

Online Simultaneous Hydrogen/Deuterium Exchange of Multitarget Gas-Phase Molecules by Electrospray Ionization Mass Spectrometry Coupled with Gas Chromatography

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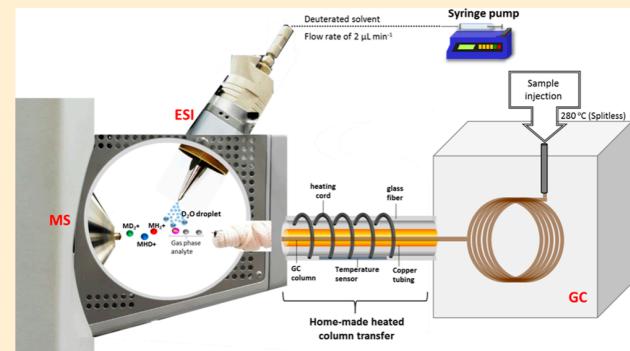
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ABSTRACT: In this study, a hydrogen/deuterium (H/D) exchange method using gas chromatography–electrospray ionization/mass spectrometry (GC–ESI/MS) was first investigated as a novel tool for online H/D exchange of multitarget analytes. The GC and ESI source were combined with a homemade heated column transfer line. GC–ESI/MS-based H/D exchange occurs in an atmospheric pressure ion source as a result of reacting the gas-phase analyte eluted from GC with charged droplets of deuterium oxide infused as the ESI spray solvent. The consumption of the deuterated solvent at a flow rate of $2 \mu\text{L min}^{-1}$ was more economical than that in online H/D exchange methods reported to date. In-ESI-source H/D exchange by GC–ESI/MS was applied to 11 stimulants with secondary amino or hydroxyl groups. After H/D exchange, the spectra of the stimulants showed unexchanged, partially exchanged, and fully exchanged ions showing various degrees of exchange. The relative abundances corrected for naturally occurring isotopes of the fully exchanged ions of stimulants, except for etamivan, were in the range 24.3–85.5%. Methylephedrine and cyclazodone showed low H/D exchange efficiency under acidic, neutral, and basic spray solvent conditions and nonexchange for etamivan with an acidic phenolic OH group. The in-ESI-source H/D exchange efficiency by GC–ESI/MS was sufficient to determine the number of hydrogen by elucidation of fragmentation from the spectrum. Therefore, this online H/D exchange technique using GC–ESI/MS has potential as an alternative method for simultaneous H/D exchange of multitarget analytes.



Hydrogen/deuterium (H/D) exchange mass spectrometry (MS) is a widely used technique for identifying metabolites^{1–3} or pharmaceutical impurities^{4,5} in drug discovery and development processes. H/D exchange MS can be implemented in various ways with the most popular being to infuse a pure compound prepared in a deuterated solvent.^{2,6} However, this method requires a process to separate and purify the target analyte from the sample. Meanwhile, an online H/D exchange MS method, which can directly analyze a sample, has also been reported. This method can be implemented using both on-column and postcolumn techniques. On-column H/D exchange MS has been performed with a deuterated solvent as a mobile phase in liquid chromatography–mass spectrometry (LC–MS)^{1–3,6–9} or capillary electrophoresis mass spectrometry (CE–MS).¹⁰ However, this method suffers from high consumption of an expensive deuterated solvent, such as D₂O, and shifts in chromatographic retention times due to the different mobile phase–analyte–stationary phase interactions

caused when two different mobile phases, such as H₂O and D₂O, are used. The postcolumn H/D exchange method has been performed by mixing LC-eluted analytes with a D₂O solvent using a tee union. In this method, the D₂O solvent is infused using a syringe pump, and H/D exchange occurs after column separation; thus, it does not cause a chromatographic retention time shift.^{11–13} However, in this method, the use of H₂O as an LC mobile phase can give rise to back-exchange, thus resulting in poor H/D exchange efficiency. To enhance the H/D exchange efficiency, a higher D₂O flow rate can be used, or the LC-eluent flow can be split into smaller branch flows. However, these methods still have the drawbacks of high consumption (100 – $400 \mu\text{L min}^{-1}$) of D₂O solvent or low analyte sensitivity due to a split LC flow.

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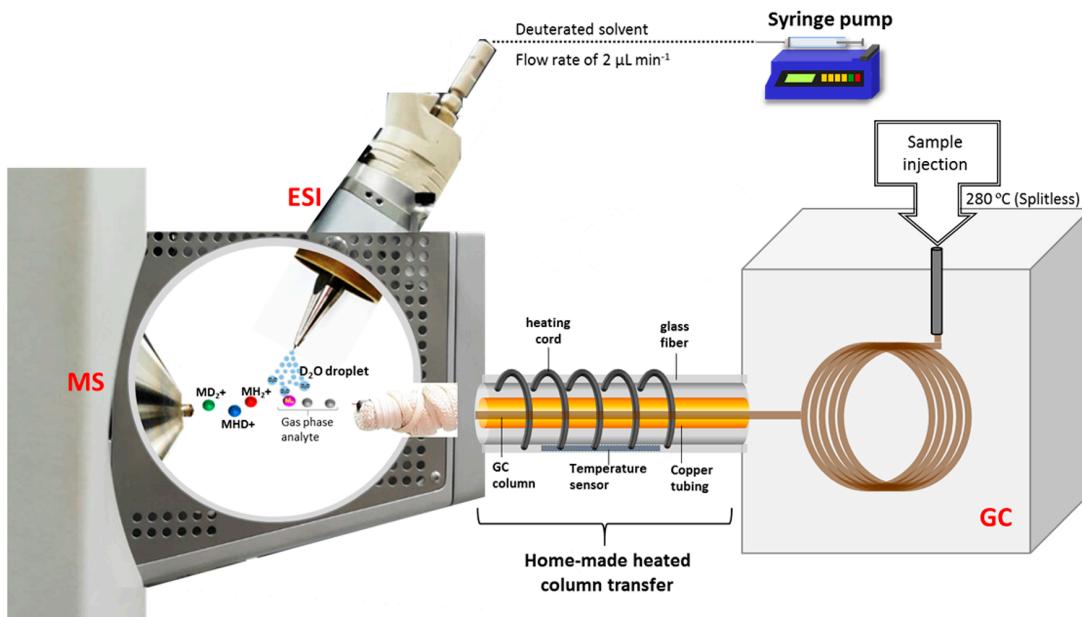


Figure 1. Overview of the H/D exchange of the gas-phase molecule using GC–ESI/MS.

