

Differentiation of regioisomeric chloroamphetamine analogs using gas chromatography–chemical ionization-tandem mass spectrometry

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Abstract In recent years, a large number of clandestinely synthesized new psychoactive substances with high structural variety have been detected in forensic samples. Analytical differentiation of regioisomers is a significant issue in forensic drug analysis, because, in most cases, legal controls are placed on only one or two of the conceivable isomers. In this study, gas chromatography–tandem mass spectrometry (GC–MS–MS) was used to differentiate the regioisomers of chloroamphetamine analogs (chloroamphetamines and chloromethamphetamines) synthesized in the authors' laboratories. Free bases, trifluoroacetyl derivatives, and trimethylsilyl derivatives were subjected to GC–MS–MS using DB-1ms, DB-5ms, and DB-17ms capillary columns, respectively. The regioisomers of chloroamphetamine analogs in all forms were well separated on the DB-5ms column. The electron ionization mass spectra of the chloroamphetamine analogs gave very little structural information for differentiation among these analogs, even after trifluoroacetyl and trimethylsilyl derivatization of the analytes. Characteristic product ions of the 2-positional isomers were observed by electron ionization-MS–MS. In contrast, chemical ionization-MS–MS of the free bases provided more structural information about chloride position on the aromatic ring when $[M+H-$

$HCl]^+$ was selected as a precursor ion. The results suggest that a combination of chromatographic analysis and MS–MS supports differentiation for regioisomers of chloroamphetamine analogs.

Keywords Chloroamphetamine analogs · Regioisomeric differentiation · GC–MS–MS · Chemical ionization

Introduction

In recent years, a large number of controlled substance analogs with high structural variety have been widely distributed as noncontrolled alternatives and have been detected in forensic samples. Because of the frequent appearance of new analogs with displacement of a functional group or an alkyl chain [1–3], structural differentiation of these new drugs is necessary in forensic laboratories for legal control against drug abuse [4–14].

4-Chloromethamphetamine (4-CMA) was first reported as a selective serotonergic depleting agent in rat brains in 1963 by Pletscher et al. [15]. Chlorinated analogs of amphetamine were subsequently synthesized and examined in the Lilly research laboratory as appetite depressants [16, 17]. It was eventually concluded that these analogs were not clinically useful and produced less central-nervous system stimulation than amphetamine or methamphetamine. After these reports were published, many researchers studied chloroamphetamines (CAPs) as factors of selective serotonin (5-hydroxytryptamine, 5-HT) diminution and as monoamine oxidase (MAO) inhibitors in brain nerve terminals [18–20]. It was also reported that 4-CAP and 4-CMA cause long-lasting depletion in the level of brain 5-HT and 5-hydroxyindole-3-acetic acid without depleting brain noradrenaline [21, 22]. Administration of

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4-CAP to rats induced tremors, rigidity, Straub tail, hind limb abduction, lateral head weaving, and reciprocal forepaw treading [23]. It was previously reported that the half-life of 4-CAP was longer than that of 4-fluoroamphetamine (4-FAP) in rat brains, and that the liposolubility of 4-CAP was higher than that of 4-FAP in vitro [24]. Because of a tendency to dissolve in the organic phase, the depletion of brain 5-HT with 4-CAP continued for at least a week [24].

In Taiwan, CAP first appeared in April 2009, and a total of 775 cases were identified up to 2011 [25]. 4-CAP was also detected in urine samples in March 2010 [26]. In Hungary, it was detected in December 2012 as a new psychoactive substance and reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and Europol for the first time [1]. Although there have been no reports of chloroamphetamine analogs (CAPs and CMAs) being abused and detected in forensic samples in Japan, 4-CAP has been controlled as a designated substance since August 2014.

For forensic purposes, differentiation of the chloro group ring position in amphetamine and methamphetamine is required because 4-CAP is controlled but 2-CAP and 3-CAP are not (Fig. 1). Although the results of infrared spectroscopy and nuclear magnetic resonance (NMR) spectrometry can provide structural information for identification of a specific isomer, these methods are not suitable for biological samples such as urine and blood because of their low sensitivity.

Gas chromatography–mass spectrometry (GC–MS) is generally used for the identification of drugs because it is

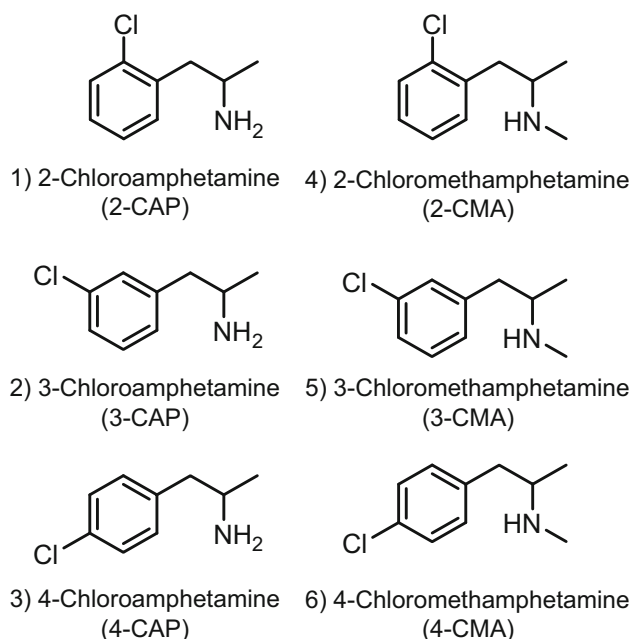


Fig. 1 Chemical structures of chloroamphetamine analog regioisomers

usually supported by an extensive electron ionization-mass spectrometry (EI-MS) database.

