# II.2.1 Amphetamines and their metabolites

*By Nariaki Takayama and Kazuichi Hayakawa*

## Introduction

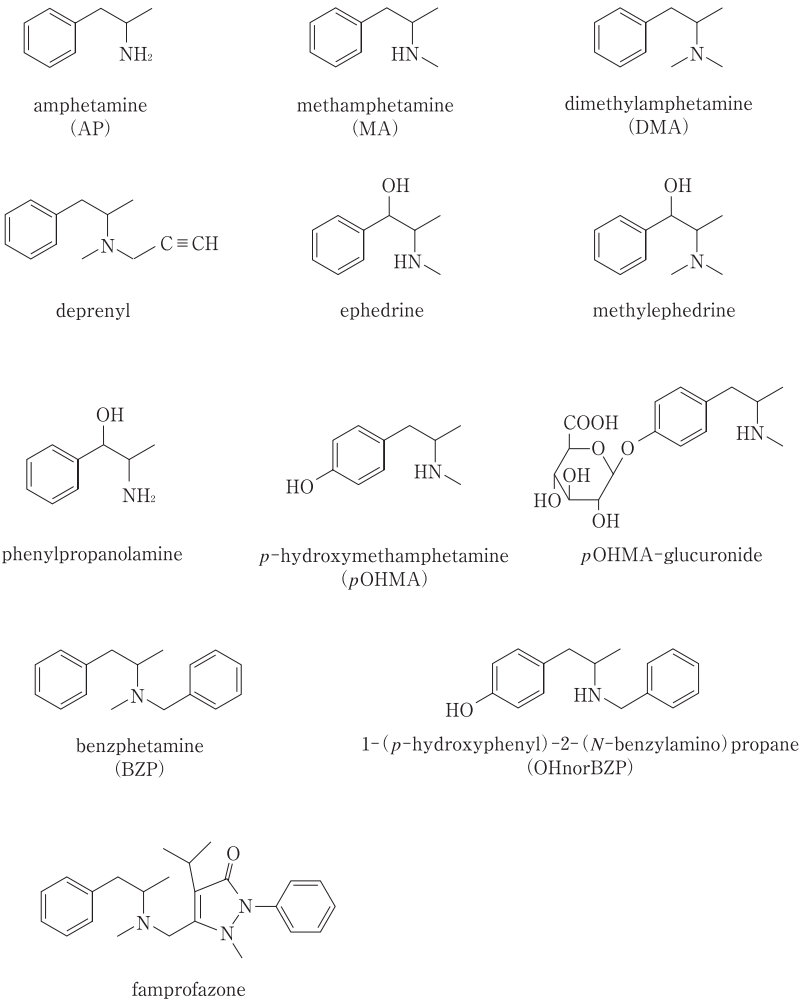
Amphetamines are a group of drugs stimulating the central nervous system; they act on the cerebral cortex to enhance psychic activities, resulting in the removal of general fatigue and drowsiness and thus in the transient improvement of working efficiency. Their abuse causes dependence, hallucination, delusion and changes in personality. Because of such harmfulness of the drugs, their use and possession are prohibited by the Stimulant Drugs Control Law in Japan [1]. The recent methamphetamine (MA) abuse is characteristically involving young generation, and causing a serious social problem; it is regarded as the 3rd peak period of MA abuse [2]. The drug is being smuggled from China, Korea Peninsula and the so-called "Golden Triangular Zone" [3], where borders of Myanmar, Thailand and Laos are intercrossing.

The stimulant drugs include amphetamine (AP, 1-phenyl-2-aminopropane), MA (1-phenyl-2-methylaminopropane) and their salts. As an ethical drug for treatment of depression, only MA hydrochloride (commercial name: Philopon) is available from Dainippon Pharmaceutical Co., Ltd., Osaka in Japan. Most of MA being abused is the one smuggled from abroad. In the Stimulant Drugs Control Law, dimethylamphetamine (DMA, 1-phenyl-2-dimethylaminopropane), deprenyl (*N*-α-dimethyl-*N*-2-propynylphenethylamine), ephedrine, phenylpropanolamine (racemic norephedrine), methylephedrine, chloroephedrine, chloromethylephedrine, phenylacetic acid, phenylacetoacetonitrile, phenylacetone are also being controlled as materials for producing MA or AP (*Figure 1.1)*.