Gas chromatography–mass spectrometry (GC–MS) with electro impact (EI) or chemical ionization (CI) based-H/D exchange has been used to identify compounds before electrospray ionization (ESI) technology became popular.^{14,15} EI provides rich spectral libraries but has the problems of reduced sensitivity by excessive fragmentation or the absence of molecular ion. In particular, the absence of molecular ion can make it difficult to identify unknown compounds that are not in the EI–MS spectral libraries. CI provides molecular ion and less fragmentation as compared to EI, but sensitivity is lower.¹⁶ Furthermore, CI with H/D exchange requires the high consumption of an expensive deuterated gas such as ND₃, CD₃OD, or D₂O. Therefore, sensitive soft ionization method for GC and economical H/D exchange method is required.

Several studies have attempted to perform H/D exchange in an atmospheric pressure ion (API) source. In-source H/D exchange was performed by Wolff et al.¹⁷ using a dual-sprayer source, which consisted of an ESI sprayer to introduce the analyte and an atmospheric pressure chemical ionization (APCI) sprayer to infuse D₂O. However, this method also involves excessive use of D₂O solvent as a D₂O-saturated atmosphere is required in the ion source. Alternatively, H/D exchange was attempted by evaporating a D₂O droplet placed on a copper plate between the ESI needle and the entrance of the mass spectrometer to minimize D₂O consumption.^{18,19} Also, H/D exchange was performed by Cho et al. and Acter et al. by atmospheric pressure photoionization (APPI)-MS or APCI-MS to identify the structures of crude oil compounds and oxygen-containing compounds.^{20,21} However, these methods using ESI, APCI, and APPI were performed by direct infusion of a sample without chromatography. Although various H/D exchange MS methods have been pursued, they still require an innovative method that enables one to routinely perform H/D exchange MS for multitarget analytes.

Recently, gas chromatography–electrospray ionization tandem mass spectrometry (GC–ESI–MS/MS), combining both the excellent chromatographic resolving power of GC and the soft ionization of ESI, has been reported.^{22,23} GC–ESI/MS,

which was implemented by simply interfacing GC and ESI–MS, was first demonstrated in 2008 by Brenner et al.²² In GC–ESI/MS, the analyte ions are formed by the interaction of a gas-phase analyte eluted by GC with a solvent sprayed at a flow rate of 0.1–5 $\mu\text{L min}^{-1}$. Our group has also shown that steroids such as trimethylsilyl derivatives can be analyzed using GC–ESI/MS with high separation efficiency and enhanced selectivity and sensitivity.^{23,24} Despite such advantages, the GC–ESI/MS setup is not yet commercially available.

In this study, we first performed in-ESI-source H/D exchange using a GC–ESI/MS configuration to identify the unknown compounds and reduce the consumption of expensive deuterated reagents. With this configuration, simultaneous H/D exchange MS of multitarget analytes in conjunction with GC is enabled with minimal use of a deuterated solvent. For H/D exchange using GC–ESI/MS, a D₂O-containing solvent is infused by a syringe pump at a flow rate of 2 $\mu\text{L min}^{-1}$, and gaseous analyte ions are generated by the interaction between gas-phase neutral analytes and D₂O droplets in the API source (Figure 1). Below, we show that GC–ESI–MS can be optimized as a new tool for simultaneous H/D exchange MS of multitarget analytes and to compare the H/D exchange efficiency of this method with that of the postcolumn method using LC–ESI–MS.

EXPERIMENTAL SECTION

Chemicals and Reagents. Isomethopentene mucate, cyclazodone, and fenetylline·HCl were purchased from the National Measurement Institute (North Ryde, Australia). Methylephedrine·HCl and mefenorex·HCl were purchased from Cerilliant Co. (Round Rock, TX). Methoxyphenamine·HCl, etamivan, and fenfluramine·HCl were purchased from Sigma-Aldrich (St. Louis, MO). Propylhexedrine·HCl, fencamfamine·HCl, and clobenzorex·HCl were purchased from Toronto Research Chemicals (Toronto, Canada). Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (Ulsan, Korea). Deuterium oxide (D₂O), formic acid, ammonium formate, ammonium acetate, and ammonia (NH₃) solution were

Table 1. Corrected Relative Abundances and Deuterium Incorporation (%D) Levels by GC–ESI/MS-Based H/D Exchange for Stimulants

Compound	Structure	MW	RT (min)	Corrected relative abundances			%D
				MH ₂ ⁺	MHD ⁺	MD ₂ ⁺	
Isomethcptene		141	2.34	33.2	96.6	70.0	59.2
Propylhexedrine		155	3.66	38.0	95.6	85.5	60.8
Methoxyphenamine		179	5.58	51.4	93.6	64.5	53.1
Methylephedrine		179	5.73	100.0	75.1	24.3	31.0
Mefenorex		211	7.07	37.1	95.0	65.0	57.1
Fencamfamine		215	7.79	52.1	91.2	59.6	51.8
Cyclazodone		216	11.01	59.9	91.7	26.8	40.7
Etamivan		223	9.11	100.0	10.3	1.8	6.2
Fenfluramine		231	4.41	38.5	94.8	63.4	56.3
Clobenxorex		259	9.66	70.0	87.5	39.5	42.2
Fenetylline		341	14.03	71.0	84.6	32.3	39.7

obtained from Sigma-Aldrich (St. Louis, MO). Ultrapure water was produced using a Milli-Q water purification system (Millipore, Bedford, MA).

