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Usefulness of the hydrogen–deuterium exchange method in the study of drug metabolism using liquid chromatography-tandem mass spectrometry

Noriko Ohashi *, Satoshi Furuuchi, Masayoshi Yoshikawa

Pharmaceutical Development Research Laboratory, Tanabe Seiyaku Company Limited, Kawagishi 2-2-50, Toda-shi, Saitama 335, Japan

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Abstract

The usefulness of the hydrogen–deuterium (H-D) exchange method in the study of drug metabolism was investigated. Metabolite samples of denopamine and promethazine prepared in vitro were introduced to a triple stage quadrupole tandem mass spectrometer via a high performance liquid chromatography (HPLC) system using a deuterated mobile phase. Mass spectra by various ionization modes and collisionally induced dissociation (CID) mass spectra were obtained. A metabolite of denopamine was observed to have a shift of 7 mass units by the H-D exchange method, and this shift proved to be a glucuronidated metabolite. Discrimination between *N*- or *S*-oxide formation and hydroxylation observed in the metabolism of promethazine was also easily accomplished by this method. In this manner, the structures of the metabolites were elucidated unequivocally by determining the number of labile hydrogen atoms by the use of the H-D exchange method, since various reactions in drug metabolism are accompanied by an increase or a decrease in the number of labile hydrogen atoms. Additional information on the structures was obtained by analysis of the CID spectra of the molecular ion species. Thus, the combination of the H-D exchange method and tandem mass spectrometry was found to be very useful for the study of drug metabolism.

Keywords: Liquid chromatography-mass spectrometry; Tandem mass spectrometry; Drug metabolism; H-D exchange

1. Introduction

The development of liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS-MS) has drastically changed the methods of drug metabolism studies. Particularly, the interface systems for electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been greatly developed and they have made it possible to ionize almost all compounds. Recently the interfaces have been improved to allow analysis at a flow rate of 1.0 ml min^{-1}. Tandem mass spectrometry has been recognized as a useful method for analysis of very small amounts of metabolites found in biological

* Corresponding author. Tel.: +81 484338111; fax: +81 484338170; e-mail: noriko-o@tanabe.co.jp

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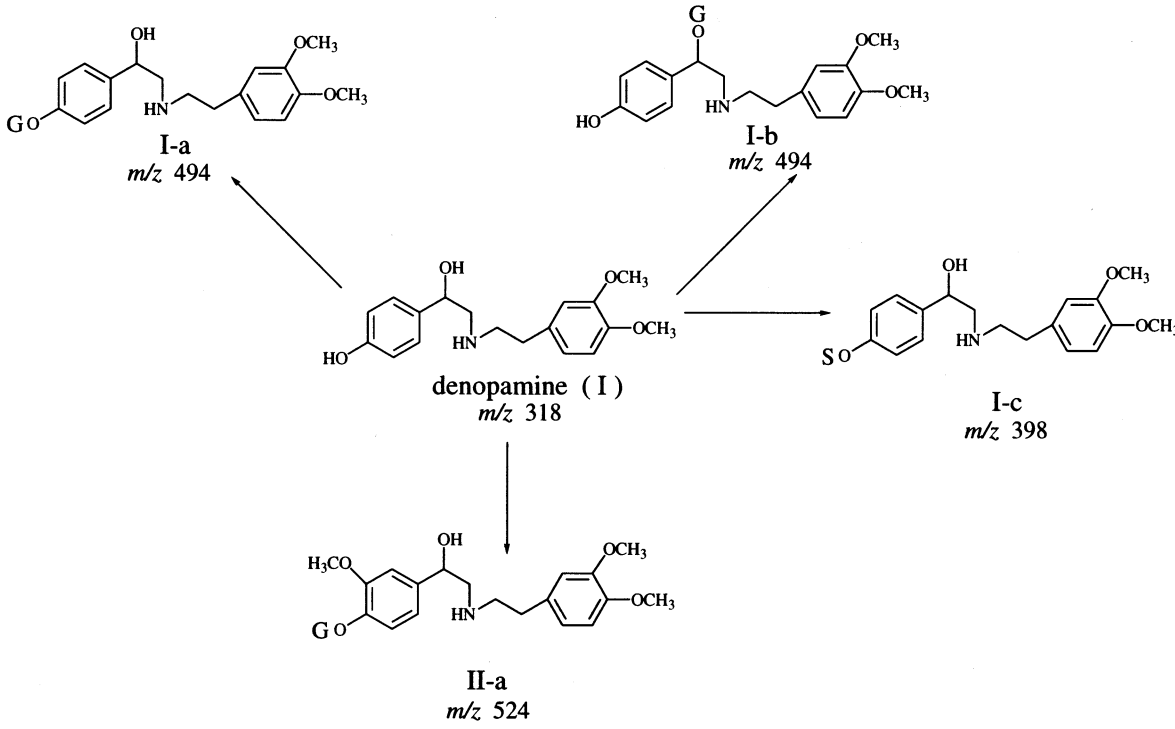