In this study, differentiation of chloroamphetamine analog regioisomers was studied using GC–MS–MS with EI and chemical ionization (CI). The effectiveness of analyte derivatization was also evaluated.

Materials and methods

Chemicals

4-Chlorophenylacetone, methylamine hydrochloride, and sodium cyanoborohydride were purchased from Tokyo Chemical Industry (Tokyo, Japan). *Ortho*- and *m*-chlorophenylacetone and trifluoroacetic anhydride (TFAA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) was obtained from Supelco (St Louis, MO, USA). All other chemicals were of analytical grade.

Chloroamphetamine synthesis

Chlorophenylacetone and ammonium acetate or methylamine hydrochloride (5 equiv.) were dissolved in methanol at room temperature and the solution was stirred for 1 h. Sodium cyanoborohydride (0.4 equiv.) at 0 °C was added to the mixture. After the reaction was complete (Fig. 2), the pH of the mixture was adjusted to 12 with a 10 % potassium hydroxide aqueous solution. The mixture was extracted with diethyl ether, washed with water, and dried over sodium sulfate. The extract was then evaporated under reduced pressure. The crude residue was purified by column chromatography (silica gel, chloroform:methanol = 10:1).

Chemical structures were elucidated using ¹H NMR spectroscopy. ¹H NMR spectra were measured in deuteriochloroform (CDCl₃) with a JNM-ECP400 spectrometer (JEOL, Akishima, Japan) at room temperature. Tetramethylsilane was used as an internal standard. The ¹H NMR data for each synthesized compound are summarized below.

2-CAP; ¹H NMR (CDCl₃) δ: 7.37–7.35 (1H, m), 7.22–7.14 (3H, m), 3.32–3.24 (1H, m), 2.86 (1H, dd),

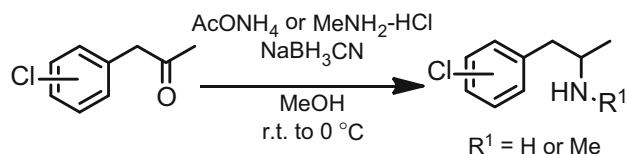


Fig. 2 Synthesis of chloroamphetamine analog regioisomers

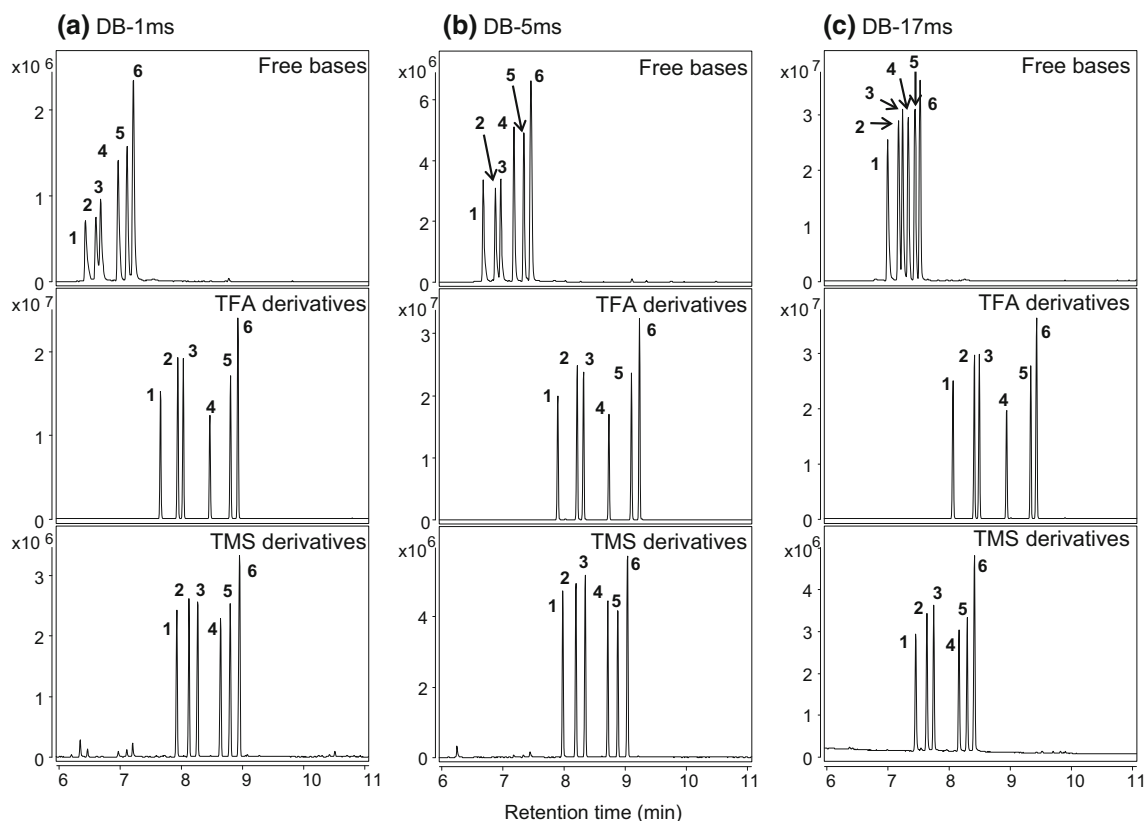


Fig. 3 Total ion current chromatograms for free bases, trifluoroacetyl (TFA) derivatives, and trimethylsilyl (TMS) derivatives of chloroamphetamine analogs separated on DB-1ms (a), DB-5ms (b), and DB-17ms (c) columns. The compound names are the same as those in Fig. 1