Standard Solution. For GC–ESI/MS analysis, mixed solutions of 11 stimulants were prepared in ethyl acetate at $10 \mu\text{g mL}^{-1}$ each. For direct-infusion MS analysis, each stimulant was prepared at $1 \mu\text{g mL}^{-1}$ in a D₂O/H₂O/acetonitrile mixed solution containing 0.1% formic acid. The ratio of D₂O to H₂O was expressed as a percentage of D₂O as follows: 100%, 83.3%, 71.4%, 50%, 33.3%, and 20% D₂O. These compositions were based on the ratio of the D₂O infusion flow rate to the LC flow rate (H₂O) reported earlier by Shah et al.¹³

GC–ESI/MS Instrumentation. GC–ESI/MS analysis was performed using a GC chromatograph (6890N GC, Agilent, Palo Alto, CA) and a linear ion-trap mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an ESI source. The GC and ESI source were combined with a homemade heated column transfer line developed previously in our laboratory.²⁴ For GC separation, the sample ($1 \mu\text{L}$) was injected into a DB-EUPAH column (20 m \times 0.180 mm ID, 0.14 μm film thickness, J&W Scientific, Rancho Cordova, CA) in the splitless mode. The oven temperature was held at 60 °C for 2 min and increased to 280 °C at a rate of 15 °C min⁻¹. The flow rate of the carrier gas (He, 99.9999%) was maintained at 1.5 mL min⁻¹ at a constant pressure. The injector and GC column transfer line were maintained at 280 °C. The ESI solvent was sprayed using a syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 2 $\mu\text{L min}^{-1}$. The capillary temperature and voltage were set to 200 °C and +35 V, respectively. Nitrogen was used as a sheath gas. Mass spectra were acquired from *m/z* 50 to 500 in the positive mode. For MS/MS analysis of stimulants, the collision energy was tuned in the range 15–25 V.

Direct-Infusion MS. Infusion MS analysis was performed using a linear quadrupole ion trap mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an ESI source. A sample solution was infused at a flow rate of 5 $\mu\text{L min}^{-1}$ using a syringe pump (Harvard Apparatus, Holliston, MA). The ESI spray potential was set to +4.0 kV, and the capillary temperature and voltage were 200 °C and +35 V, respectively. A sheath gas was passed at 5 arbitrary units (arb). Mass spectra were acquired in the positive ion mode in the *m/z* range 50–500.

RESULTS AND DISCUSSION

Optimization of In-ESI-Source H/D Exchange. The configuration of GC–ESI/MS and an overview of the H/D exchange of gas-phase molecule in the ESI source are shown in Figure 1. The experimental parameters for online in-ESI-source H/D exchange coupled with GC–ESI/MS were optimized using propylhexedrine with a secondary amine as a representative analyte among the 11 target analytes of interest. As summarized in Table 1, propylhexedrine has only one exchangeable hydrogen in the secondary amino (–NH) group, and, therefore, two hydrogens, including ionizing H⁺, can potentially be exchanged in the H/D exchange experiments. Indeed, propylhexedrine was observed with three peaks: MH₂⁺ at *m/z* 156, MHD⁺ at *m/z* 157, and MD₂⁺ at *m/z* 158. Here,

the MH_2^+ , MHD^+ , and MD_2^+ peaks represent the unexchanged, partially exchanged, and fully exchanged propylhexedrine ions, respectively. In this experimental setup, the gas-phase analytes, which were eluted through the GC capillary and the following heated transfer line, underwent a multitude of collisions under atmospheric conditions with gaseous D_2O molecules or D_2O -containing droplets sprayed through the ESI source. As a result of such collisions, partial or full H/D exchange of propylhexedrine occurred.

It was found that the degree of H/D exchange depends on three experimental parameters: spray potential, sheath gas flow rate, and the temperature of the desolvating capillary. First, the spray potential was tuned in the range from +2.1 to +4.0 kV, with the sheath gas flow rate set to a fixed value. Below a spray potential of +2.1 kV, it was difficult to acquire stable mass spectral signals. As shown in Figure 2, the degree of H/D

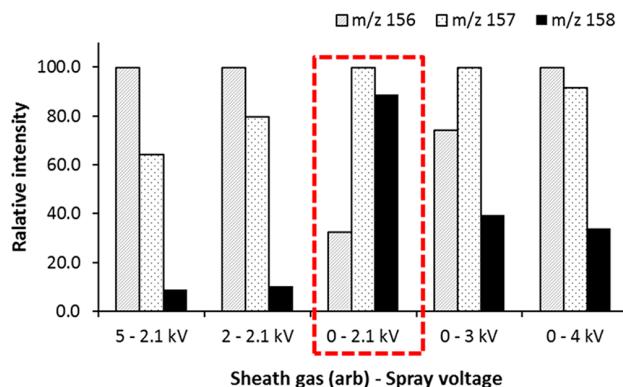


Figure 2. Degree of H/D exchange for propylhexedrine according to spray voltage and sheath gas flow rate in GC–ESI/MS: The D_2O :acetonitrile (50:50, v/v) solution with 0.1% formic acid as spray solvent was used at a flow rate of $2 \mu\text{L min}^{-1}$ without sheath gas.

