR. UV and radioactivity (^{14}C) chromatograms were obtained in the same chromatographic run by injecting 20 μl of the M3 crude sample (Fig. 2). Only the chromatographic UV peak at 8.0 min was radioactive and thus associated with M3. Stop-flow mode was carried out with the remainder of the 200 μl sample (160 μl), stopping on the chromatographic UV peak at ca. 8.27 min. This injected sample was estimated to be ca. 4 μg based on radioactivity.

^{1}H NMR spectra were acquired after 37 min and one day to improve the signal-to-noise ratio. This also ensures that no peaks belonging to the metabolite were below the detection limit in the 37 min acquisition. The presence of the *p*-fluorophenyl ring with the characteristic splitting pattern indicated that the compound was drug related. The downfield shift of the *ortho* protons at 7.91 ppm suggested the presence of a carbonyl substituent (Table 1). The presence of a singlet at 4.85 ppm, integrating for ca. two protons, was consistent with a methylene that was flanked by the carbonyl and a hydroxyl group (Table 1). Based on the three suggested structures from the reduction of M3 with $NaBH_4$, only one was consistent with the ^{1}H NMR data. These features thus led to proposing the structure for M3 as the *p*-fluoro-α-hydroxyacetophenone (Fig. 3).

3.2. Identification of M3 metabolite from incubations of [^{14}C]M1 and [^{14}C]M2 and with microsomes containing recombinant human CYP isozymes

Further studies were carried out using LC–NMR as the structural analytical tool to determine which human CYP isozymes were involved in the

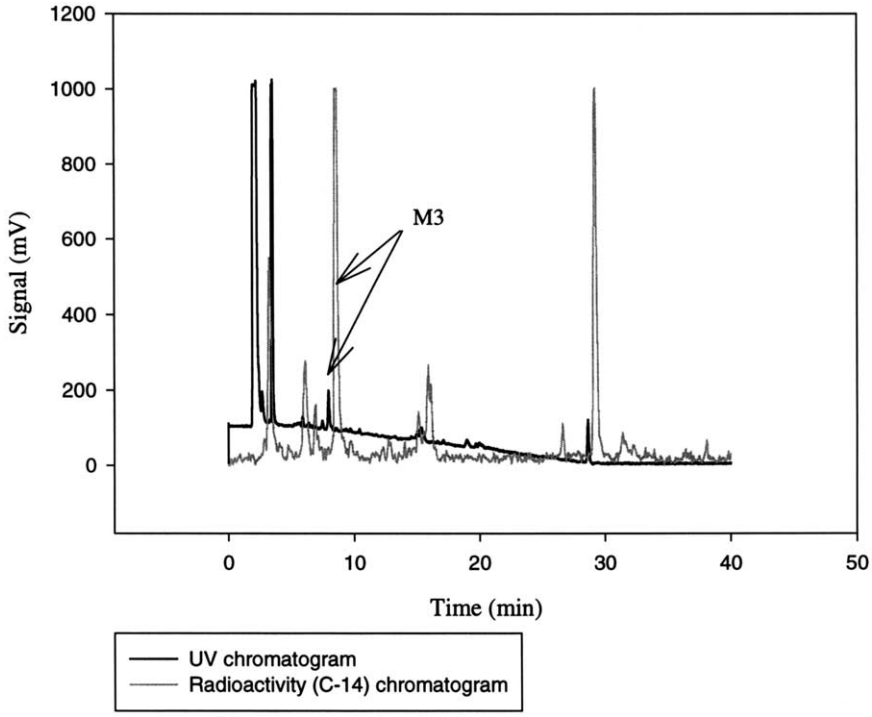
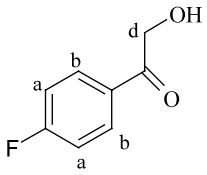


Fig. 2. UV-radioactivity (C-14) chromatograms of M3 metabolite.

Table 1
^{1}H NMR chemical shifts (ppm) of M3 metabolite from microsomal incubations



M3

Microsomal incubations	δ (ppm) protons		
	Ha	Hb	Hd
M1 with rat liver microsomes	7.19 (t[a], 2H, $J = 8.8$ Hz)	7.91 (dd, 2H, $J = 5.6$, 8.7 Hz)	4.85 (s, 2H)
M1 with CYP2C19	7.19 (t, 2H, J = 8.7 Hz)	7.91 (dd, 2H, $J = 5.5$, 8.8 Hz)	4.85 (s, 2H)
M1 with CYP1A2	7.20 (t, 2H, $J = 8.8$ Hz)	7.92 (dd, 2H, $J = 5.5$, 8.8 Hz)	4.85 (s, 2H)
M1 with CYP3A4	7.19 (t, 2H, $J = 8.8$ Hz)	7.91 (dd, 2H, $J = 5.4$, 8.8 Hz)	4.85 (s, 2H)
M2 with CYP2C19	7.20 (t, 2H, $J = 8.7$ Hz)	7.92 (dd, 2H, $J = 5.5$, 8.7 Hz)	4.85 (s, 2H)
M2 with CYP1A2	7.20 (t, 2H, $J = 8.8$ Hz)	7.92 (dd, 2H, $J = 5.4$, 8.8 Hz)	4.86 (s, 2H)
M2 with CYP3A4	7.20 (t, 2H, $J = 8.8$ Hz)	7.92 (dd, 2H, $J = 5.5$, 8.8 Hz)	4.85 (s, 2H)
p-FPG[b] with no CYP	7.18 (t, 2H, $J = 8.8$ Hz)	7.90 (dd, 2H, $J = 5.6$, 8.8 Hz)	4.84 (s, 2H)