Table 1 Retention indices of chloroamphetamine analogs in different chemical forms on different separation columns

Analyte	Retention index								
	Free base			TFA derivative			TMS derivative		
	DB-1ms	DB-5ms	DB-17ms	DB-1ms	DB-5ms	DB-17ms	DB-1ms	DB-5ms	DB-17ms
2-CAP	1281	1310	1524	1426	1449	1651	1449	1455	1568
3-CAP	1299	1331	1546	1462	1488	1696	1473	1481	1591
4-CAP	1307	1341	1555	1473	1500	1707	1490	1498	1605
2-CMA	1340	1365	1566	1522	1549	1772	1537	1544	1658
3-CMA	1356	1383	1579	1566	1594	1822	1557	1563	1675
4-CMA	1368	1395	1590	1582	1613	1837	1576	1584	1690

TFA trifluoroacetyl, TMS trimethylsilyl, CAP chloroamphetamine, CMA chloromethamphetamine

$J = 13.2, 6.0$ Hz), 2.68 (1H, dd, $J = 13.2, 8.0$ Hz), 1.14 (3H, d, $J = 6.4$ Hz).

3-CAP; ^1H NMR (CDCl_3) δ : 7.21–7.18 (3H, m), 7.08–7.06 (1H, m), 3.21–3.13 (1H, m), 2.68 (1H, dd, $J = 13.2, 5.6$ Hz), 2.51 (1H, dd, $J = 13.2, 8.0$ Hz), 1.12 (3H, d, $J = 6.4$ Hz).

4-CAP; ^1H NMR (CDCl_3) δ : 7.28–7.26 (2H, m), 7.13–7.10 (2H, m), 3.19–3.11 (1H, m), 2.67 (1H, dd,

$J = 13.6, 5.6$ Hz), 2.52 (1H, dd, $J = 13.2, 7.6$ Hz), 1.05 (3H, d, $J = 6.4$ Hz).

2CMA; ^1H NMR (CDCl_3) δ : 7.36–7.34 (1H, m), 7.23–7.13 (3H, m), 2.97–2.87 (2H, m), 2.71–2.64 (1H, m), 2.44 (3H, s), 1.05 (3H, d, $J = 6.4$ Hz).

3-CMA; ^1H NMR (CDCl_3) δ : 7.24–7.18 (3H, m), 7.10–7.06 (1H, m), 2.83–2.69 (2H, m), 2.60–2.55 (1H, m), 2.40 (3H, s), 1.05 (3H, d, $J = 5.6$ Hz).

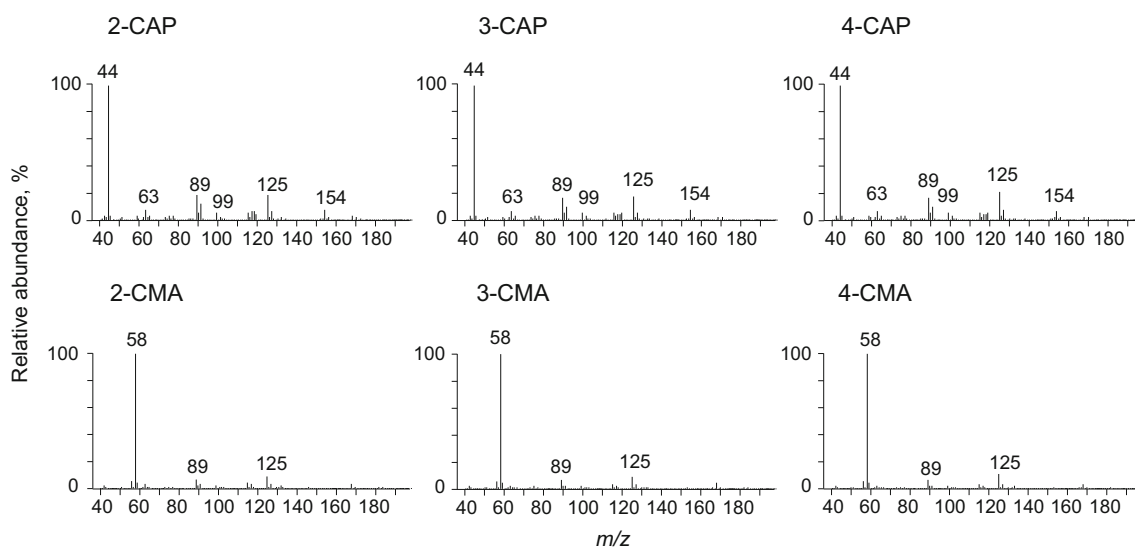


Fig. 4 Electron ionization (EI) mass spectra for free bases of chloroamphetamine analogs