exchange was the highest at a spray potential of +2.1 kV and zero sheath gas flow. As the spray potential was increased, the degree of H/D exchange decreased accordingly. Specifically, the relative abundances of the peaks at m/z 156, 157, and 158 were 32.6, 100, and 88.7, respectively, at a spray potential of +2.1 kV and changed to 74.3, 100, and 39.4 at +3.0 kV and 100, 91.9, and 34.1 at +4.0 kV. Second, the sheath gas flow rate was varied from 0 to 5 (arbitrary units) at a spray potential of +2.1 kV. As the sheath gas flow rate increased from 0 to 5, the degree of H/D exchange dramatically decreased (Figure 2). Thus, in the

following online H/D exchange experiments coupled with GC–ESI/MS, a spray potential of +2.1 kV and no sheath gas were used, although there was some loss of chromatographic peak shape due to the absence of sheath gas. In GC–ESI/MS, the ionization occurs by ion evaporation after dissolution process of the gas-phase analyte into the charged droplet or by a gas-phase proton transfer reaction between protonated solvent and analyte.²² In this study, the ionization mechanism is likely to be ion evaporation rather than a gas-phase proton transfer reaction. This ionization mechanism is also supported by the fact that sheath gas and a high spray potential decrease the exchange yield. In other words, the gaseous molecules from the GC capillary might be dissolved into the charged H_2O or D_2O droplet and then protonated (or deuterated) before the formation of single ions according to the ESI mechanism. Third, the temperature of the desolvating capillary was tuned in the range 150 °C–350 °C at a spray potential of +2.1 kV without sheath gas. The relative abundances of the MD_2^+ ion (m/z 158) were 68.6% at 150 °C, 96.4% at 200 °C, 95.1% at 250 °C, 94.1% at 300 °C, and 91.7% at 350 °C. The H/D exchange efficiency was relatively low at 150 °C but not significantly different at 200 °C or higher. These results are not in line with the APCI and APPI results reported by Acter et al.,²¹ but are consistent with the previously reported ESI result, indicating that the capillary temperature has less impact on small m/z ions.²⁵ However, because the source dissociation of some stimulants increased at higher temperatures, 200 °C was used as the temperature for the desolvating capillary in this study.

In-ESI-Source H/D Exchange of Stimulants. Online in-ESI-source H/D exchange coupled with GC–ESI/MS was conducted for 11 stimulants with secondary amino (–NH) or hydroxyl (–OH) groups to investigate their H/D efficiencies. The 11 stimulants under examination are listed in Table 1: isomethcptene, propylhexedrine, methoxyphenamine, methylephedrine, mefenorex, fencamfamine, cyclazodone, etamivan, fenfluramine, clobenzorex, and fenetylline. All stimulants contain only one exchangeable NH or OH group, allowing a maximum of two H/D exchanges.

The H/D exchange efficiency for a spray solvent in GC–ESI/MS was investigated using $\text{D}_2\text{O}:\text{CH}_3\text{CN}$ (50:50, v/v) solutions with various modifiers, based on the experiments by Shah et al.,¹³ as follows: pure solution, 0.1% formic acid, 0.1% NH_3 , 10 mM ammonium formate, and 10 mM ammonium acetate. The relative abundances of the MD_2^+ ions of stimulants, except fenetylline, were the highest in 0.1% formic

Table 2. Relative Abundances of the Fully Exchanged MD_2^+ Ion According to the Spray Solvents in GC–ESI/MS

compound	MD_2^+	modifier of spray solvent				
		formic acid	ammonium formate	ammonium acetate	ammonia	pure
isomethcptene	m/z 144	80.0	50.9	38.4	36.3	75.0
propylhexedrine	m/z 158	96.4	51.8	45.5	32.6	62.0
methoxyphenamine	m/z 182	76.2	53.9	33.7	27.0	61.1
methylephedrine	m/z 182	33.7	20.2	19.7	33.6	31.5
mefenorex	m/z 214	90.0	52.3	51.7	66.2	71.9
fencamfamine	m/z 218	75.0	47.2	41.5	42.1	51.0
cyclazodone	m/z 219	39.6	9.8	16.8	16.1	6.1
etamivan	m/z 226	3.2	1.9	2.1	2.9	5.0
fenfluramine	m/z 234	76.5	30.4	31.0	43.8	60.7
clobenzorex	m/z 262	78.6	61.1	61.6	84.7	67.9
fenetylline	m/z 344	50.6	45.2	46.5	100.0	30.4

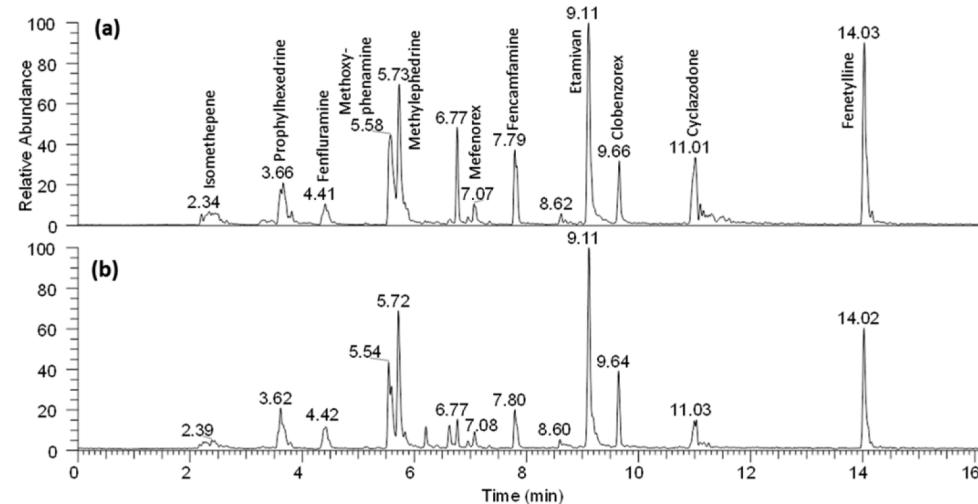


Figure 3. Total ion GC-ESI/MS chromatograms obtained using (a) D₂O and (b) H₂O as the spray solvents.