Unchanged MA, its metabolite AP and *p*-hydroxymethamphetamine (*p*OHMA)[a] are usually detected from urine of MA abusers. Recently, the abuse of DMA, a material for producing amphetamines, has been reported; DMA is partly metabolized into MA, AP and *p*OHMA, which are excreted into urine together with unchanged DMA [4, 5]. Therefore, it is now essential to test the presence of DMA[b] for urine of abusers in order to discriminate between DMA and MA abusers. Benzphetamine (BZP) is commercially available in USA as a slimming drug (Didrex); a major part of the drug is metabolized into 1-(*p*-hydroxyphenyl)-2-(*N*-benzylamino)propane (OHnorBZP), and a minor part of it into MA, which are both excreted into urine. Thus, the detection of OHnorBZP has also become required for urine specimens of MA abusers [6, 7].

The optical isomers of MA exist, because of the presence of asymmetric carbon in the MA structure. The medical drug Philopon is the *d*-isomer of MA. MA being abused is largely its *d*-form, but the *l*-form has occasionally become detectable from specimens of abusers [8]. However, according to the Control Law in Japan, the optical isomers are not discriminated; both *d*- and *l*-forms are the objects of legal control. The effective ingredient of a nasal decongestant Vicks Inhaler, being sold in U.S.A., is *l*-MA. Selegiline (*l*-deprenyl, FP®, Fujimoto Pharmaceutical, Osaka, Japan) started to be used for treatment of Parkinson's disease from

**Figure 1.1**




**Chemical structures of amphetamines and their related compounds to be dealt with in this chapter.**

December, 1998 in Japan; it is metabolized into *l*-MA followed by *l*-AP to be excreted into urine [9][b]. Famprofazone (*Figure 1.1*), an effective ingredient of an analgesic Gewodin® being sold in Germany, is also metabolized into racemic MA to be excreted into urine. Nowadays, chiral analysis of amphetamines has become necessary, because of the above reasons.

For the final identification of a trace drug or poison in human specimens, the measurements of mass spectra are essential. Many methods for detection and determination of MA, AP and related compounds were reported using GC, HPLC, GC/MS and LC/MS. In this chapter, GC and HPLC are first presented as usual methods for analysis of MA, AP and related compounds; the methods by GC/MS and LC/MS are also described as the final confirmatory tests. In addition, a new capillary electrophoresis (CE) method for the compounds is also introduced.

## Reagents and their preparation

- *d*-MA hydrochloride is obtainable from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan or Sigma, St. Louis, MO, USA with a proper legal procedure; *d*-AP sulfate: it can be synthesized by the method of Blackburn and Burghard [10] also with a legal procedure; *dl*-AP sulfate (previous commercial name: Zedrin®) was obtained from Takeda Chem. Ind. Ltd., Osaka, Japan; *l*-MA hydrochloride and *l*-AP sulfate donated from the Ministry of Health, Labor and Welfare of Japan; *p*OHMA from Sigma; trifluoroacetic anhydride (TFAA) obtainable from Wako Pure Chemical Industries, Ltd., Osaka, Japan and other manufacturers; OHnorBZP was synthesized by the method of Niwaguchi et al. [11].
- A 30-mL volume of chloroform is mixed with 10 mL isopropanol to prepare a solvent (3:1, v/v) to be used for extracting MA and metabolites from urine.
- A 10-mg aliquot of diphenylmethane (DPM, Nacalai Tesque, Kyoto, Japan and other manufacturers) is dissolved in 100 mL ethyl acetate to prepare stock solution (100 μg/mL). The solution is diluted 100-fold with ethyl acetate to prepare internal standard (IS) solution (1 μg/mL).
- A 10-mg aliquot of 2-phenylethylamine hydrochloride (PEA, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan and other manufacturers) and 10 mg of 1-amino-4-phenylbutane hydrochloride (APB, Tokyo Kasei Kogyo and other manufacturers) are dissolved in 100 mL of ultra-pure water to be used as IS mixture solution (100 μg/mL each) for CE analysis.
- A 5.8-mL volume of phosphoric acid is mixed with ultra-pure water to prepare 10 mL of stock solution (8.5 M). A 0.1-mL volume of the stock solution is dissolved in ultra-pure water to prepare 300 mL solution (2.8 mM) as a mobile phase for the pretreatment-HPLC analysis.
- A 7.7-g aliquot of ammonium acetate is dissolved in 100 mL ultra-pure water to prepare stock solution (1 M). The stock solution is diluted 50-fold and 100-fold with ultra-pure water to prepare a mobile phase (20 mM) for the Simon's reaction-HPLC and a mobile phase (10 mM) for the LC/MS analysis, respectively.
- A 0.91-g aliquot of Tris(hydroxymethyl)aminomethane (Tris, Wako Pure Chemical Industries and other manufacturers) is dissolved in 100 mL ultra-pure water, and the pH of the solution is adjusted to 2.5 by adding 20% phosphoric acid solution. A 1.3-g aliquot of 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD, Wako Pure Chemical Industries and other manufacturers) and 0.34 g of β-cyclodextrin (β-CD, many manufacturers) are dissolved in the above solution to prepare an electrolyte (75 mM Tris/10 mM DM-β-CD/3 mM β–CD) for the CE analysis.
- A 9-mL volume of chloroform is mixed with 1 mL methanol and 0.1 mL of 25% ammonia solution to prepare a developing solvent (90:10:1, v/v) for TLC (the volume of the development container, 380 mL).
- A 5-mL volume of 20% sodium carbonate aqueous solution is mixed with 5 mL of 1% sodium nitroprusside (Wako Pure Chemical Industries and other manufacturers) aqueous solution just before use as the Simon's reagent.
- Pure acetaldehyde solution (Wako Pure Chemical Industries and other manufacturers) is placed in a airtight container to obtain acetaldehyde gas to be used for color development of a TLC plate.