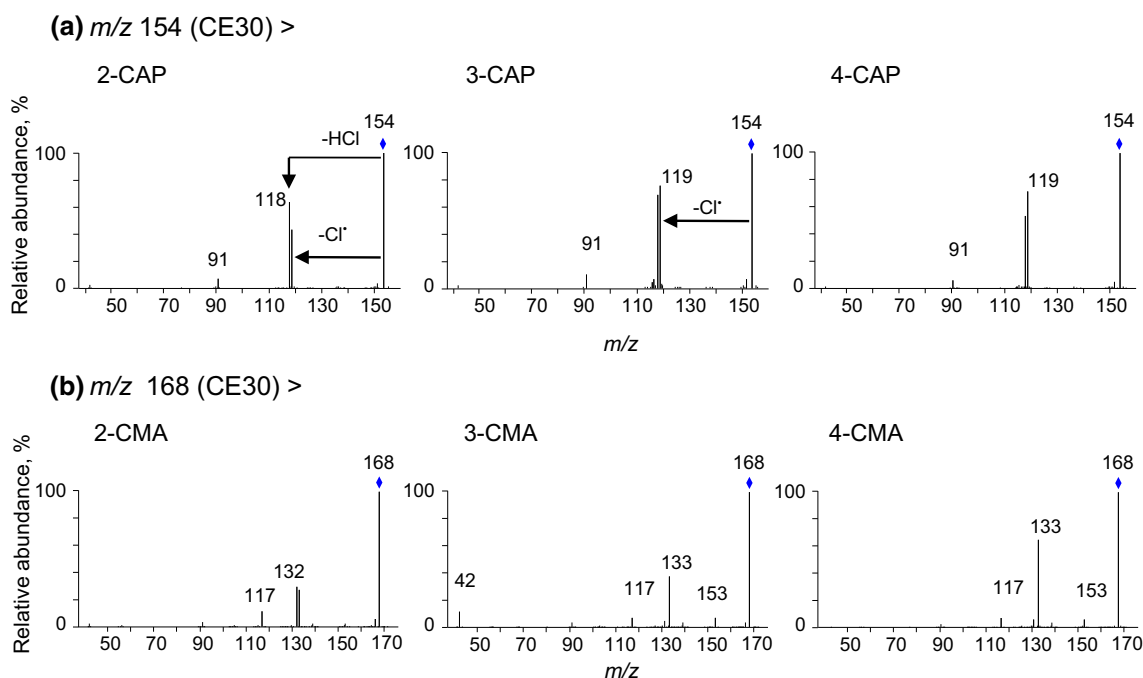


Fig. 5 EI product ion spectra for free bases of chloroamphetamines (CAPs) (precursor ions at m/z 154; **a**) and chloromethamphetamines (CMAs) (precursor ions at m/z 168; **b**)

4-CMA; ^1H NMR (CDCl_3) δ : 7.27–7.25 (2H, m), 7.13–7.11 (2H, m), 2.83–2.70 (2H, m), 2.59 (1H, dd, $J = 13.2, 6.4$ Hz), 2.39 (3H, s), 1.05 (3H, d, $J = 5.6$ Hz).

Preparation of sample solution

Stock standard solutions of all six compounds as corresponding hydrochloride salts were prepared in distilled

water to give a concentration of 1.0 mg/ml. The solutions were further diluted with distilled water to give appropriate concentrations.

Extraction procedure

One milliliter of 0.5 mg/ml aqueous solution containing hydrochloride salts of chloroamphetamine analogs was alkalinized with 20 μl of 2 M sodium hydroxide aqueous

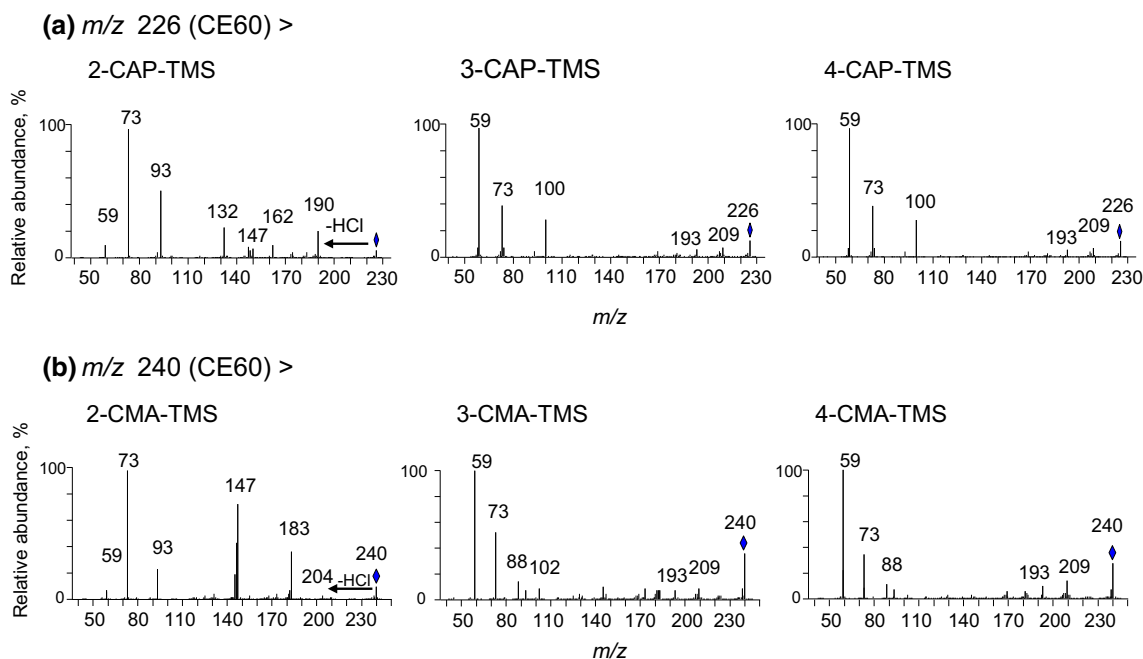


Fig. 6 EI product ion spectra for TMS derivatives of CAPs (precursor ions at m/z 226, $[M-CH_3]^+$; **a**) and CMAs (precursor ions at m/z 240, $[M-CH_3]^+$; **b**)