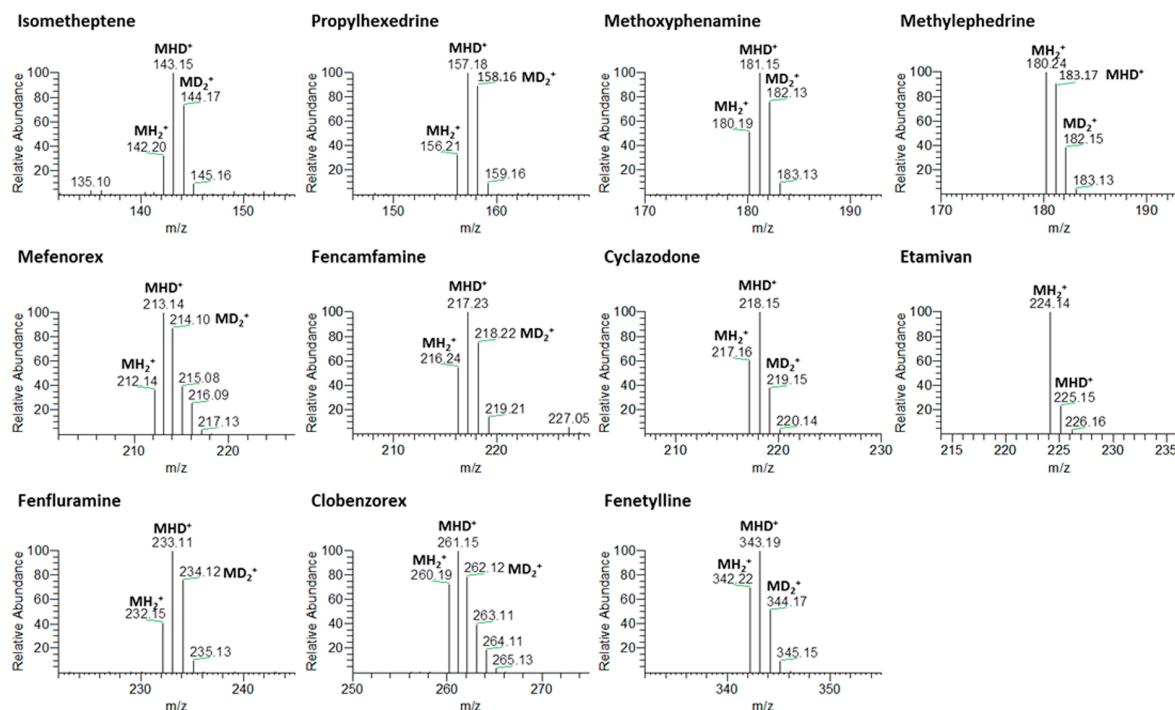


Figure 4. GC-ESI/MS spectra of stimulants after H/D exchange.

acid solution (e.g., 96.4% for propylhexedrine), followed by a pure solution (62.0%), a 10 mM ammonium formate solution (51.8%), a 10 mM ammonium acetate solution (45.5%), and a 0.1% NH₃ solution (32.6%). For fenetylline, the relative abundance of the MD₂⁺ ion was the highest at 100% in 0.1% NH₃ solution. This was sufficient to observe the MD₂⁺ ion in the mass spectrum of the 0.1% formic acid solution. The fully exchanged MD₂⁺ ion for etamivan was not produced under any of these conditions. Therefore, the D₂O solution with 0.1% formic acid as the spray solvent was used for simultaneous H/D exchange of stimulants. The relative abundances of the MD₂⁺ ions of stimulants according to spray solvents are summarized in Table 2.

Figure 3a shows a representative chromatogram acquired with D₂O as a spray solvent. For comparison, a chromatogram

acquired with H₂O spray solvent is shown in Figure 3b. In both experiments, H₂O:CH₃CN or D₂O:CH₃CN (50:50, v/v) solution with 0.1% formic acid was used as a spray solvent, which is infused at a flow rate of 2 $\mu\text{L min}^{-1}$. Comparison of these two chromatograms clearly indicates that the retention times of the 11 stimulants acquired using the D₂O solvent (Figure 3a) are almost identical to those acquired using H₂O as a solvent (Figure 3b). As the retention times of the current experiments are related solely to the gas-chromatographic retention times of the analytes, the identical retention times of the unexchanged and deuterated stimulants could be readily understood.

To examine the degrees of in-ESI-source H/D exchange for the 11 stimulants, it is necessary to see the individual mass spectra. Figure 4 shows the mass spectra for all 11 stimulants.

Table 3. Relative Abundances Corrected by Considering the Naturally Occurring Heavy Isotopes for the Fully Exchanged MD_2^+ Ion According to Percentage D_2O in Direct-Infusion MS Analysis in Comparison to GC-ESI/MS

compound	MD_2^+	GC-ESI/MS	percentage D_2O					
			100%	83.3%	71.4%	50%	33.3%	20%
isomethcptene	<i>m/z</i> 144	70.0	98.8	95.6	92.2	42.9	26.6	10.6
propylhexedrine	<i>m/z</i> 158	85.5	99.1	95.6	91.7	42.2	25.8	9.8
methoxyphenamine	<i>m/z</i> 182	64.5	98.1	94.8	90.2	45.0	25.9	9.6
methylephedrine	<i>m/z</i> 182	24.3	23.0	20.5	16.9	8.5	5.4	2.1
mefenorex	<i>m/z</i> 214	65.0	95.0	89.5	80.2	37.3	23.6	7.2
fencamfamine	<i>m/z</i> 218	59.6	98.4	93.1	87.6	39.4	23.9	9.4
cyclazodone	<i>m/z</i> 219	26.8	4.2	8.0	4.2	2.7	6.7	1.9
etamivan	<i>m/z</i> 226	1.8	2.7	6.5	3.4	2.1	2.0	2.0
fenfluramine	<i>m/z</i> 234	63.4	98.2	93.7	89.8	46.3	23.8	8.7
clobenzorex	<i>m/z</i> 262	39.5	65.6	50.1	34.1	15.3	15.4	3.9
fenetylline	<i>m/z</i> 344	32.3	27.1	21.9	15.1	8.1	9.3	4.8

