Online Hydrogen-Deuterium Exchange and a Tandem-Quadrupole Time-of-Flight Mass Spectrometer Coupled with Liquid Chromatography for Metabolite Identification in Drug Metabolism*

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Abstract

A method for metabolite identification in drug discovery and development utilizing online hydrogen-deuterium (H-D) exchange and a tandem-quadrupole time-of-flight mass spectrometer coupled with liquid chromatography has been developed for the first time. For purposes of evaluating this method, nimodipine is incubated with rat, dog, monkey, and human hepatic microsomes over a 60-min period. This procedure involves the replacement of H_2O with D_2O in the mobile phase. The equilibration time required to change (switch) from H_2O in the mobile phase into D_2O is approximately 10 min. There is no significant difference between the retention times in H_2O or D_2O mobile phase. The results show that the separations of nimodipine and its metabolites and the subsequent H-D exchanges were achieved online. This leads to the identification of nimodipine metabolites formed in vitro. Five metabolites of nimodipine are identified and characterized. These metabolites are formed by dehydrogenation, demethylation of methoxy group, cleavage of the ester groups by hydrolysis or oxidation, and hydroxylation of methyl groups. The combination of the H-D exchange process and the assay system is found to be a powerful tool for the study of metabolite identification. The method appears to be highly desirable for identification of metabolites produced by dehydrogenation, oxidation, and dealkylation, which are discussed herein. This method also greatly enhances throughput, which in turn facilitates the ability to rapidly provide metabolite elucidation.

Introduction

Structural information on metabolites can serve to accelerate the drug discovery and development cycles. In recent years, drug discovery efforts have moved from development to discovery using high-throughput technology for screening compounds, which in turn has led to strong demand for more rapid methods for metabolite identification (1). Metabolite identification is a crucial part of the process of determining the following: the phase I metabolites likely to be formed in vivo, differences between species in drug metabolism, major circulating metabolites, phase I and II pathways of metabolism, pharmacologically-active metabolites, and determination of metabolizing enzyme inhibition or induction (or both) (2,3). The ability to produce this information earlier in the discovery phase is becoming increasingly important as a basis for judging whether a drug candidate merits further development. Identification of potential metabolic liabilities or issues and provision of a metabolism perspective to guide synthesis efforts so as to block or enhance metabolism to optimize the pharmacokinetic and safety profiles of newly synthesized drug candidates are major benefits of such early identification.

Hydrogen-deuterium (H-D) exchange methods have long been recognized as a valuable means to study structure and conformational changes in proteins and nucleic acids, and are often able to access structural information that other methods cannot. Such methods work well with samples in various states: in solution and in the membrane protein-bound, crystalline, molten globular, and lyophilized states in many biological complexes (4–8). H-D exchange methods are useful for determination of the presence, number, and position of H-D exchangeable functional groups on metabolite structures and thus serve as an aid for structural elucidation of metabolites, as well as aiding in differentiation of compounds. It has been reported that H-D exchange can be used to discriminate between N- or S-oxide formation and monohydroxylation; also, conjugation such as glucuronide can be easily identified with this technique (9). However, in this study the samples were dried under a nitrogen stream and then reconstituted in D_2O mobile phase for the H-D exchange.

Instrumental techniques such as NMR and mass spectrometry (MS) are critical in such determinations. NMR spectroscopic techniques are most often used to study H-D in metabolite iden-

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tification in drug development. NMR is a good choice, but MS has advantages over NMR with respect to the sensitivity, smaller sample size, and the fact that its sample analysis time is much faster. Thus, several studies have been carried out using H-D exchange in conjunction with MS. For nearly every ionization method (10–19), a procedure has been developed to perform H-D exchange experiments including electron impact, chemical ionization, atmospheric pressure chemical ionization, fast atom bombardment, thermospray, gas chromatography (GC)–MS, and electrospray ionization. Also, a method has been reported that used deuterated buffer for NMR and MS analyses to identify the metabolites formed in protein films with ethylene dibromide, trichloroacetic acid, and tetrachloroethylene (20). In recent years, a few more efforts have been reported in which MS was used to investigate H-D in metabolite identification using offline systems (21). Recently, the deuterium exchange of labile hydrogen atoms for structure elucidation of unknown impurities and degradation products was reported (22).