Fig. 1. Major urinary metabolites of denopamine in the dog. G, glucuronic acid; S, sulfuric acid.

fluids. Although much structural information is obtained from the collisionally induced dissociation (CID) mass spectra, additional information could be obtained by the hydrogen–deuterium (H-D) exchange method which determines the number of labile hydrogen atoms in fragments of ions. In mass spectrometry as well as in nuclear magnetic resonance (NMR), some studies on the H-D exchange method have been reported utilizing fast atom bombardment (FAB)-MS with glycerol-*O*-d_3 as the matrix [1] and chemical ionization (CI)-MS with deuterated ammonia (ND_3) as the reagent gas [2]. These methods, however, are not necessarily found to be practical because the H-D exchange efficiencies may not be high and large amounts of deuterated reagents are needed. On the other hand, H-D exchange methods using LC-MS have been reported in CI [2], thermospray (TSP) [3–5], ESI [6,7] and FAB [1], and relatively high exchange efficiencies have been achieved. TSP mass spectra of deuterated molecular ion species obtained by the so-called 'sandwiched slug injection technique' have been reported by Siegel [3]. ESI mass spectra of angiotensins I and II obtained with a microcolumn LC-MS using deuterium oxide in the mobile phase have been reported by Karlsson [6]. H-D exchange methods are useful for the determination of the number of H-D exchangeable functional groups on metabolite structures and thus serves as an aid for structural elucidation of fragments of ions.

In this study, H-D exchange efficiencies with samples dissolved in D_2O or methanol-d_1 were compared between the flow injection method using TSP, APCI, and ESI and the direct probe insertion method using EI and FAB. Profile analysis of the metabolites of a model drug was also carried out using deuterium oxide as the mobile phase component in an LC-MS system to examine the usefulness of the H-D exchange method in the study of drug metabolism.