## Instrumental conditions

### GC analysis

Column: a DB-5 fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent Technologies, Folsom, CA, USA).

GC conditions; instrument: an Agilent 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA,USA); detectors: an FID and a nitrogen-phosphorus detector (NPD); column temperature: 40° C (1 min)→60° C/min→100° C (1 min)→20° C/min→250° C (5 min); injection temperature: 200° C; detector temperature: 250° C; injection volume: 1 μL (splitless injection); carrier gas: He; its flow rate: 1.2 mL/min.

### GC/MS analysis

#### i. Usual GC/MS conditions

Column: a DB-5MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent Technologies); instrument: a QP-5050A GC/MS instrument (Shimadzu Corp., Kyoto, Japan); column temperature: 80° C (3 min)→10° C/min→200° C (5 min); injection temperature: 200° C; interface temperature: 250° C; injection volume: 1 μL (splitless injection); carrier gas: He; its flow rate: 1.5 mL/min; ionization mode (electron energy): EI (70 eV); detector voltage: 1.5 kV; scan range: *m/z* 60–250

#### ii. Chiral GC/MS conditions

Column: a β-DEX225 fused silica capillary column coated with cyclodextrin on its inside surface (30 m × 0.25 mm i.d., film thickness 0.25 μm, Supelco, Bellefonte, PA, USA); instrument: an Agilent 5973N GC/MS instrument (Agilent Technologies); column temperature: 60° C (1 min)→6° C/min→120° C→2° C/min→150° C→5° C/min→180° C (3 min); injection temperature: 210° C; interface temperature: 210° C; injection volume: 1 μL (splitless injection); carrier gas: He; its flow rate: 1.0 mL/min; ionization mode (electron energy): EI (70 eV); electron multiplier voltage: 1,094 V; scan range: *m/z* 35–350