Recently we have developed an automated assay for identification of metabolites from in vitro microsomal incubations for use in the early stage of drug discovery (23). This is a generic approach that involves integrated system setup, bioanalysis, and data handling to maximize sample throughput and speed up the process for identification of metabolites. The assay utilizes a robotic liquid handler (Genesis workstation), PALLAS MetabolExpert 10.0 software to predict possible metabolites, exact mass measurement via a tandem-quadrupole time-of-flight (QTOF)-MS coupled with liquid chromatography (LC), and MetaboLynx software to find potential metabolites and Advanced Chemistry Development/MS (ACD/MS) software to predict hypothetical metabolite chemical structures that provided useful tools to assist the prediction of the metabolic pathway of potential drug candidates. This method greatly enhances throughput to provide this guidance to the synthetic chemist.

In this paper, extended efforts to refine and enhance high-throughput methodology for metabolite identification in drug discovery by incorporating the H-D exchange procedure have been extended. Isopropyl 2-methoxyethyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate (nimodipine) is a potent calcium antagonist, which selectively inhibits serotonin- and thromboxane-induced contractions in animal and cerebral arteries (24–31). Nimodipine was used to evaluate the method. Figure 1 shows the chemical structure of nimodipine with the labile H exchange, as indicated by asterisks. The arrows indicate the positions of the possible metabolites as predicted using PALLAS MetabolExpert software. Reported are the results of a study aimed at finding a method to replace some of the protons present in nimodipine with deuterium atoms to facilitate the characterization of metabolites formed in vitro and to determine its potential feasibility for use with other drug candidates.

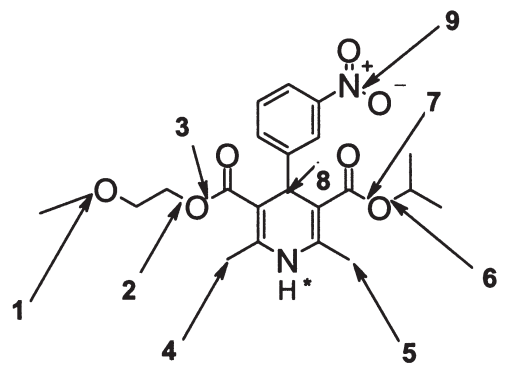

Figure 1. The chemical structure of nimodipine used in this study with the labile H exchange as indicated by an asterisk: (1) cleavage, (2) cleavage, (3) cleavage, (4) hydroxylation, (5) hydroxylation, (6) cleavage, (7) cleavage, (8) hydroxylation, and (9) cleavage.

Experimental

Chemicals and materials

Isopropyl-2-methoxyethyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate (nimodipine) was obtained from Sigma Chemical (St. Louis, MO). Rat (Wistar, male), dog (beagle, female), monkey (rhesus, male), and human (pool, male) liver microsomes were obtained from a commercial source (Xenotech, Kansas City, KS). Magnesium chloride, potassium phosphate, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase nicotinamide adenine dinucleotide phosphate (NADPH) were also from Sigma. All other chemicals were reagent grade. The analytical column Synergi, MAX-RP (150 × 2 mm, 4 µm) was from Phenomenex (Torrance, CA).

Software

PALLAS MetabolExpert 10.0 software for Windows was obtained from CompuDrug Chemistry AHSystems Group (Bowie, MD) (32–34). PALLAS has its own structure drawing interface, as well as import/export functions and has a graphical input interface for transformations. The result is graphical, and the user can easily rearrange the metabolic tree. MetabolExpert 10.0 software was used to predict metabolites for dextromethorphan, alprenolol, and propranolol, identifying sites on the molecule where metabolic transformation may occur. The MetaboLynx 3.5 software (Micromass UK, Manchester, U.K.) was used to search for and identify metabolite peaks in the mass spectra and to determine the elemental composition of metabolites.