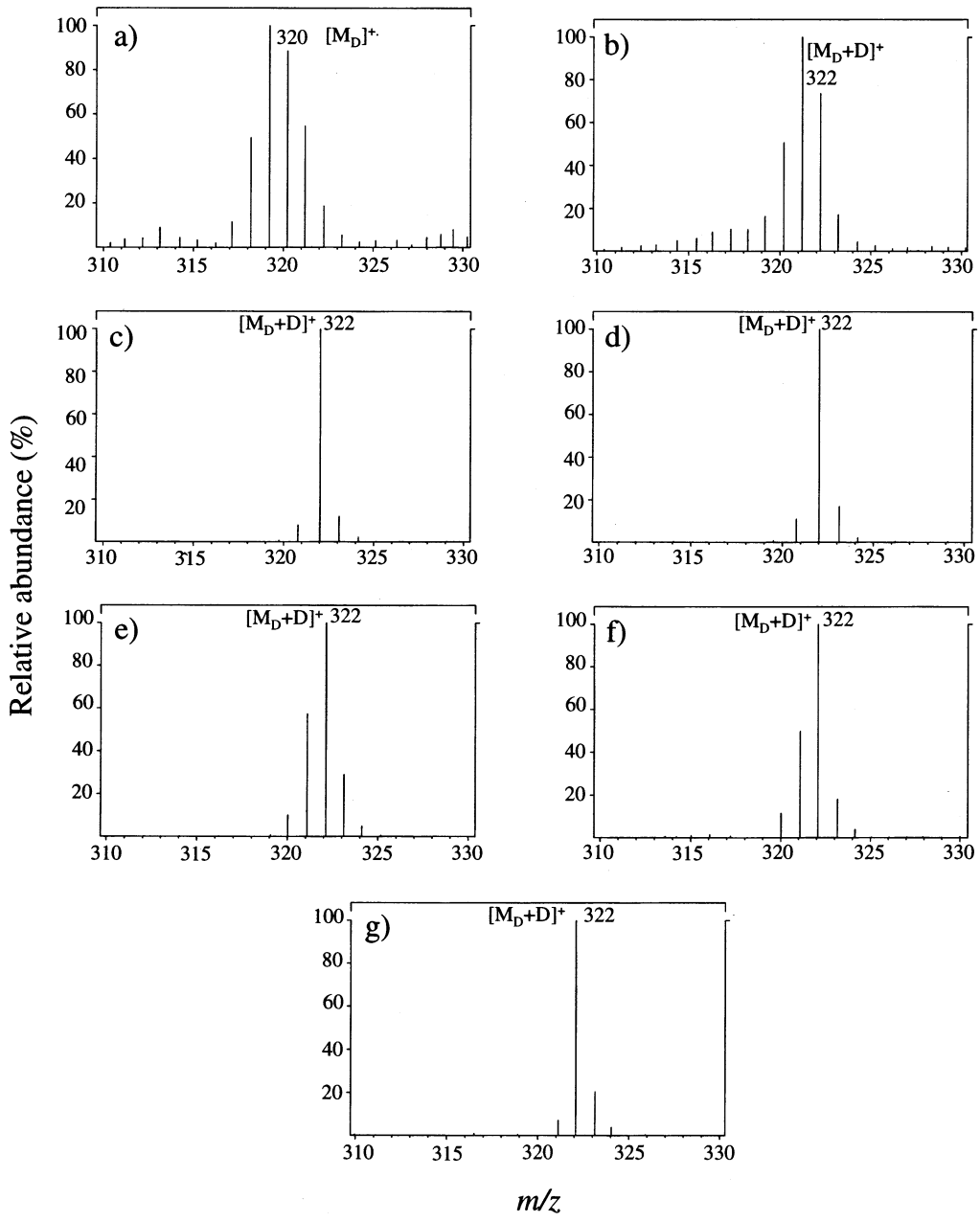

Fig. 2. Mass spectra of the molecular species of denopamine following H-D exchange utilizing various introduction methods and ionization modes. (a) EI (direct inlet), (b) FAB (direct inlet), (c) TSP (flow injection), (d) APCI (flow injection), (e) ESI (flow injection), (f) ESI (syringe pump), and (g) ESI (HPLC).

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile, methanol and ammonium acetate were obtained from Nacalai Tesque (Kyoto, Japan), deuterium oxide (99.9 at.% D), methanol-d_1 (99.7 at.% D) and glycerol-*O*-d_3 (98 at.% D) from ISOTEC (Miamisburg, USA), and *p*-chlorobenzensulfonamide (*p*-CBSA) from Aldrich (Milwaukee, USA). Metabolites of denopamine (Tanabe Seiyaku, Japan), a β_1-selective inotropic cardiotonic agent [8], were prepared by incubating denopamine (50 μM) with dog hepatocytes for 24 h at 37°C. Promethazine, a histamine H_1 antagonist, and its metabolites prepared with human liver microsomes were kindly provided by Mr Nakamura of Hokkaido University, Sapporo, Japan.

2.2. Apparatus

All mass spectra in the positive ion mode were obtained using a TSQ 700 triple stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with EI, FAB, TSP, APCI, and ESI systems or units. A Waters HPLC model 600MS system (Milford, MA) was used.

2.3. Sample introduction methods

2.3.1. Flow injection method

A sample loop of 200 μl was installed in a Rheodyne (model 7725) injection system. Denopamine was dissolved in a mixture of methanol and 0.02 M ammonium acetate in D_2O (5:995 v/v) at a concentration of 16 μM, and introduced directly into the interface carrier stream. The carrier solvent system of a non-deuterated mixture consisting of 0.02 M ammonium acetate and acetonitrile (1:1 v/v) was used at a flow rate of 1.0 ml min^{-1}.

Table 1
H-D exchange ratios of denopamine-d_3 obtained by various combinations of ionization and introduction methods

Ionization	Introduction	H-D exchange (%)
EI	Direct inlet	48.3
FAB	Direct inlet	42.8
TSP	Flow injection	94.9
APCI	Flow injection	93.7
ESI	Flow injection	68.9
ESI	Syringe pump	72.3
ESI	HPLC	96.1

2.3.2. Syringe pump method

A Harvard model 22 syringe pump (Harvard Apparatus, South Natik, MA) was used to infuse samples into the electrospray source at a flow rate of 20 μl min^{-1}.