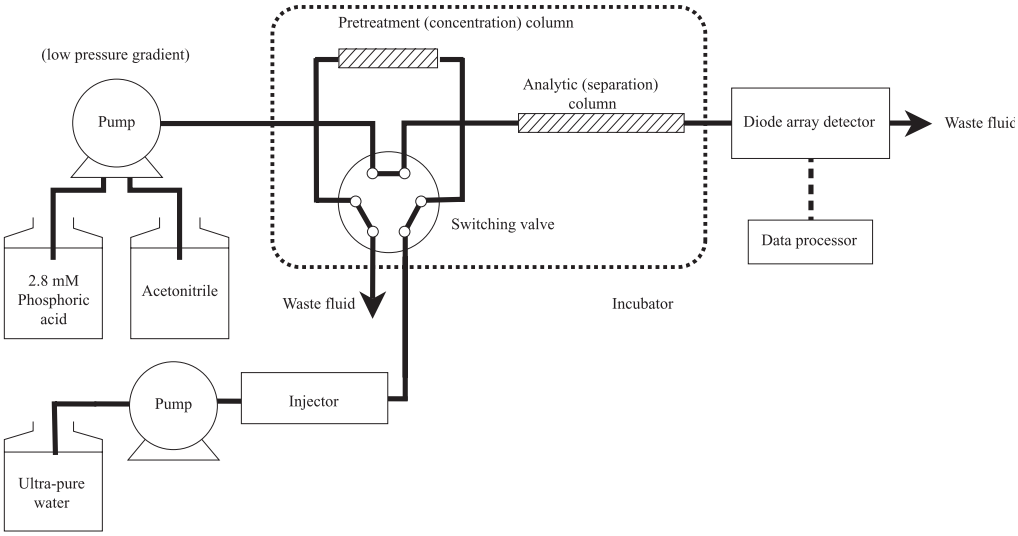
### HPLC analysis

#### i. Usual pretreatment-HPLC conditions

Column: a silica-based ODS column, Develosil ODS-UG-5 (250 × 4.6 mm i.d., Nomura Kagaku, Aichi, Japan); instrument: LC-10Avp (Shimadzu Corp.); mobile phase (gradient elution conditions): acetonitrile/2.8 mM phosphate buffer solution (gradient from 10:90, v/v, to 50:50 for 10 min and hold at 50:50 for 20 min); flow rate: 1.0 mL/min; column temperature: 50° C; injection volume: 10 μL; detector: a diode array detector (DAD, detection wavelength 205–350 nm); pretreatment column: a polyvinyl alcohol resin column, Shim-pack SPC-RP3 (30 × 4.0 mm i.d., Shimadzu GLC Center, Tokyo, Japan)

The specimen solution and ultra-pure water are introduced into the pretreatment column at a flow rate of 1.0 mL/min for 2 min to trap a target compound in the column; by

**Figure 1.2**




**Outline of the system for the usual pretreatment-HPLC.**

switching the valve, the mobile phase is poured in the opposite direction through the pretreatment column to bring the target compound to the separation (analytical) column. The outline of the system is shown in *Figure 1.2*

### ii. Simon's reaction-HPLC conditions

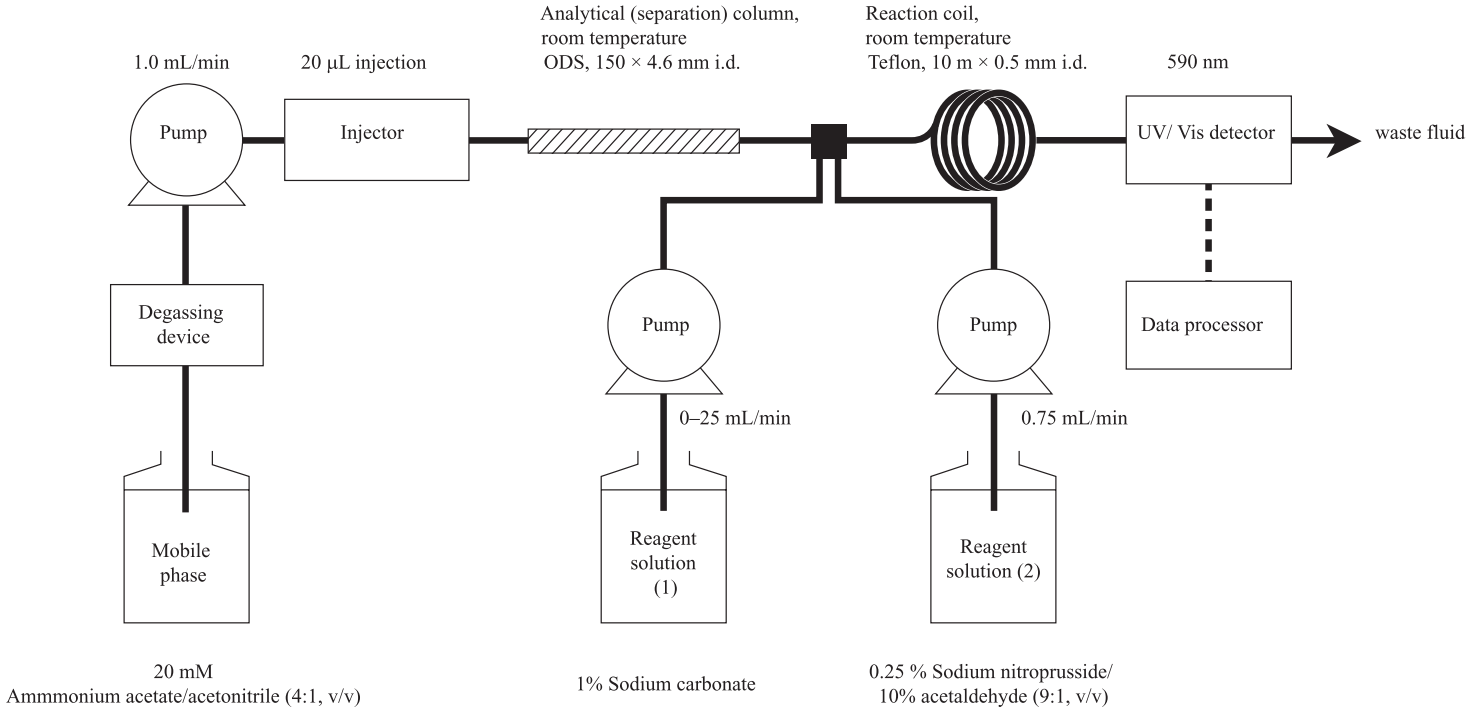
The Simon's reaction is made in the post-column mode; the two kinds of reagents are mixed with the post-column mobile phase. The outline of the system is shown in *Figure 1.3*. Column: a silica-based ODS column, L-column ODS (150 × 4.6 mm i.d., Chemicals Evaluation and Research Institute, Tokyo,Japan); instrument: LC-10A (Shimadzu Corp.); mobile phase (isocratic elution mode): acetonitrile/20 mM ammonium acetate solution (20:80, v/v); flow rate: 1.0 mL/min; reaction solution (1): 1% sodium carbonate aqueous solution, 0.25 mL/min; reaction solution (2): 0.25% sodium nitroprusside aqueous solution/10% acetaldehyde aqueous solution (90 : 10, v/v), 0.75 mL/min; reaction coil: 10 m × 0.5 mm i.d.; injection volume: 20 μL; detector: a UV-visible detector (detection wavelength 590 nm); column and reaction coil temperature: room temperature