Microsomal incubations

Metabolites formed from nimodipine were generated using rat (Wistar, male), dog (beagle, female), monkey (rhesus, male), and human (pool, male) liver microsomes over a 60-min incubation period. The dimethyl sulfoxide (DMSO) concentration was 0.2% (v/v). The incubation was terminated with acetonitrile. Microsomal incubations were performed in the presence of an NADPH-generating system composed of 3mM $MgCl_2$, 1mM NAPDH, 5mM glucose-6-phosphate, 1mM EDTA, and 1 unit/mL glucose-6-phosphate dehydrogenase in a 0.1M potassium phosphate buffer (pH 7.4); all concentrations are relative to the final incubation volume. Compounds were diluted to obtain a final incubation concentration of 20µM. Final protein concentrations were 0.5 mg/mL. Incubations were conducted at 37°C in duplicate with samples taken at 0 and 60 min. Control reactions (no

NADPH, n = 1) were conducted as described previously, but with the substitution of an equal volume of distilled water for NADPH in the NADPH-generating system. At each time point, 200 µL of sample was added to 700 µL of acetonitrile to terminate the reaction. The plates were then centrifuged (1900 × g), and the supernatants were transferred to a new plate to be assayed by LC–MS.

LC–MS

A general method was used to perform LC–MS and LC–MS–MS experiments. LC–MS was carried out by coupling a HP1100 system to Micromass QTOF quadrupole orthogonal acceleration TOF mass spectrometer (Micromass UK). The instrument was operated in positive ion mode. The source and desolvation temperatures were set to 150 and 300°C, respectively. The nitrogen desolvation and nebulizer gas flow rates were set to 400 and 90 L/h, respectively. The cone voltage for nimodipine was set to 10 V, and for MS–MS experiments the collision energy used was 25 eV (for nimodipine). Prior to performing all experiments, the instrument was calibrated for positive mode using poly-(propylene glycol)s. This was chosen for accurate mass measurements (monoisotopic lock mass) of potential metabolites. High-performance liquid chromatography (HPLC) was carried out using the Phenomenex column, Synergi, MAX-RP (150-× 2-mm, 4 µm). The HPLC column was maintained at 40°C. The gradient program was carried out in 30 min with water or deuterium oxide containing 5.0mM ammonium acetate pH 4.0 (mobile phase A) and acetonitrile (mobile phase B) at 0.25 mL/min. Both solvents were degassed online. The gradient program was conducted as follows: an initial 85% A, hold for 2 min at 85% A, followed by a linear gradient for 13 min to 35% A, linear gradient for 5 min to 30% A, linear gradient for 1 min to 25% A, linear gradient for 3 min to 2% A, linear gradient for 2 min to 85% A, and the column was then equilibrated for 4 min at 85% A. Exact mass analysis was performed on TOF-MS at resolutions in the region of 8000 full width half maxima.