solution before being shaken with 1 ml of ethyl acetate for 3 min. After centrifugation, a portion of the extract was diluted by a factor of ten. The resulting solution was subjected to GC–MS.

Trifluoroacetylation

Up to 40 μ l of ethyl acetate extract was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 200 μ l of ethyl acetate and 200 μ l of TFAA, and the mixture was heated for 15 min at 50 $^{\circ}$ C. After

being cooled to room temperature, the mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 1000 μ l of ethyl acetate and subjected to GC–MS.

Trimethylsilylation

Ten microliters of extracted ethyl acetate solution was evaporated to dryness under a gentle stream of nitrogen. The dry residue was dissolved in 100 μ l of acetonitrile, and 100 μ l of BSTFA with 1 % TMCS content was added to

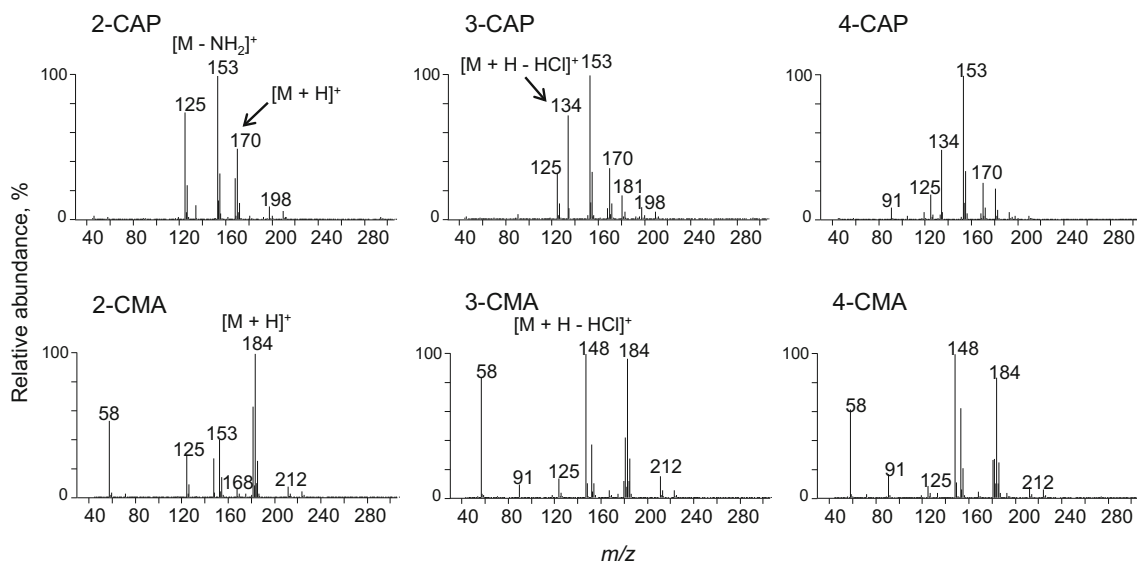


Fig. 7 Chemical ionization (CI) mass spectra for free bases of chloroamphetamine analogs