## LC/MS analysis

### i. Usual LC/MS conditions

Column: L-column ODS (150 × 2.1 mm i.d., Chemicals Evaluation and Research Institute); instrument: M-8000 LC/MS (Hitachi, Tokyo, Japan); mobile phase (isocratic elution mode): methanol/10 mM ammonium acetate solution (15:85, v/v); flow rate: 0.2 mL/min; column temperature: 40° C; injection volume: 10 μL; ionization mode: sonic spray ionization (SSI); polarity: positive electric charge mode; drift and focus voltage: 30 V; chamber voltage: 1.2 kV: assisting gas: nitrogen, 2.4 kg/cm$^2$; target gas: He; scan range: $m/z$ 70–250

**Figure 1.3**




**Outline of the system of the Simon's reaction-HPLC and its analytical conditions.**

#### ii. Chiral LC/MS conditions

Column: a silica-based ODS column bound with cyclodextrin, Chiral Drug Column (150 × 2.0 mm i.d., Shiseido, Tokyo, Japan); instrument: an Alliance LC/MS instrument attached with ZMD (Waters, Milford, MA, USA); mobile phase (isocratic elution mode): methanol/acetonitrile/10 mM ammonium acetate solution (30:10:60, v/v); flow rate: 0.25 mL/min; column temperature: 40° C; injection volume: 10 μL; ionization mode: electrospray ionization (ESI); polarity: positive electric charge mode; capillary voltage: 3.5 kV; cone voltage: 30 V; sheath gas: nitrogen (400° C, 400 L/h); scan range: *m/z* 80–350

### CE analysis

Capillary: 80.5 cm (effective length 72 cm) × 75 μm i.d. fused silica bubble cell (Agilent Technologies, Waldbronn, Germany)

CE conditions; instrument: an Agilent CE system (Agilent Technologies); capillary temperature: 25° C; impressed voltage: +30 kV; sample injection: 50 mbar × 3 s; detection wavelength: 195 nm; electrolyte: aqueous solution containing 75 mM Tris/10 mM DM-β-CD/3 mM β-CD

## Specimens and procedures

### Specimens

Urine specimens obtained from abusers of MA and DMA and from selegiline users were used. The mean concentration of MA in urine of MA abusers was reported to be 66 μg/mL (mean of 68 subjects) [12].

### Procedures

i. A 10-mL volume of urine is alkalinized by adding 1 mL of 15% sodium carbonate solution, and extracted with 10 mL of chloroform/isopropanol three times. For urine of CE analysis, 100 μL of the mixture solution of PEA and APB (100 μg/mL each) is added beforehand.
ii. The combined organic layer is dehydrated by adding about 1 g of anhydrous sodium sulfate and filtrated through a filter paper. A small amount of the organic filtrate is subjected to GC analysis (› *Figure 1.4*, an analysis example for urine of a DMA abuser).
iii. The organic filtrate is evaporated to dryness after adding 1–2 drops of 10% hydrochloric acid solution, under a stream of nitrogen or with a rotary evaporator.
iv. The resulting residue is dissolved in 0.5 mL ultra-pure water to serve as specimens of analysis [13]. Parts of the aqueous solution can be directly subjected to the usual pretreatment-HPLC (› *Figure 1.5*, an analysis example for urine of a DMA abuser), the Simon's reaction-HPLC (› *Figure 1.6*, an analysis example for urine of an MA abuser to which OHnorBZP had been spiked), the usual LC/MS (› *Figure 1.7*, an analysis example for urine of a DMA abuser), the chiral LC/MS (› *Figure 1.8*, an analysis example for urine of

an MA abuser) and the CE (*Figure 1.9*, an analysis examples for urine specimens of an MA abuser and a selegiline user).