2.3.3. HPLC methods

Samples were dissolved in the mobile phase. For the H-D exchange studies, D_2O was used as a mobile phase component; solvent A was 0.02 M ammonium acetate in D_2O and solvent B was acetonitrile. The flow rate was 1.0 ml min^{-1}. Separation was performed on a TSK gel ODS-80 Ts column (4.6 mm × 150 mm, 5 μm, Tosoh, Tokyo, Japan) maintained at 40°C. For the analysis of denopamine and its metabolites, a linear gradient was run as follows: A:B = 85:15–60:40 (20–40 min). For the analysis of metabolites of promethazine, the mobile phase was a mixture consisting of solvents A and B (56:44 v/v).

2.3.4. Ionization methods

Denopamine and *p*-CBSA dissolved in methanol-d_1 (3 ~ 5 mM) were introduced via the direct inlet into the ion source of EI or FAB.In EI-MS, a direct insertion probe was used to evaporate the samples into the ion source, which was heated at 180°C. The temperature of the probe tip was set to 100°C and the ionization energy was 70 eV. In FAB-MS, the sample was added to the matrix of glycerol-*O*-d_3. A standard saddle field FAB gun (Ion Tech, Teddington, UK) was operated at a 2 mA current and 7 keV energy and xenon was used as the bombarding gas (1×10^{-5} Torr). The ion source temperature was maintained at 50°C. In TSP-

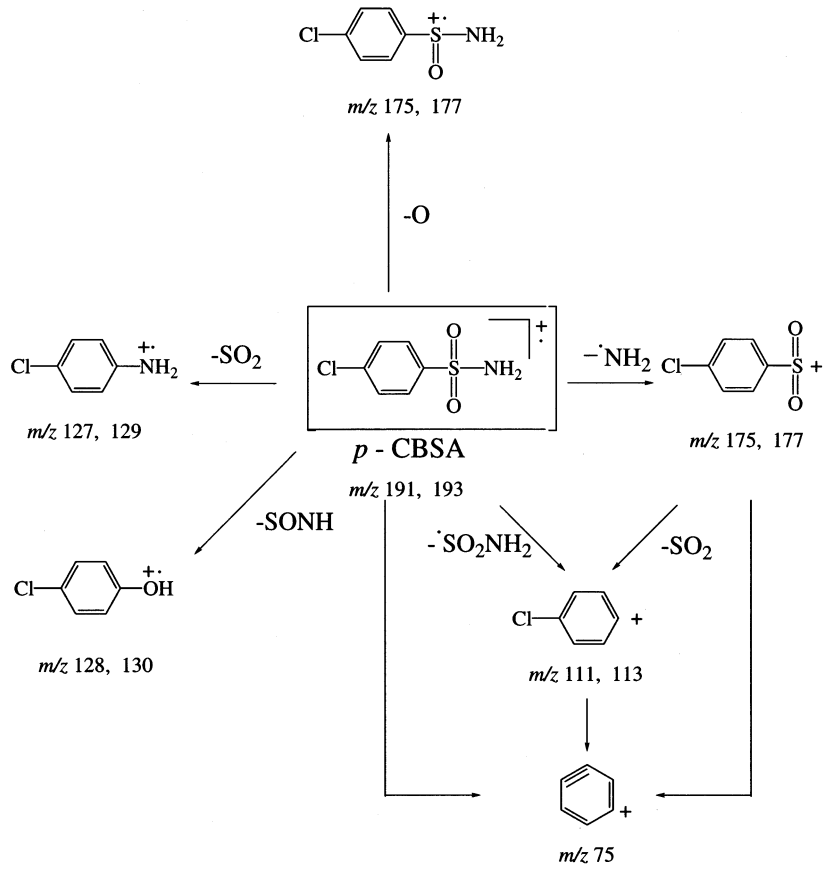

Fig. 3. Fragmentation pathways of *p*-CBSA.

MS, the vaporizer temperature was maintained at 95°C and the ion source temperature was set at 220°C. The voltages of the discharge electrode and repeller were set at 1500 and 0 V, respectively. In APCI-MS analysis, the temperatures of the vaporizer and heated capillary were maintained at 400 and 150°C, respectively. The voltage of the corona discharge was set to 4.5 kV. In ESI-MS, a potential of 4.5 kV was applied to the ESI needle. The heated capillary was maintained at 250°C. APCI-MS and ESI-MS were carried out using nitrogen as the sheath (70 psi) and auxilary (15 l min^{-1}) gasses to assist nebulization. CID was carried out using argon as the collision gas at a pressure of 1.0×10^{-3} Torr and at a collision energy of -20 eV.