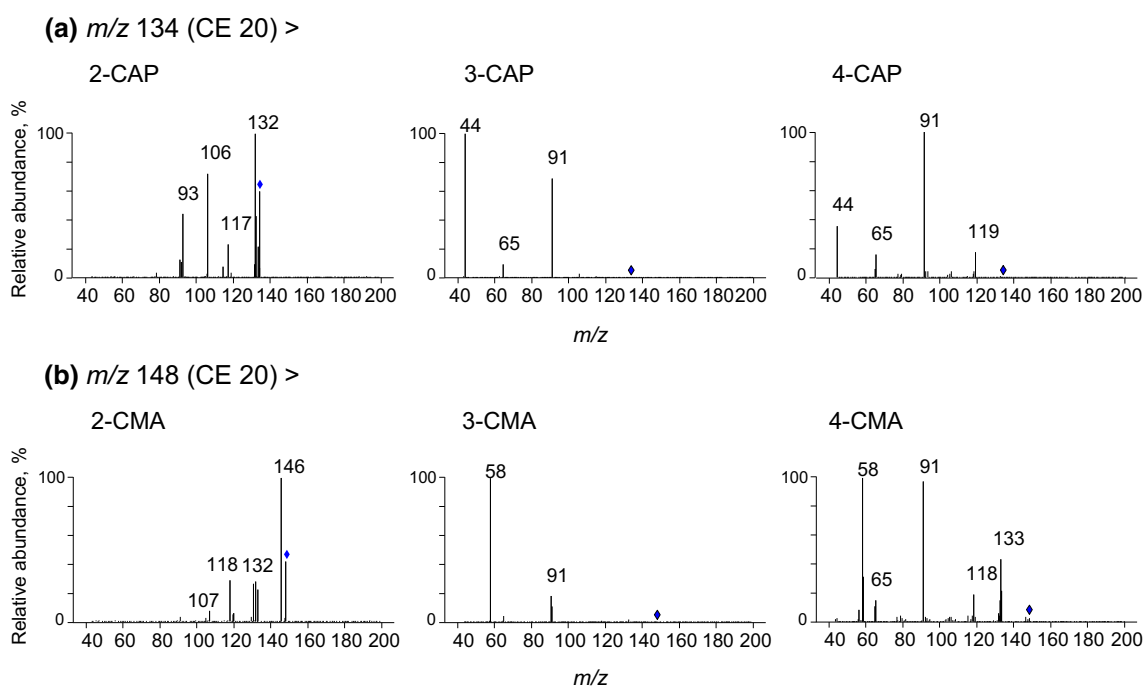


Fig. 8 CI product ion spectra for free bases of CAPs (precursor ions at m/z 134, $[M+H-HCl]^+$; **a**) and CMAs (precursor ions at m/z 148, $[M+H-HCl]^+$; **b**)

the solution. The mixture was heated for 30 min at 80 °C and subjected to GC–MS analysis after being cooled to room temperature.

GC–MS conditions

GC–MS was performed on a 7000 Triple quadrupole GC–MS system (Agilent, Santa Clara, CA, USA) equipped with a DB-1ms, a DB-5ms, or a DB-17ms capillary column (30 m \times 0.25 mm I.D., film thickness 0.25 μ m, Agilent J&W, Folsom, CA, USA). The oven temperature was maintained at 80 °C for 1 min following injection and then raised to 300 °C at a rate of 15 °C/min. The injection port and interface temperature were set at 250 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. One microliter of sample solution was injected in splitless mode.

For EI mode, the ionization energy was 70 eV, the ion source temperature was 230 °C, and the scan mass range was m/z 40–400. For CI mode, the ionization energy was 70 eV, the ion source temperature was 250 °C, and the scan mass range was m/z 43–400.

Results and discussion

Gas chromatography

Figure 3 shows total ion current chromatograms of free bases, trifluoroacetyl (TFA) derivatives, and trimethylsilyl

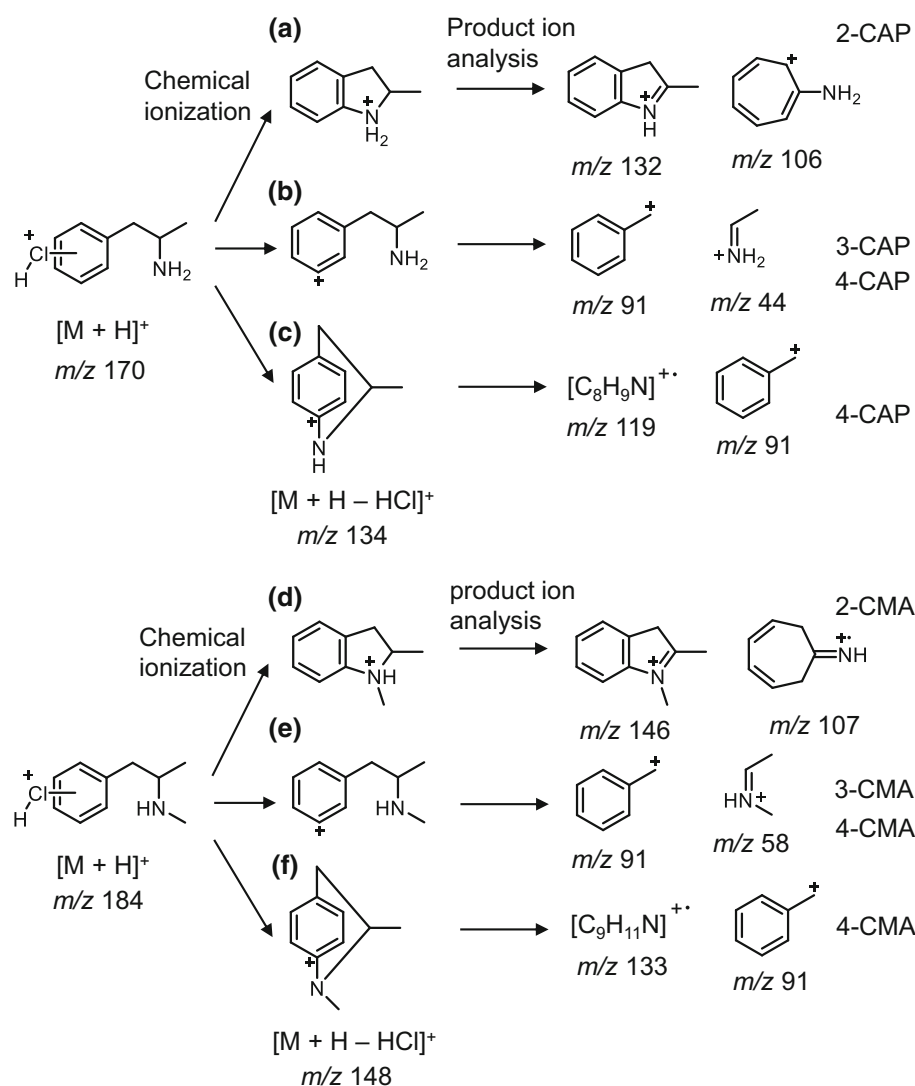
(TMS) derivatives of chloroamphetamine analogs. Retention indices of the analogs are shown in Table 1. The regioisomers of chloroamphetamine analogs in all the forms of free bases, TFA derivatives, and TMS derivatives were eluted in the order of 2-positional isomers > 3-positional isomers > 4-positional isomers. Free bases of six analytes were well separated from each other on the DB-5ms column, although the peak shapes showed tailing (Fig. 3b). Their TFA and TMS derivatives were separated completely with symmetric peaks on all three separation columns.