v. For analysis requiring derivatization, the following procedure is used. A 0.1-mL volume of the above aqueous solution is evaporated to dryness under a stream of nitrogen in a screw cap vial; ethyl acetate and TFAA, 0.2 mL each, are added to the residue, capped airtightly and vortex-mixed.

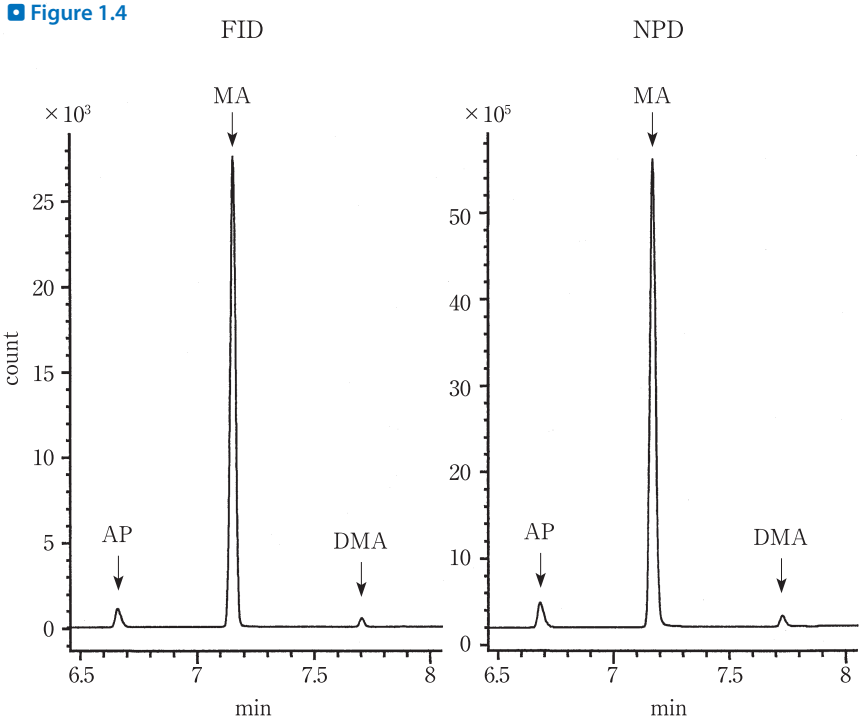
vi. The vial is heated at 55° C for 15 min; the content is evaporated to dryness under a gentle stream of nitrogen, dissolved in 0.5 mL of the IS solution containing 1 μg/mL DPM and subjected to the usual GC/MS [13] (*Figure 1.10*, an analysis example for urine of an MA abuser) and to the chiral GC/MS (*Figure 1.11*, an analysis example for urine of an MA abuser).

## Assessment and some comments on the methods

### Characteristics and cautions for each method

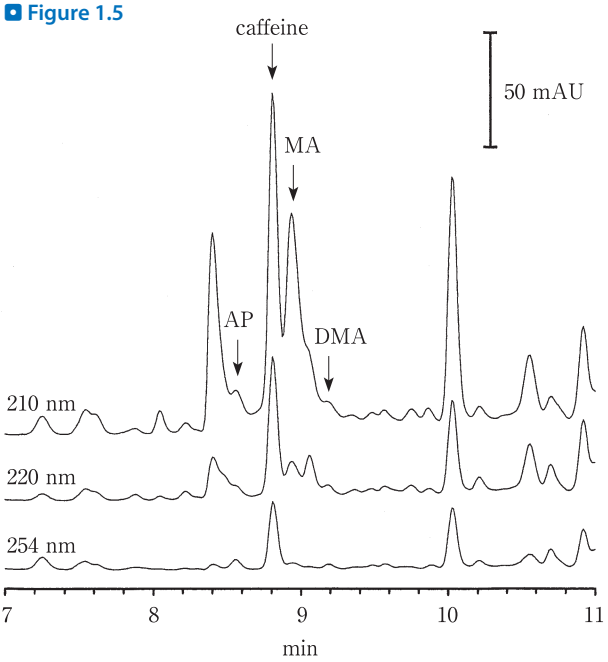
Free base of amphetamines can be analyzed by GC with an FID and an NPD without any derivatization (*Figure 1.4*); the organic extract can be directly injected into GC.