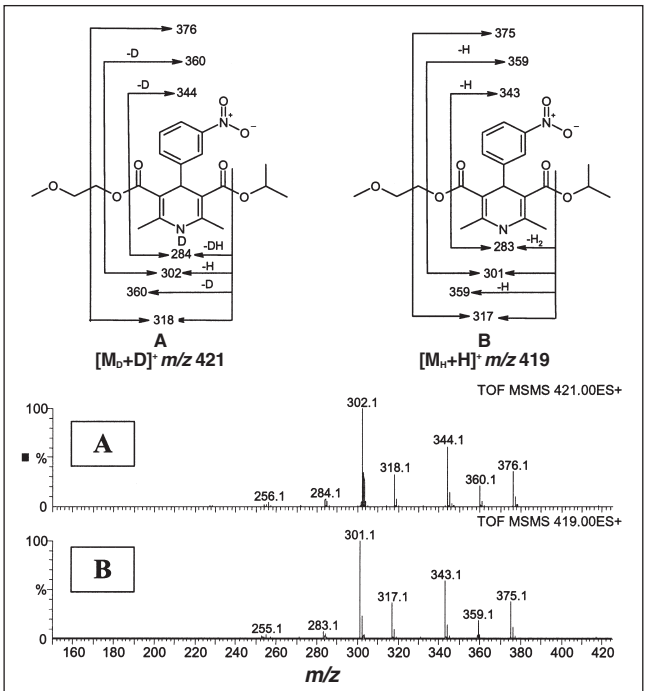

Figure 2. QTOF-MS–MS spectra of nonmetabolized nimodipine in (A) H_2O and (B) D_2O.

Results and Discussion

H-D exchange experiments

Table I shows the exchange of labile hydrogens in nimodipine and its metabolites formed in vitro. The labile hydrogens ranged from none to two, which provides a significant means of identification, particularly with dehydrogenation metabolites, suggesting that the dehydrogenation took place on the pyridine moiety, resulting in a loss of one labile hydrogen.

Table I. Exchange of Labile Hydrogens in Nimodipine and Metabolites Formed In Vitro

Compound	Labile hydrogen	Mass [M_H+H]+ (m/z)	Mass [M_D+D]+ (m/z)	
			Predicted	Measured
Nimodipine	1	419	421	421
M-1	1	359	361	361
M-2	2	435	438	438
M-3	1	403	405	405
M-4	2	405	408	408
M-5	0	417	418	418

* Abbreviations: M_H = the molecular weight in H_2O and M_D = the molecular weight in D_2O.

Characterization of biotransformation products using QTOF-MS–MS

The structures of nimodipine and its metabolites were elucidated by QTOF-MS–MS analysis under the LC–MS–MS conditions described in the methodology.

Nimodipine synthetic reference material

A spectrum of nimodipine was acquired, which revealed a protonated molecular ion [M_H+H]$^+$ at m/z 419. M_H represents the molecular weight in H_2O. The product-ion spectrum of m/z 419 showed fragment ions of m/z 375, 359, 343, 301, and 283. When H_2O was replaced with D_2O in the mobile phase, the full-scan mass spectrum of nimodipine revealed a molecular ion [M_D+D]$^+$ at m/z 421 (M_D represents the molecular weight in D_2O), 2 mass units higher than [M_H+H]$^+$. The increase of 2 mass units is in agreement with the presence of one exchangeable hydrogen atom in nimodipine, as indicated by the asterisk in Figure 1, whereas the other mass unit was contributed by the charge D+. The product-ion spectrum of m/z 421 showed the fragment ions of m/z 376, 360, 344, 302, and 284. The nonmetabolized nimodipine gives an identical retention time and MS–MS characterization as nimodipine synthetic reference material. The proposed TOF-MS–MS fragmentation for nonmetabolized nimodipine in D_2O and H_2O is shown in Figure 2.

Metabolite M-1

The full-scan mass spectrum of M-1 revealed a

protonated molecular ion $[M_H+H]^+$ at *m/z* 359, 60 amu lower than nimodipine, suggesting that this metabolite was a cleaved product. The molecular ion $[M_D+D]^+$ was 2 amu greater than $[M_H+H]^+$ M-1, indicating the presence of one exchangeable hydrogen atom in M-1. Nimodipine has one exchangeable hydrogen atom, and the cleavage will result in another exchangeable hydrogen atom. The proposed TOF-MS–MS fragmentation for M-1 in D_2O and H_2O suggested that M-1 formed from the cleavage, as well as dehydrogenation of nimodipine.