In all mass spectra, three major ion peaks were observed, representing the unexchanged (MH_2^+), partially exchanged (MHD^+), and fully exchanged (MD_2^+) stimulant ions. It should be noted that the second isotopic peaks of the fully exchanged stimulant ions are also observed in low abundances; for example, see the peak at *m/z* 145.16 for isomethcptene. Except for methylephedrine and etamivan, the partially exchanged (MHD^+) stimulant ions were the most abundant. In the case of methylephedrine and etamivan, the unexchanged (MH_2^+) stimulant peaks were most abundant. For some stimulants, the fully exchanged stimulants are more abundant than the unexchanged stimulant ions: for example, isomethcptene, propylhexedrine, methoxyphenamine, mefenorex, fencamfamine, and fenfluramine.

The degree of in-ESI-source H/D exchange was determined by calculating the %D.^{26,27} For this calculation, the relative abundances of the H/D-exchanged ions MH_2^+ , MHD^+ , and MD_2^+ should be corrected by considering the naturally occurring heavy isotopes. In these corrections, only the abundances of the first (M) and second (M+1) isotopes were considered, except for mefenorex and clobenzorex, which contain one Cl atom. The higher isotopes, that is, the third (M+2) and fourth (M+3) isotopes and above, were not generally considered for simplicity. Theoretical isotope contributions were calculated using the Xcalibur program. The relative second (M+2) isotope contributions of the stimulants were as follows: isomethcptene, 10.3%; propylhexedrine, 11.4%; methoxyphenamine, 12.5%; fencamfamine, 16.9%; fenfluramine, 13.5%; fenetylline, 21.7%; cyclazodone, 13.9%; methylephedrine, 12.5%; and etamivan, 13.7%. However, for mefenorex and clobenzorex with Cl, due to the unique Cl isotopic pattern, the third (M+2) isotopes cannot be ignored. The relative abundance contributions of the second (M+1) and third (M+2) isotopes for mefenorex were 13.6% and 32.8%, and those for clobenzorex were 17.9% and 33.5%, respectively. On the basis of these higher isotope abundance contributions, the relative abundances of the H/D-exchanged ions, that is, $A(\text{MH}_2^+)$, $A(\text{MHD}^+)$, and $A(\text{MD}_2^+)$, were corrected. For example, for isomethcptene (MW 141) with a second (M+1) isotope contribution of 10.3%, the corrected relative abundances of MH_2^+ , MHD^+ , and MD_2^+ , which were 33, 100, and 90, respectively, before corrections, became 33, 97 (=100 - (33 × 0.103)), and 70 (=80 - (97 × 0.103)). These relative abundances correspond to a % distribution of 17:48:35 for the peaks at *m/z* 142, 143, and 144.

From this distribution, the %D is calculated to be 59.2% (=48% × 1/2 + 35% × 2/2).

Table 1 summarizes the corrected relative abundances of the H/D-exchanged ions and the %D levels for the 11 stimulants. The %D levels of the stimulants were in the range 31.0%–60.8%, except for etamivan. In etamivan, an acidic phenolic OH group is exchanged, contrary to the other compounds. Methylephedrine and fenetylline contain a further basic center, where protonation may occur besides the exchanged OH or NH group as a reason for low exchange, which could explain the lower exchange yields. The H/D exchange efficiency of amines and alcohol can be reduced at low pH. However, the H/D efficiencies of methylephedrine and etamivan were not improved by spray solvents with neutral or basic pH. The observed %D levels for the –NH or –OH functional group in the stimulants were generally lower than those reported by Wolff et al. (32%–90%)¹⁷ but similar to those reported by Tolonen et al. (44%–59%),¹² where H/D exchanges were performed in solution. This result strongly suggests that the present experimental configuration for online in-ESI-source H/D exchange coupled with GC-ESI/MS is also suitable for simultaneous H/D exchange of multitarget analytes.

Comparison of In-ESI-Source and Solution H/D Exchange. The H/D exchange efficiency of the in-ESI-source H/D exchange method coupled with GC-ESI/MS was compared to that of the solution H/D exchange method. The solution H/D exchange was performed by directly infusing H/D-exchanged stimulants prepared in $\text{D}_2\text{O}/\text{H}_2\text{O}/\text{acetonitrile}$ solutions containing 0.1% formic acid with varying percentages of D_2O : 100%, 83.3%, 71.4%, 50%, 33.3%, and 20% D_2O in aqueous solution. The relative abundances of MD_2^+ obtained by the in-ESI-source and solution H/D exchange methods are summarized in **Table 3**. All relative abundances of the MD_2^+ ions are corrected by considering the isotopic contributions of the naturally occurring heavy isotopes. The relative abundances of MD_2^+ from the in-ESI-source H/D exchange method were generally lower than those from the solution H/D exchange method for 100%, 83.3%, and 71.4% D_2O but higher for those with 50%, 33.3%, and 20% D_2O . In the case of methylephedrine, etamivan, and cyclazodone, the solution H/D exchange was limited. The relatively low H/D exchange efficiency of the in-ESI-source exchange method is presumably due to the limited exchange time of in-ESI-source H/D exchange.^{10,11}

Structural Elucidation Using Deuterated MS/MS Spectrum. MS/MS of deuterated compounds can provide valuable information for interpreting fragmentation pathways