**Figure 1.4**



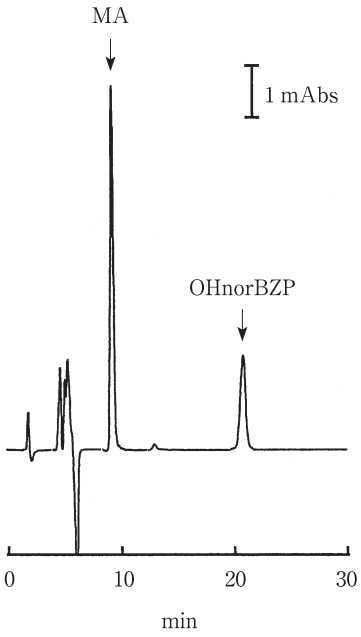

**Detection of methamphetamine (MA), amphetamine (AP) and dimethylamphetamine (DMA) from urine of a DMA abuser by GC-FID and GC-NPD. The drugs were not derivatized.**

**Figure 1.5**



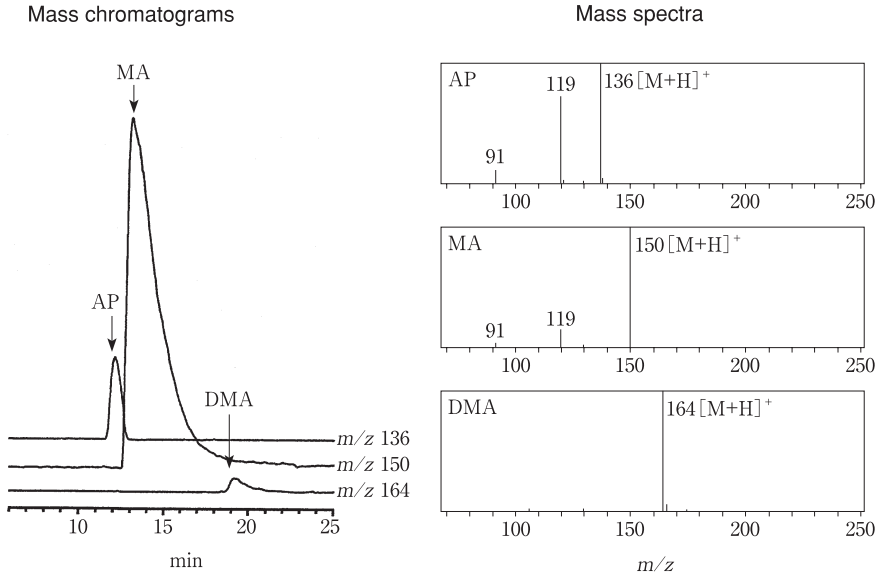

**Detection of MA, AP and DMA from urine of a DMA abuser by the usual pretreatment-HPLC.**

**Figure 1.6**



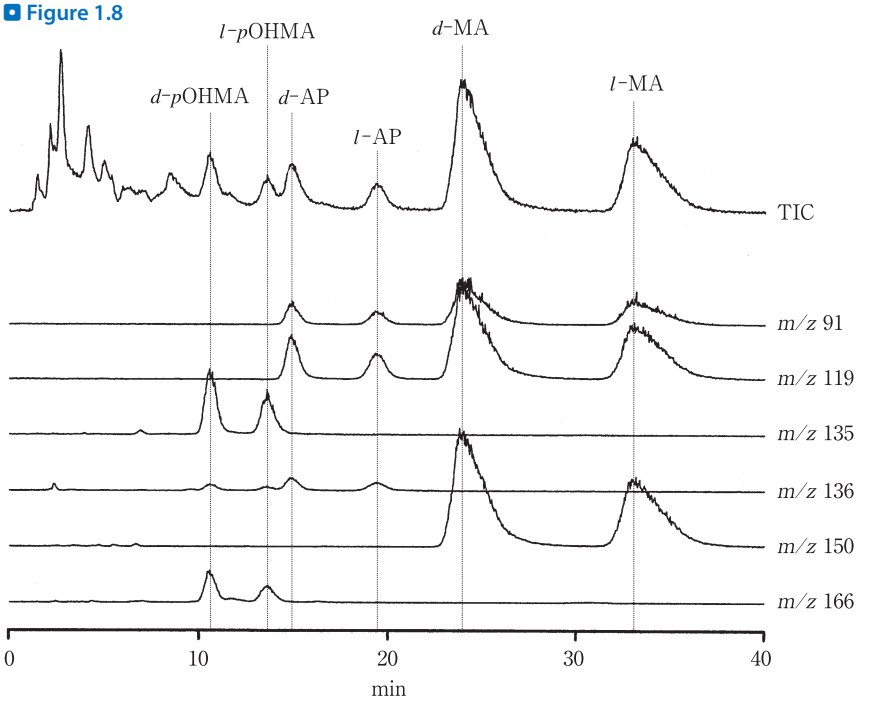