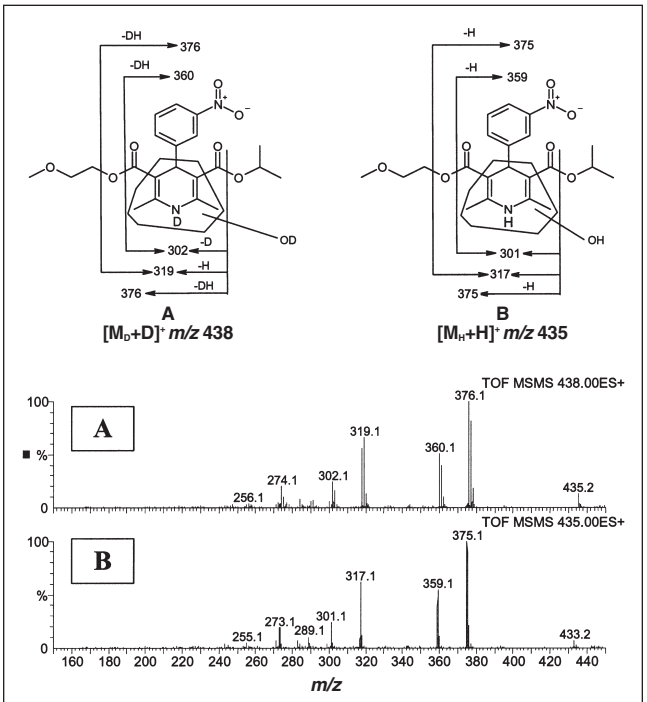

Figure 3. QTOF-MS–MS spectra of metabolite M-2 in (A) D_2O and (B) H_2O.

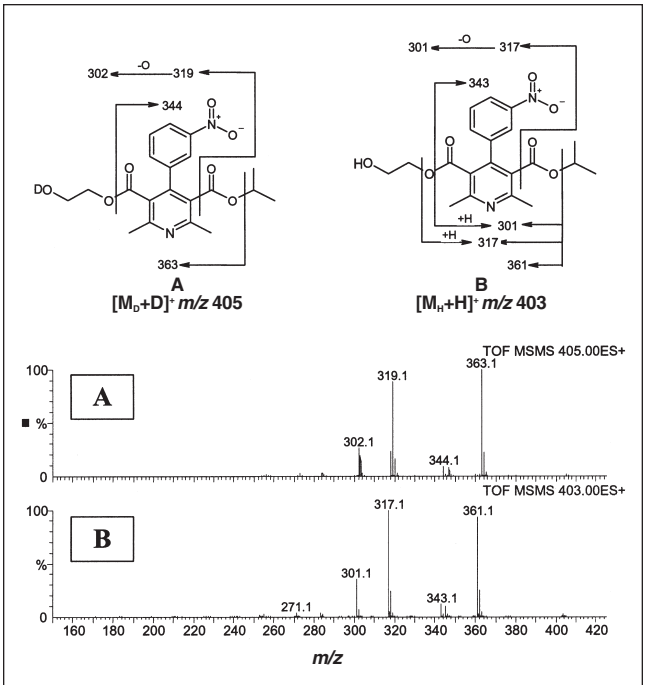

Figure 4. QTOF-MS–MS spectra of metabolite M-3 in (A) D_2O and (B) H_2O.

The H-D experiment supports this hypothesis.

Metabolite M-2

The full-scan mass spectrum of M-2 revealed a protonated molecular ion $[M_H+H]^+$ at *m/z* 435, 16 amu higher than nimodipine, indicative of the addition of an oxygen atom. The product-ion spectrum of $[M_H+H]^+$ at *m/z* 435 showed the fragment ions of *m/z* 375, 359, 317, and 301. When H_2O was replaced with D_2O in the mobile phase, the full-scan mass spectrum of nimodipine revealed a molecular ion $[M_D+D]^+$ at *m/z* 438, 3 mass units higher than $[M_H+H]^+$ M-2, indicating the presence of two exchangeable hydrogen atoms in M-2. The product-ion spectrum of *m/z* 438 showed the fragment ions of *m/z* 376, 360, 319, and 302. The proposed TOF-MS–MS fragmentation for M-2 in D_2O and H_2O is shown in Figure 3. Based on these data, M-2 was identified as monohydroxy of nimodipine.