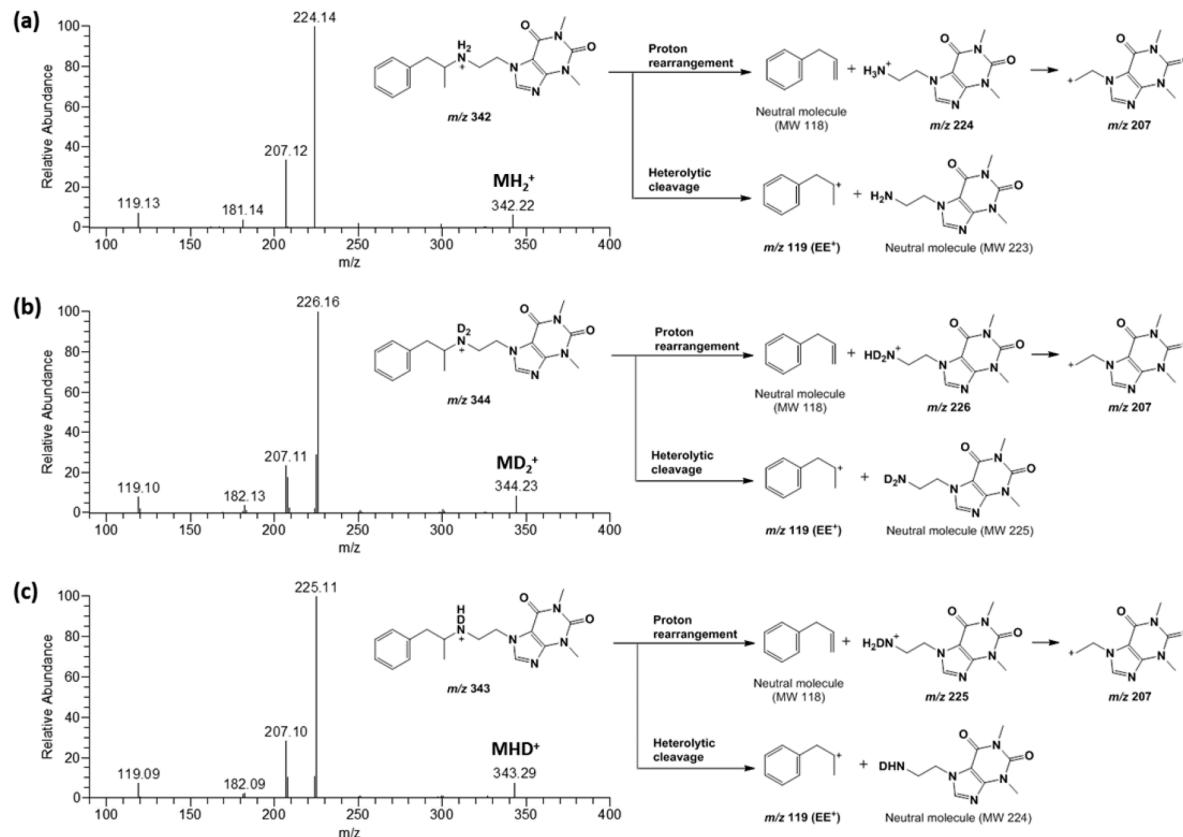


Figure 5. Product ion spectra and fragmentation pathways of (a) unexchanged MH_2^+ , (b) fully exchanged MD_2^+ , and (c) partially exchanged MHD^+ ions of fenetylline obtained by GC-ESI/MS-based H/D exchange.

and further elucidating structures based on fragment ions whose mass values are affected by deuterium inclusion. Thus, collision-induced dissociation (CID) spectra were obtained for the H/D-exchanged stimulant ions. For example, Figure 5 shows the CID spectra of unexchanged (MH_2^+), partially exchanged (MHD^+), and fully exchanged (MD_2^+) fenetylline ions generated by online in-ESI-source H/D exchange in GC-ESI/MS. For fenetylline, it is likely that a proton resides on the secondary nitrogen, due to its high proton affinity. The protonated fenetylline (m/z 342) can undergo unimolecular dissociation upon collisional activation via two fragmentation pathways (right-hand side of Figure 5a). First, heterolytic cleavage between the protonated nitrogen and the adjacent carbon gave rise to an even-electron cation (EE^+) at m/z 119 and a neutral molecule of 223 Da. Alternatively, hydrogen rearrangement (H-shift) can accompany heterolytic cleavage, producing EE^+ at m/z 224 and a neutral molecule of 118 Da. The produced EE^+ at m/z 224 can further experience NH_3^+ loss to give rise to a fragment at m/z 207. The suggested fragmentation pathways are consistent with the CID spectra of the fully and partially exchanged fenetylline ions. In particular, the production of the two fragment peaks at m/z 226 and 207 in the CID spectrum of MD_2^+ supports the protonation site at the secondary nitrogen and the heterolytic bond cleavages occurring between the protonated nitrogen and adjacent carbons (Figure 5b). The suggested fragmentation pathways adequately explain why the mass shift occurred only with the fragment peak at m/z 224 ($\rightarrow m/z$ 226) and not for that at m/z 207. The CID mass spectrum for the partially exchanged fenetylline ion (Figure 5c) is also consistent with the

suggested fragmentation pathways, showing the mass shift of the fragment at m/z 224 to m/z 225 and no mass shift of the fragment at m/z 207, which indicates the loss of NDH_2 .