Electron ionization-mass spectrometry

The EI mass spectra of free bases of chloroamphetamine analogs are shown in Fig. 4. The mass spectra of the isomers were very similar. Chloroamphetamine analogs showed the same major fragment ions based on benzyl bond cleavage (m/z 125) and α -cleavage (m/z 44 or 58). Their TFA and TMS derivatives also gave very little structural information for differentiation among these analogs (data not shown).

Tandem MS was applied under various conditions of collision energy. The characteristic fragmentation of 2-positional isomers was observed clearly from the product ion spectra of chloroamphetamine analogs with moderate collision energy (CE = 30, Fig. 5). The fragment ions at m/z 118 and 132 based on desorption of hydrochloride came from precursor ions at m/z 154 and 168. Meanwhile, 3- and 4-positional isomers produced fragment ions at

Fig. 9 Plausible structures of the precursor ions for CI-tandem mass spectrometry of CAPs and CMAs and their cleavage pathways. The precursor ions were m/z 134 for CAPs and m/z 148 for CMAs, which corresponded to $[M+H-HCl]^+$



m/z 119 and 133 based on chloride radical desorption from precursor ions rather than fragment ions of hydrochloride loss under the same collision energy. The loss of hydrochloride by the 2-positional Cl group on an aromatic ring in tandem MS was in accordance with the content of a previous report by Katiaho et al. [27]. The ratio of the fragment ion at m/z 42 for 3-CMA was higher than that for 4-CMA. The difference between 3-CMA and 4-CMA was more obvious because of strong collision energy (data not shown). The results obtained here suggest that careful analysis of tandem EI-MS with changes in collision energy supports the differentiation of CMA regioisomers.

TMS derivatives of 2-positional isomers showed a significant number of fragment ions at m/z 132 and 190 for 2-CAP and m/z 147 and 204 for 2-CMA in product ion spectra (Fig. 6). However, the spectra for TMS derivatives of 3- and 4-positional isomers were almost equivalent. It was considered that the product ions at m/z 132 and 147 were produced by desorption of $[Si(CH_3)_2-Cl]$ from the

precursor ion, $[M-CH_3]^+$. The ions at m/z 190 and 204 came from hydrochloride desorption of the precursor ions.

Chemical ionization-mass spectrometry

The CI mass spectra for free bases of chloroamphetamine analogs are shown in Fig. 7. CI-MS of CAPs provided the same major fragment ions at m/z 153 and 170. In the spectra of CMAs, $[M+H]^+$ at m/z 184 was also produced as the base peak. However, the mass spectra of 2-positional isomers were different from those of 3- and 4-positional isomers, the spectra of which were almost the same. Their fragment ion desorption of chloride ions (m/z 134 or 148) was also higher than that of benzyl cations (m/z 125). The 2-positional isomers produced an equivalent or higher ratio of benzyl cations than fragment ion produced by desorption of chloride ions. TFA and TMS derivatives were examined, but only TMS derivatives of 2-CAP were differentiated by a