Metabolite M-3

The full-scan mass spectrum of M-3 revealed a protonated molecular ion $[M_H+H]^+$ at *m/z* 403, 16 amu lower than nimodipine, suggesting that this metabolite was a cleaved product. The product-ion spectrum of $[M_H+H]^+$ at *m/z* 403 showed the fragment ions of *m/z* 361, 343, 317, and 301. When H_2O was replaced with D_2O in the mobile phase, the full-scan mass spectrum of nimodipine revealed a molecular ion $[M_D+D]^+$ at *m/z* 405, 2 mass units higher than $[M_H+H]^+$ M-3, indicating the presence of one exchangeable hydrogen atom in M-3. The product-ion spectrum of *m/z* 405 showed the fragment ions of *m/z* 363, 344, 319, and 302. Nimodipine has one exchangeable hydrogen atom, with the cleavage producing another exchangeable hydrogen atom. This eliminates the possibility that M-3 is caused by direct cleavage of nimodipine. It is possible that M-3 can be formed from the dehydrogenation and cleavage of nimodipine. The proposed TOF-MS–MS fragmentation for M-3

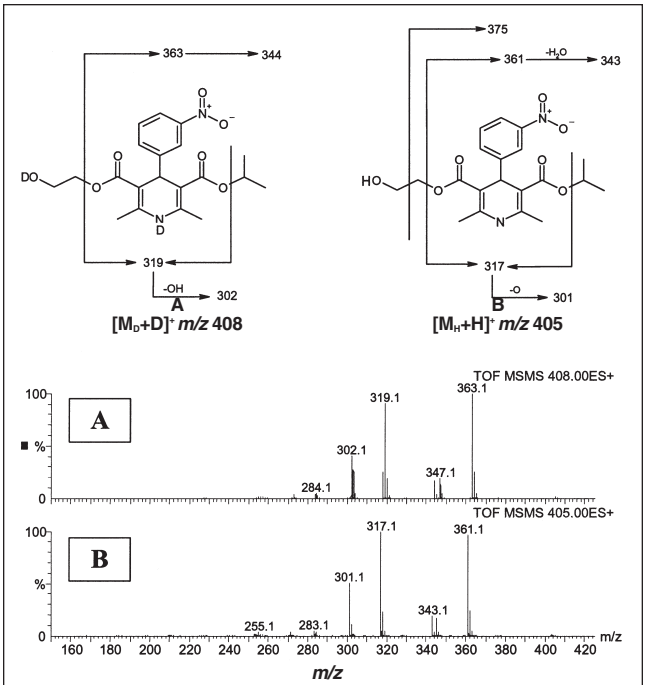

Figure 5. QTOF-MS–MS spectra of metabolite M-4 in (A) D_2O and (B) H_2O.

in D_2O and H_2O is shown in Figure 4.

Metabolite M-4

The full-scan mass spectrum of M-4 revealed a protonated molecular ion $[M_H+H]^+$ at *m/z* 405, 14 amu lower than nimodipine, suggesting that this metabolite was a cleaved product. The product-ion spectrum of $[M_H+H]^+$ at *m/z* 405 showed the fragment ions of *m/z* 375, 361, 343, 317, and 301. When H_2O was replaced with D_2O in the mobile phase, the full-scan mass spectrum of nimodipine revealed a molecular ion $[M_D+D]^+$ at *m/z* 408, 3 mass units higher than $[M_H+H]^+$ M-3, indicating the presence of two exchangeable hydrogen atoms in M-4. The product-ion spectrum of *m/z* 408 showed the fragment ions of *m/z* 363, 344, 319, and 302. Nimodipine has one exchangeable hydrogen atom; the cleavage will produce a second. This suggests the possibility that M-1 can be formed from the cleavage of nimodipine. The proposed TOF-MS–MS fragmentation for M-4 in D_2O and H_2O is shown in Figure 5.