3. Results and discussion

3.1. H-D exchange ratios of denopamine by various introduction methods and ionization modes

Denopamine (Fig. 1, I) possesses three labile hydrogen atoms, one each in the alcoholic hydroxy, phenolic hydroxy and secondary amino groups. Fig. 2 shows the mass spectra of denopamine following H-D exchange using various combinations of introduction methods (direct probe insertion, flow injection, syringe pump and HPLC) and ionization modes (EI, FAB, TSP, APCI, and ESI). The percentage D values were calculated by a method described previously [9] and the results were summarized in Table 1. The percentage D values in the EI-MS and FAB-MS analyses were relatively low,

being 48 and 43%, respectively, probably due to back-exchange. The H-D exchange efficiency in the flow injection-ESI mode was 69% and did not change with the volume of the injection loop (20–1000 μl) and the concentration of ammonium acetate (0.05–0.2 M) in D_2O. When Siegel carried out an H-D exchange method, the so-called 'sandwiched slug injection technique', by use of ammonium acetate-d_6, the H-D exchange efficiencies were more than 95% [3]. In his procedure, the sample was dissolved in a small volume of deuterated solvent, then the microliter sample was injected between two 50 μl slugs of D_2O into the non-deuterated solvent carrier stream. In our study, a relatively high H-D exchange efficiency was obtained when the loop was filled with the sample dissolved in the D_2O solution of non-deuterated ammonium acetate. However, in the flow injection method, the methanol used as the carrier solvent instead of acetonitrile lowered the H-D exchange efficiency significantly, suggesting the occurrence of H-D back-exchange into the sample from the solvent in the presence of an excess of methanol. The percentage D value in the syringe pump-ESI method was 72%. On the other hand, exchange efficiencies of more than 93% were obtained in the flow injection-TSP and APCI modes, indicating that heating may contribute to the high efficiencies of H-D exchange in these analysis modes.

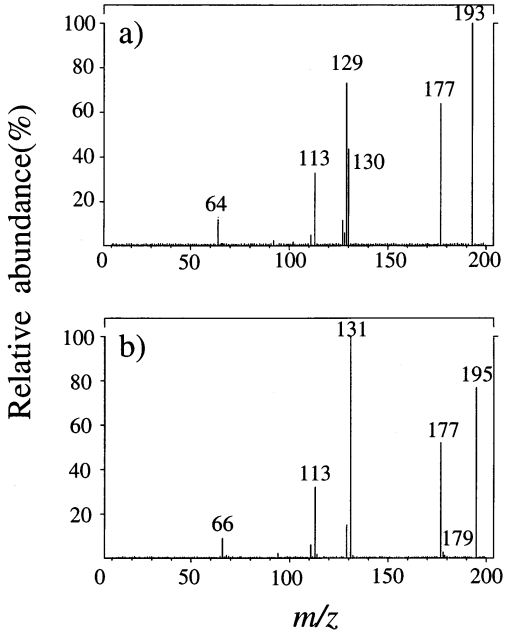

Fig. 4. Product ion mass spectra of (a) *p*-CBSA and (b) *p*-CBSA-d_2.

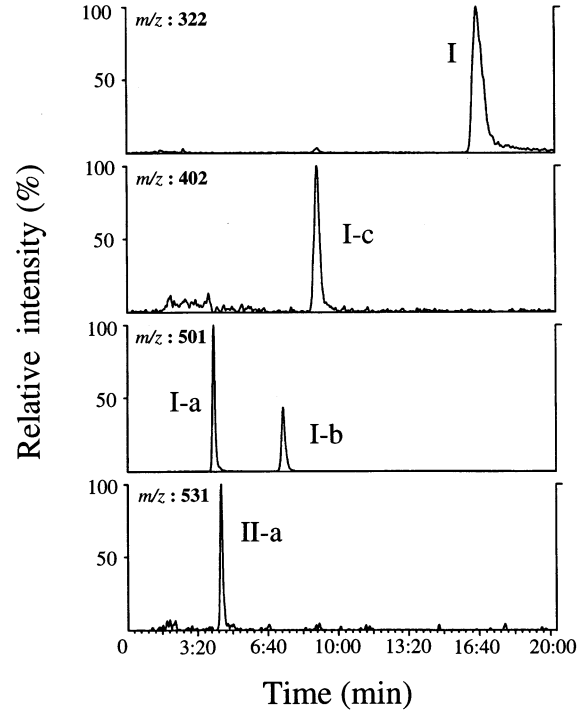

Fig. 5. ESI mass chromatograms of denopamine and its metabolites using a deuterated mobile phase (cf. Fig. 1).