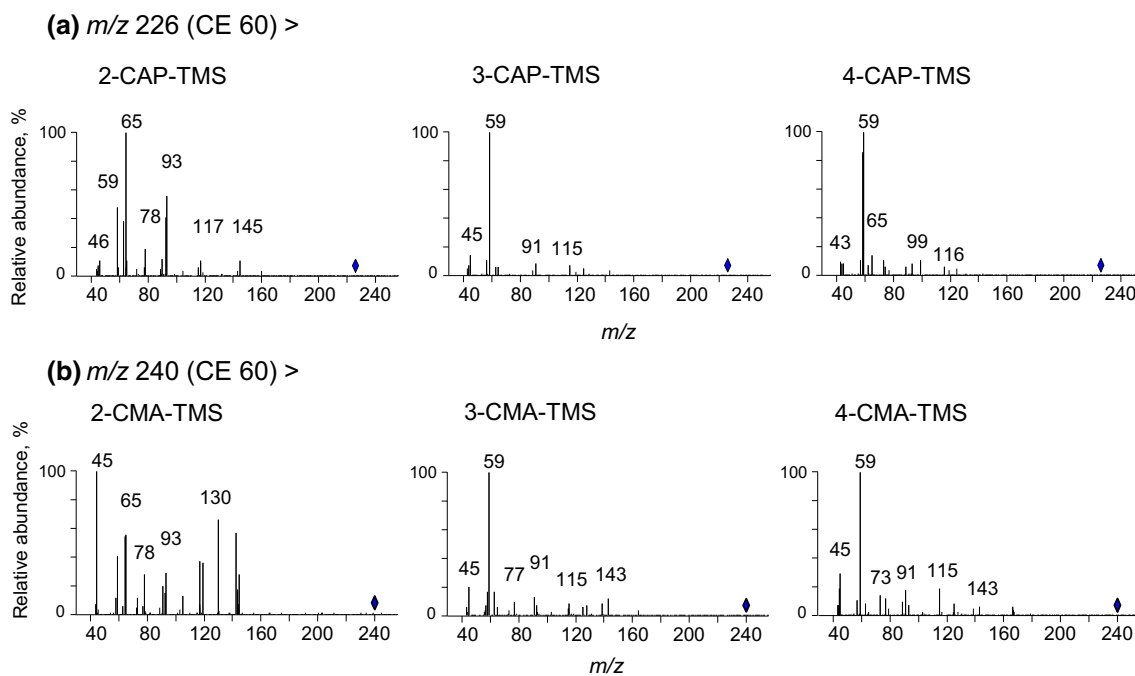


Fig. 10 CI product ion spectra for TMS derivatives of CAPs (precursor ions at m/z 226, $[M-CH_3]^+$; **a**) and CMAs (precursor ions at m/z 240, $[M-CH_3]^+$; **b**)

characteristic ion at m/z 206, $[M+H-HCl]^+$ (data not shown).

In contrast, product ion analysis of the free bases provided more structural information about chloride position on the aromatic ring when an optimal precursor ion was selected. Figure 8a shows the product ion spectra of CAP free bases obtained from the precursor ions at m/z 134, corresponding to $[M+H-HCl]^+$. Three CAP isomers showed different spectrum patterns. In particular, 2-CAP was markedly different from the other isomers. Only 2-CAP gave the ions at m/z 132 (neutral loss of hydrogen molecule), 106, and 93. In addition, 2-CAP did not give the ions at m/z 44 (α -cleaved iminium cation) and 91 (tropylium cation), which were observed for both 3-CAP and 4-CAP. These differences suggested that the precursor ions at m/z 134 in the CI mass spectra were composed of different chemical structures between 2-CAP and the others. The precursor ion of 2-CAP was presumed to be 2-methylindolinium ion (Fig. 9a). This structure was supported by the ion at m/z 106 in the product ion spectrum because this ion corresponded to anchimeric-stabilized amine tropylium cation generated by rearrangement with loss of ethylene from 2-methylindolinium ion. This pathway was as reported in a previous study by Westphal et al. [28] on the differentiation of fluorinated amphetamines using GC-CI-MS-MS.

The ions at m/z 91 and 44 in the product ion spectra of 3-CAP and 4-CAP suggested that their precursor ions

included the cations that were produced by simple elimination of HCl (Fig. 9b). These cations were cleaved to tropylium cation and iminium cation in product ion analysis (Fig. 9b). However, the ratio of m/z 91 against m/z 44 in 4-CAP was higher than that in 3-CAP, and their ratios were highly reproducible with a relative standard deviation (RSD) of less than 2%. Moreover, the ion at m/z 119 was observed only in the product ion spectrum of 4-CAP. These findings suggested that the ions at m/z 134 in the CI spectra of 4-CAP was partially derived from a para-cyclophane-like cation (Fig. 9c). The ion at m/z 119 in the product ion spectrum of 4-CAP was produced by loss of methyl cation from the para-cyclophane-like cation. Moreover, considering the difference in the ratio of m/z 91 and 44 between 4-CAP and 3-CAP, the tropylium cation of 4-CAP may be partially derived from the para-cyclophane-like cation.

Product ion spectra of CMAs differed between isomers with the same tendency as CAPs. Figure 8b shows the product ion spectra of CMA free bases obtained from the precursor ions at m/z 148, corresponding to $[M+H-HCl]^+$. 2-CMA showed the ions at m/z 146, 132, and 107, which were not observed in the spectra of 3-CMA and 4-CMA. 2-CMA did not give the ions at m/z 58 (α -cleaved iminium cation) and 91 (tropylium cation), which were observed for both 3-CMA and 4-CMA. The ratios of m/z 91 and 58 were different between 3-CMA and 4-CMA with high reproducibility (RSD less than 2%). 4-CMA gave a characteristic ion of m/z 133, corresponding to the

loss of methyl cation from the precursor ion. These findings suggested the ions at m/z 148 in the CI spectra originated from 2-methylindolinium for 2-CMA (Fig. 9d), a cation which was produced by simple elimination of HCl for 3-CMA (Fig. 9e), and the simply eliminated cation and para-cyclophane-like cation for 4-CMA (Fig. 9e, f).

The CI product ion spectra of TMS derivatives of 2-positional isomers were clearly different from those of 3- and 4-positional isomers (Fig. 10). In contrast, the CI-MS–MS of TFA derivatives gave almost the same spectra (data not shown). These results indicate that product ion spectrometry with optimal precursor ions based on CI-MS–MS can be used to differentiate between chloroamphetamine analogs.

Conclusions

Six ring-substituted chloroamphetamine analogs were successfully separated using GC–MS with DB-1ms, DB-5ms, and DB-17ms columns. EI-MS provided structural information corresponding to the position of a chloride atom on the aromatic ring. All regioisomers of chloroamphetamine analogs as free bases could be differentiated using CI-MS–MS on $[M+H-HCl]^+$. The results suggest that a combination of chromatographic analysis and tandem MS allow the differentiation of regioisomers of chloroamphetamine analogs.

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Conflict of interest There are no financial or other considerations that could lead to a conflict of interest in relation to this study.

Ethical approval This article does not contain any studies with human participants or animals.

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