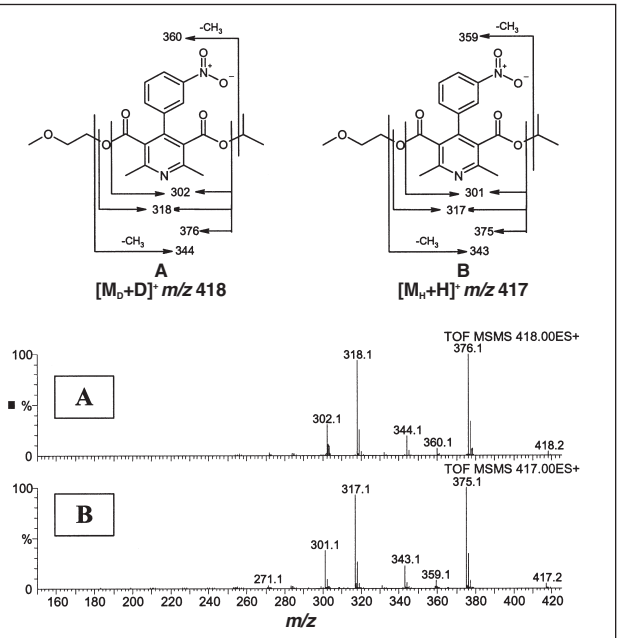

Figure 6. QTOF-MS–MS spectra of metabolite M-5 in (A) D_2O and (B) H_2O.

Metabolite M-5

The full-scan mass spectrum of M-5 revealed a protonated molecular ion $[M_H+H]^+$ at *m/z* 417, 2 amu lower than nimodipine, suggesting that the nimodipine underwent dehydrogenation. The product-ion spectrum of $[M_H+H]^+$ at *m/z* 417 showed the fragment ions of *m/z* 375, 359, 343, 317, and 301. When H_2O was replaced with D_2O in the mobile phase, the full-scan mass spectrum of nimodipine revealed a molecular ion $[M_D+D]^+$ at *m/z* 418, 1 mass unit higher than $[M_H+H]^+$ M-5, indicating there is no exchangeable hydrogen atom in M-5. The product-ion spectrum of *m/z* 418 showed the fragment ions of

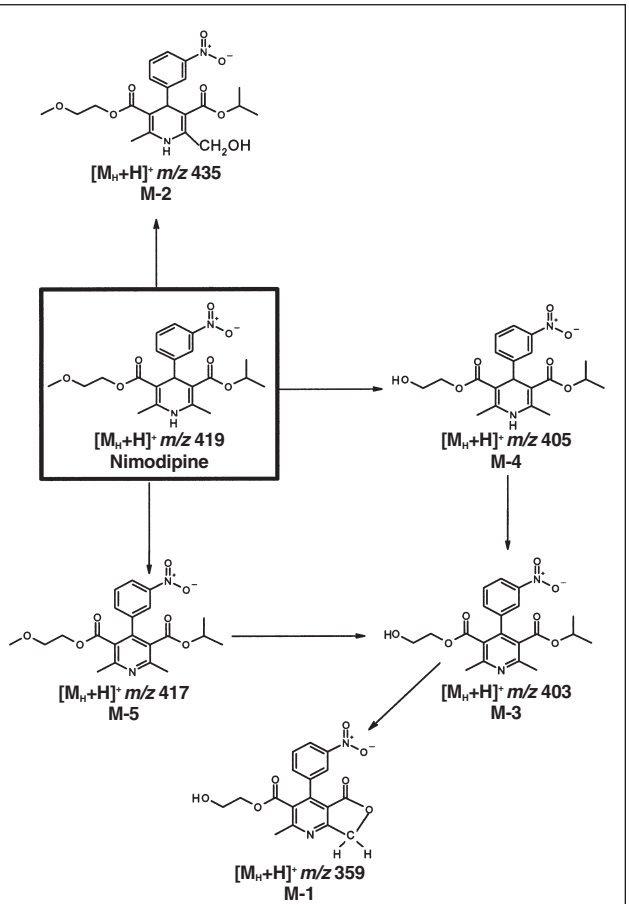

Figure 7. Proposed metabolic pathways of nimodipine in hepatic microsomal incubations in H_2O.