The greatest H-D exchange efficiency (96.1%) was obtained by the HPLC method using the deuterated solvent and no difference in efficiency was recognized among the various ionization modes examined (data not shown). Thus, it appeared that the H-D exchange efficiency depended on the temperature and H:D ratio in the surroundings of the samples during the ionization period.

3.2. Discrimination between the loss of an amino group and that of an oxygen atom in fragmentation

Although the H-D exchange efficiencies in the EI and FAB modes seem to be lower than those of other ionization modes, the H-D exchange

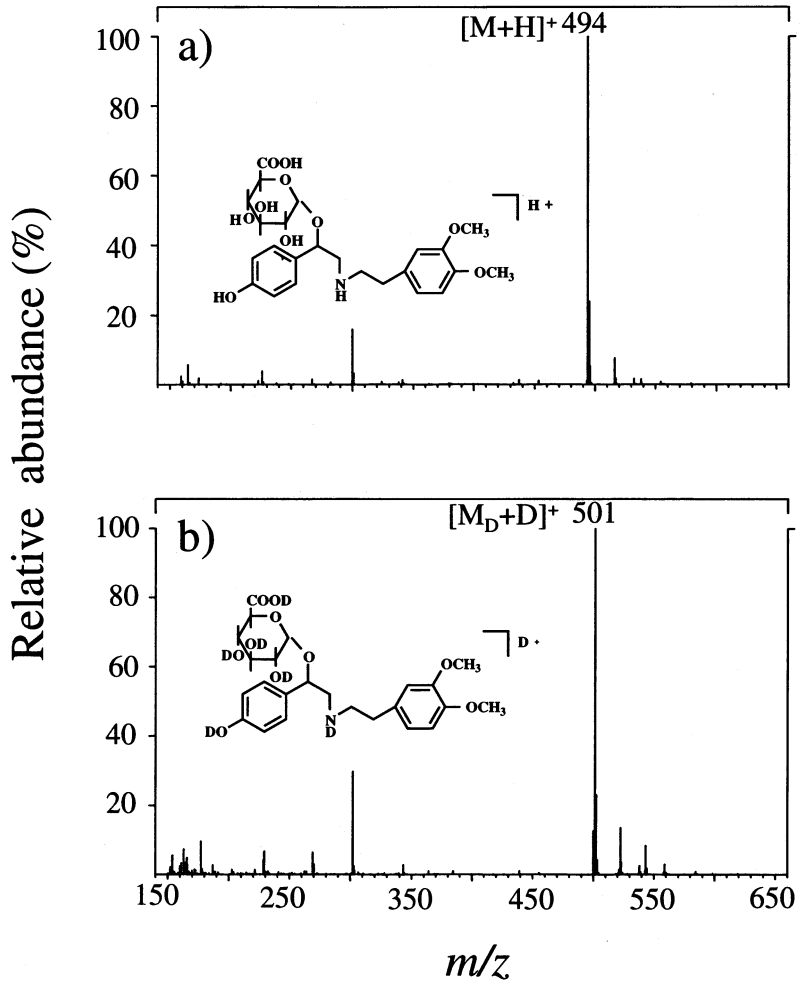

Fig. 6. ESI mass spectra of the permethylated derivative of alcoholic *O*-glucuronide of denopamine. (a) a non-deuterated mobile phase and (b) a deuterated mobile phase.

method is very useful in structural analysis because additional information is obtained from the structures of fragment ions in the CID spectrum produced from the selected ions, $[M_D]^{+\cdot}$ or $[M_D + D]^+$. An example of this method is shown in the analysis of *p*-CBSA (Fig. 3), a metabolite of a certain drug [10,11]. The H-D exchange efficiency of *p*-CBSA-d_2 was about 55% in the EI-MS. In the CID spectrum of *p*-CBSA (Fig. 4a), an ion corresponding to $[M\text{-}16]^{+\cdot}$ was seen at *m/z* 177, which was ascertained to be produced by the loss of an amino group rather than that of an oxygen atom, because the shift of 18 mass units corresponding to the loss of ND_2 was observed in the CID spectrum of *p*-CBSA-d_2 (Fig. 4b). The detailed fragmentation pathways elucidated by using the H-D exchange method are shown in Fig. 3.