[a] Signal splitting patterns: s = singlet, dd = doublet of doublets, t = triplet.
[b] p-FPG = p-fluorophenylglyoxal.

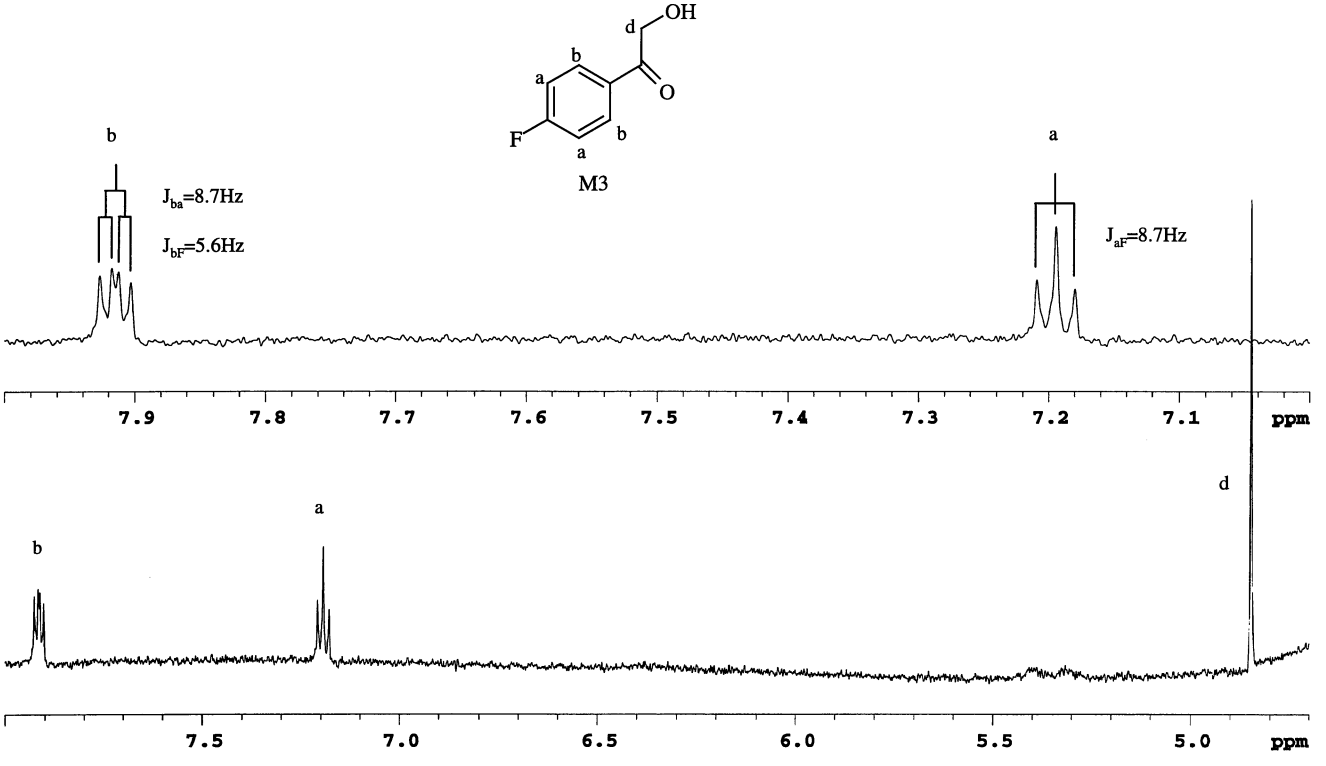


Fig. 3. Expanded sections of the ^{1}H NMR spectrum of metabolite M3 acquired for one day.

formation of the M3 metabolite. [^{14}C]M1 and [^{14}C]M2 were incubated with microsomes containing human recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4. Only human recombinant CYP2C19, CYP1A2 and CYP3A4 metabolized M1 and M2 to the very polar metabolite, as judged by HPLC analysis (data not shown). The supernatants from the incubation mixtures of M1 or M2 independently with CYP2C19, CYP1A2 and CYP3A4 were semi-purified by HPLC and LC–NMR analysis was carried out to determine the structure of the very polar metabolite common in these six incubations. The ^{1}H NMR spectra showed that all six fractions had the same spectroscopic features as M3 (Table 1). These results indicated that M1 and M2 were metabolized by microsomes containing recombinant human CYP2C19, CYP3A4 and CYP1A2 to M3.