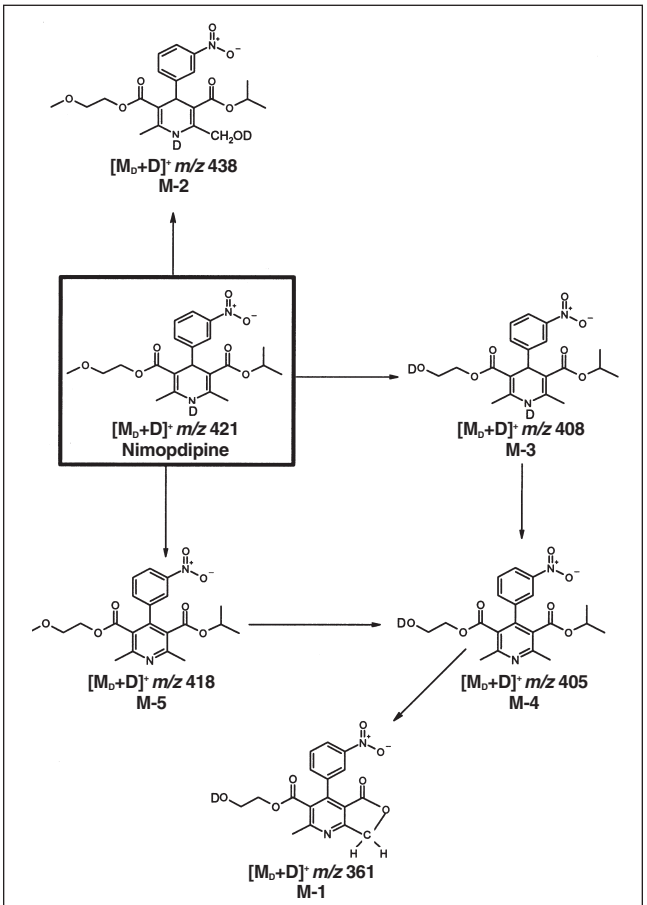

Figure 8. Proposed metabolic pathways of nimodipine in hepatic microsomal incubations in D_2O.

m/z 376, 360, 344, 318, and 302. Nimodipne has only one exchangeable hydrogen atom on the pyridine moiety. This suggests that the dehydrogenation took place on the pyridine moiety and resulted from the loss of the exchangeable hydrogen atom. The proposed TOF-MS–MS fragmentation for M-5 in D_2O and H_2O is shown in Figure 6.

Conclusion

The feasibility of carrying out online H-D exchange via LC–QTOF-MS has been investigated. Five metabolites of nimodipine formed in each of four species of liver microsomes; all were identified and characterized by LC–QTOF (Figures 7 and 8). Thus, there was a good correlation across the four species studied. Nimodipine metabolized by means of dehydrogenation, demethylation of methoxy group, cleavage of the ester groups by hydrolysis or oxidation, and hydroxylation of methyl groups. H-D exchange provided significant information for all five metabolite identifications. One advantage of the H-D exchange method is that with LC–MS–MS it offers an easy estimation of the number of labile hydrogen atoms in such groups as –SH, –OH, –NH, $–NH_2$, and –COOH. This number is useful in comparing metabolite structure with that of the parent drug to determine the presence or absence of the above groups. H-D exchange experiments have facilitated structural elucidation and interpretation of fragmentation processes, as well. The generic method has proved to be simple, easy, fast, sensitive, robust, and reliable, as well as enhancing throughput, which in turn facilitates the ability to rapidly provide characterization of metabolites in vitro. This time saving, combined with the particular benefits of QTOF such as higher resolution, makes the method a valuable tool for structure elucidation. This method is valuable when used with low-resolution instruments, as well. The results indicated that this method should be particularly desirable for identification of metabolites produced by dehydrogenation, oxidation, and dealkylation.

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