3.3. Profile analysis of the metabolites of denopamine

Major urinary metabolites of denopamine (I) in the dog are shown in Fig. 1. Because the alcoholic *O*-glucuronide was resistant to hydrolyses with various *β*-glucuronidases, the structures of the

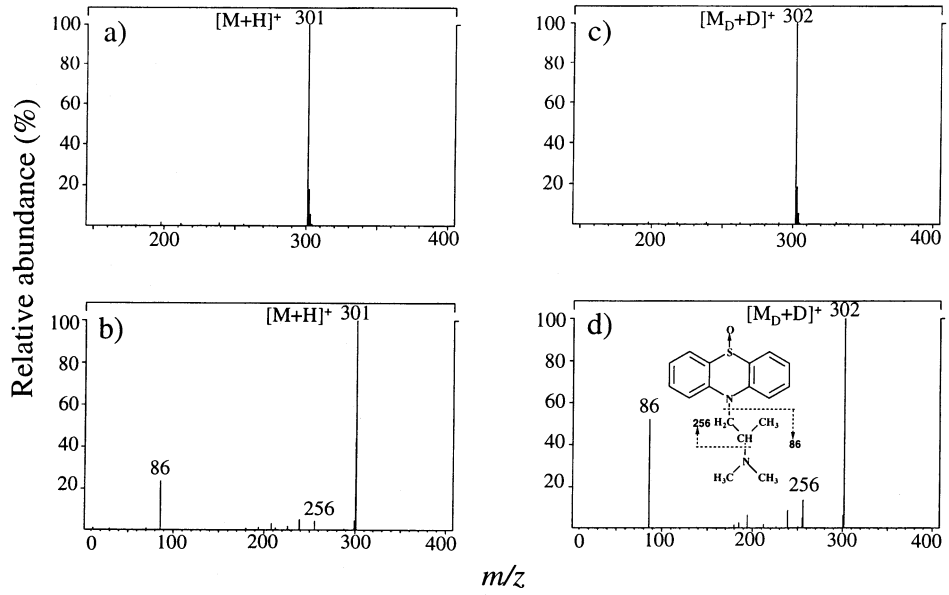

Fig. 7. ESI mass spectra (a and c) and product ion mass spectra (b and d) of M1, a metabolite of promethazine. (a, b) a non-deuterated mobile phase and (c, d) a deuterated mobile phase.

urinary metabolites of denopamine were analyzed by GC-MS [12] as their permethylated derivatives. ESI mass chromatograms of denopamine and its in vitro metabolites obtained by using the H-D exchange method are shown in Fig. 5. $[M+H]^+$ ions of the unchanged drug (I) and six metabolites were observed at m/z 318 (I), 398 (I-c; sulfate), 494 (I-a and -b; alcoholic and phenolic *O*-glucuronides, respectively), and 524 (II-a; phenolic *O*-glucuronide of 3-methoxy-I). ESI-MS of the alcoholic *O*-glucuronide (I-b) obtained by using the H-D exchange method are shown in Fig. 6. $[M_D+D]^+$ at m/z 501 using the H-D exchange method was observed with a shift of 7 mass units from $[M+H]^+$ (m/z 494–501) and this shift shows that it is a glucuronidated metabolite. Additional structural information on this alcoholic *O*-glucuronide was obtained by comparing its CID spectrum with those of the phenolic *O*-glucuronides (data not shown).

Although this technique has the disadvantage of requiring relatively expensive deuterated solvents as the mobile phase, the higher cost performance is achieved in the profile analysis of the metabolites especially in the case of a profile having various metabolic routes.