MS/MS can be usefully applied to distinguish isomers in the mixture. The structural isomers methoxyphenamine and methylephedrine were clearly distinguished by interpretation of CID spectra, as shown in Figure 6. For the two isomers, it is likely that a proton resides on the secondary nitrogen for methoxyphenamine and hydroxyl oxygen for methylephedrine. Protonated methoxyphenamine (m/z 180) undergoes heterolytic cleavage between protonated nitrogen and adjacent carbon, producing EE^+ at m/z 149 and a neutral molecule of 31 Da (Figure 6a). On the other hand, protonated methylephedrine (m/z 180) produces EE^+ at m/z 162 and a neutral molecule of 18 Da by the heterolytic cleavage between protonated oxygen and adjacent carbon (Figure 6c). The CID mass spectra for MD_2^+ (m/z 182) of the two isomers in Figure 6b and d are also consistent with the suggested fragmentation pathways, showing that there was no mass shift of the fragments at m/z 149 for methoxyphenamine and m/z 162 for methylephedrine, which indicates the loss of ND_2CH_3 (33 Da) and D_2O (20 Da), respectively.

CONCLUSIONS

Herein, simultaneous H/D exchange using GC-ESI/MS was newly investigated and optimized. The GC-ESI/MS-based H/D exchange method was applied to the 11 stimulants with secondary amino or hydroxyl groups. In this method, the H/D exchange for gas-phase stimulants occurred in the ESI source. The in-ESI-source H/D exchange efficiency obtained by GC-

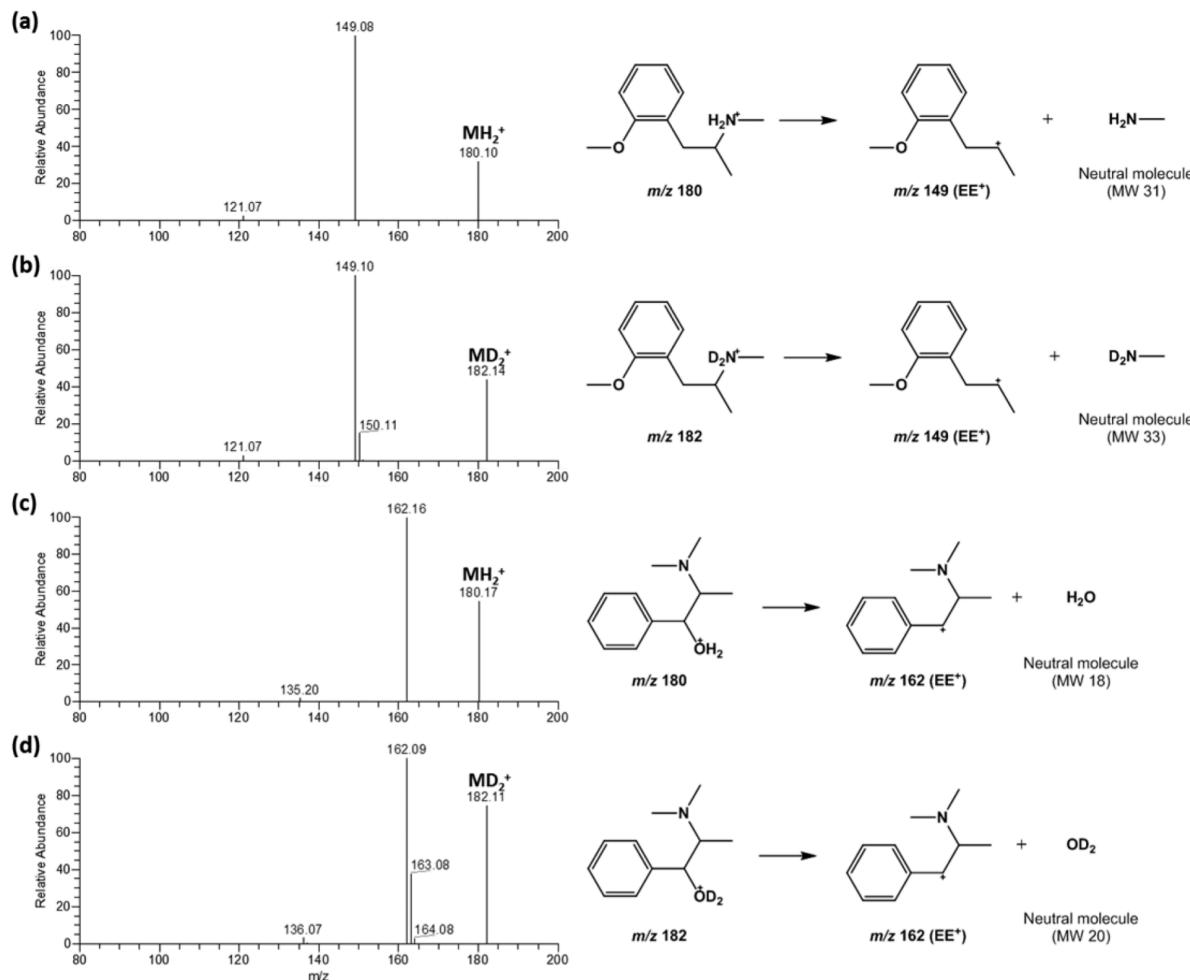


Figure 6. Product ion spectra and fragmentation pathways of (a) unexchanged MH_2^+ and (b) fully exchanged MD_2^+ ions for methoxyphenamine and (c) unexchanged MH_2^+ and (d) fully exchanged MD_2^+ ions for methylephedrine obtained by GC-ESI/MS-based H/D exchange.

ESI/MS was relatively lower than those obtained by solution H/D exchange, but was sufficient to determine the number of hydrogen by the interpretation of the fragmentation mechanism for the product ion spectrum. The online H/D exchange by GC-ESI/MS has the disadvantage of being restricted to small volatile molecules due to the limitations of GC. However, the proposed method offers advantages such as low consumption of deuterated solvent, simultaneous H/D exchange of multitarget analytes, and direct analysis of samples without the purification of interesting peaks.

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The authors declare no competing financial interest.

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