The proposed mechanism of the formation of M3 from M1 or M2 could be through CYP-mediated *O*-dealkylation of the 1-(3,5-bis(trifluoromethylphenyl))ethanol moiety, followed by equilibrium of the hemiacetal-aldehyde forms of the morpholine ring (Fig. 4). The aldehyde or open form of the morpholine ring could either lose the aminoethanol tether after oxidation on the benzylic position in the case of M1 or hydrolysis in the case of M2 giving rise to the *p*-fluorophenylglyoxal, which could be further reduced to the *p*-fluoro-α-hydroxyacetophenone or M3.

3.3. Identification of M3 metabolite from incubation of p-fluorophenylglyoxal with microsomes lacking CYP monooxygenase

In order to determine that the reduction of *p*-fluorophenylglyoxal could not occur with CYP isozymes, *p*-fluorophenylglyoxal was incubated with control microsomes from insect cells. This incubation revealed a new peak by HPLC, which by LC–NMR (stop-flow at 8.26 min) had the same spectroscopic features as M3 (Table 1). These results suggested that the microsomes contained enzymes to reduce the proposed *p*-fluorophenylglyoxal metabolite generated by the CYP isozymes.

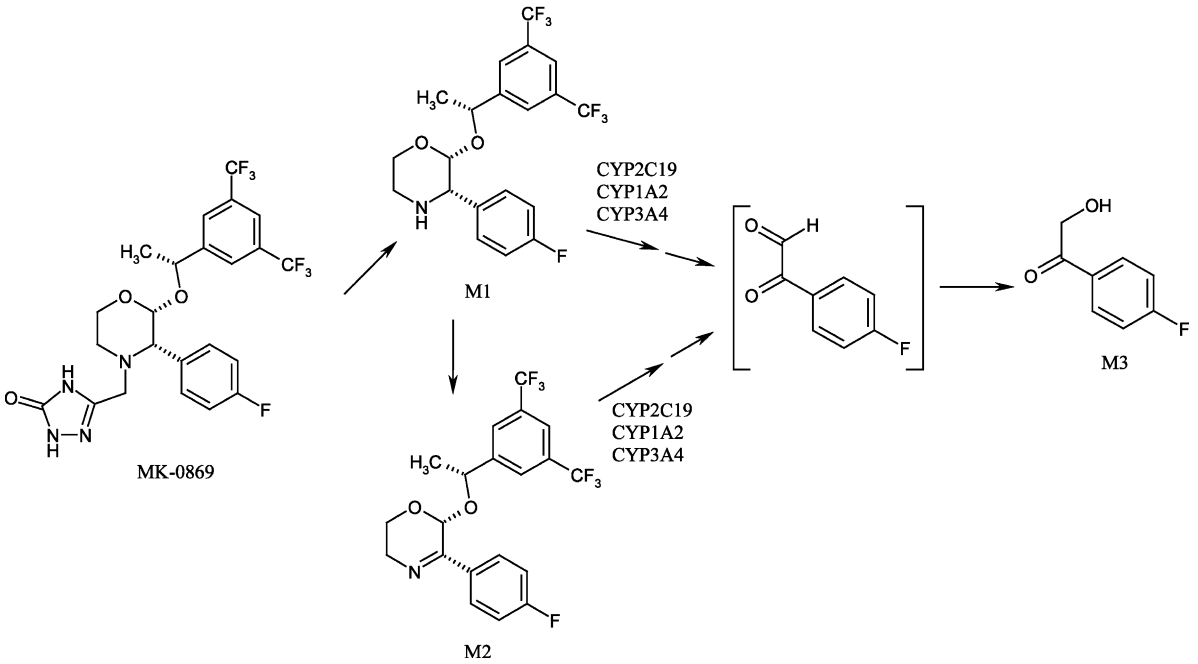


Fig. 4. Metabolic pathway for the M3 metabolite of MK-0869.

4. Conclusions

In this report we have shown that LC–NMR is a suitable technique for the structural identification of volatile metabolites. It will probably be the main technique of use especially when the molecular weights of metabolites are less than 200 Da and where LC–MS might provide little or no structural information. Connecting several detectors on-line to the LC–NMR system increases the ability to target the correct metabolite peak when UV is not sufficient. We were able to propose the structure of metabolite M3 as *p*-fluoro-α-hydroxyacetophenone by the use of chemical derivatization and LC–NMR studies. We identified the very polar metabolite M3 from the independent incubations of the primary metabolites of MK-0869 (M1 and M2) with microsomes containing human recombinant CYP2C19, CYP1A2 and CYP3A4. These results suggested that the formation of M3 from M1 and M2 proceeds through an *O*-dealkylation metabolic pathway.

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