3.4. Discrimination between N- or S-oxide formation and hydroxylation in metabolism

The H-D exchange method was found to be very useful for discrimination between *N*- or *S*-oxide formation and hydroxylation, the well known general metabolic pathways. The ESI-MS and CID-MS of the metabolites of promethazine [13,14], which were obtained by incubation with human liver microsomes, are shown in Figs. 7 and 8. In each of the two metabolites (M1 and M2), $[M+H]^+$ observed at m/z 301 indicated an addition of 16 mass units to $[M+H]^+$ of promethazine, suggesting an oxidated metabolite, i.e. addition of an oxygen or a hydroxyl group to the phenothiazine, but discrimination between these oxidation types could not be accomplished simply by analysis of their CID spectra. Therefore, the H-D exchange method was used to differentiate between these possibilities. The ESI-MS using the deuterated solvent as a component of the HPLC mobile phase are shown in Figs. 7 and 8. The molecular ion species of M1 and M2 were observed at m/z 302 $[M_D+D]^+$ and 303 $[M_D+D]^+$, respectively (Fig. 7C and Fig. 8C). These

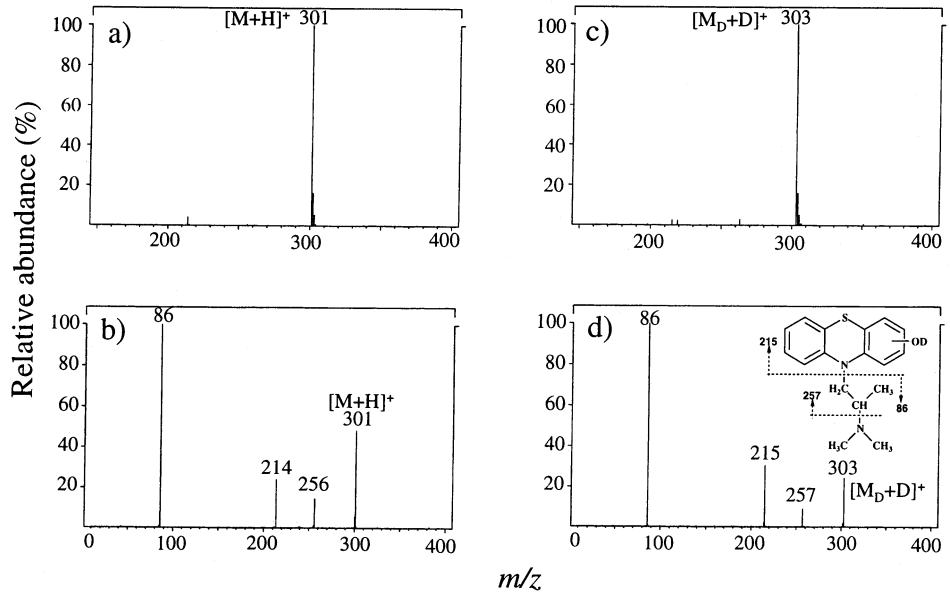

Fig. 8. ESI mass spectra (a and c) and product ion mass spectra (b and d) of M2, a metabolite of promethazine. (a, b) A non-deuterated mobile phase and (c, d) a deuterated mobile phase.

ions showed a shift of 1 or 2 mass units from the corresponding ions of the non-deuterated molecules, demonstrating the presence of no or one labile hydrogen. Therefore, M1 was assumed to be an *S*-oxidated metabolite and M2 a hydroxylated metabolite. In this experiment, the acidic solvent at pH 3 containing 5% CH_3COOH used as the mobile phase was observed to have no effect on the H-D exchange ratio.

4. Conclusions

In the study of drug metabolism, LC-MS-MS can be a powerful instrument. In the present study, the H-D exchange method was evaluated in a study to rapidly elucidate the structures of denopamine and promethazine metabolites. The values of the H-D exchange method can be summarized as follows: (1) Use of the H-D exchange method with LC-MS-MS offers an easy estimation of the number of labile hydrogen atoms in such groups as -OH, -NH, -NH_2, -COOH, and comparison of this number in the metabolite with that of the parent drug gives information about the presence or absence of the above groups in the metabolite structure. However, attention is needed in this case to the presence of an active methylene or methyne group in the parent compound or metabolite, which may cause keto-enol isomerization. (2) Discrimination between the loss of an amino group and that of an oxygen atom in mass fragmentation is easily achieved. (3) Especially, discrimination between *N*- or *S*-oxide formation and hydroxylation can be made. (4) LC-MS and LC-MS-MS using D_2O as the mobile phase component of HPLC can rapidly provide a metabolic profile. Thus, the combination of the H-D exchange method and tandem mass spectrometry was found to be very useful for the study of drug metabolism.

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