**Detection of MA and spiked OHnorBZP (a metabolite of benzphetamine) from urine of an MA abuser by the Simon's reaction-HPLC. The concentration of OHnorBZP spiked was 4 µg/mL.**

**Figure 1.7**



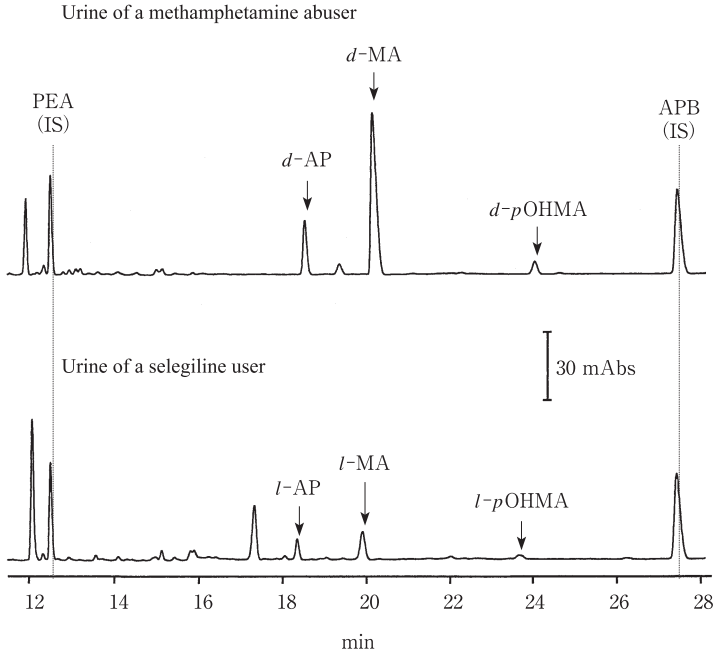

**Detection of MA, AP and DMA from urine of a DMA abuser by the usual LC/MS.**

**Figure 1.8**



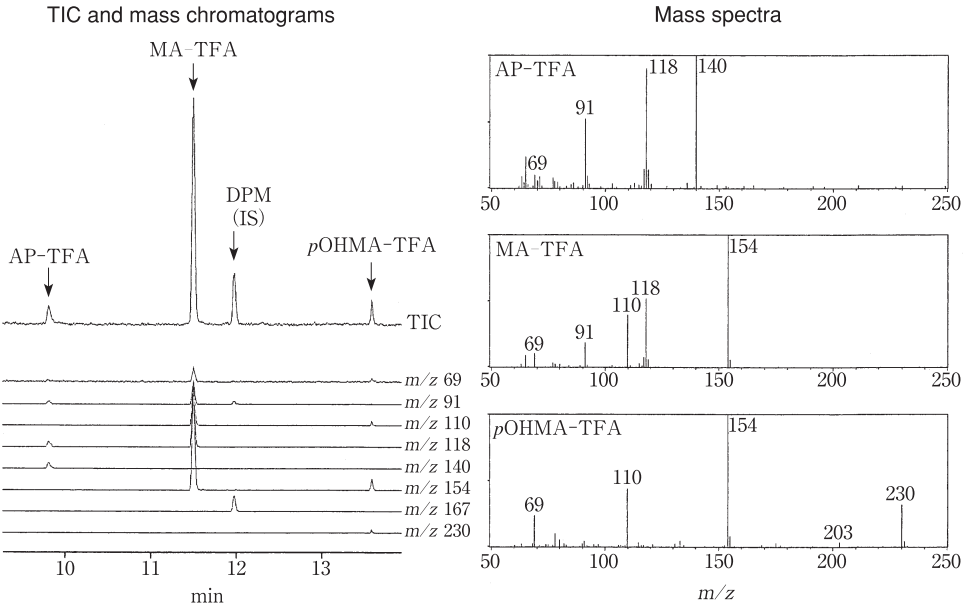

**Chiral analysis of *d*-/*l*-isomers of MA, AP and *p*-hydroxymethamphetamine (*p*OHMA) in urine of an MA abuser by chiral LC/MS.**

**Figure 1.9**



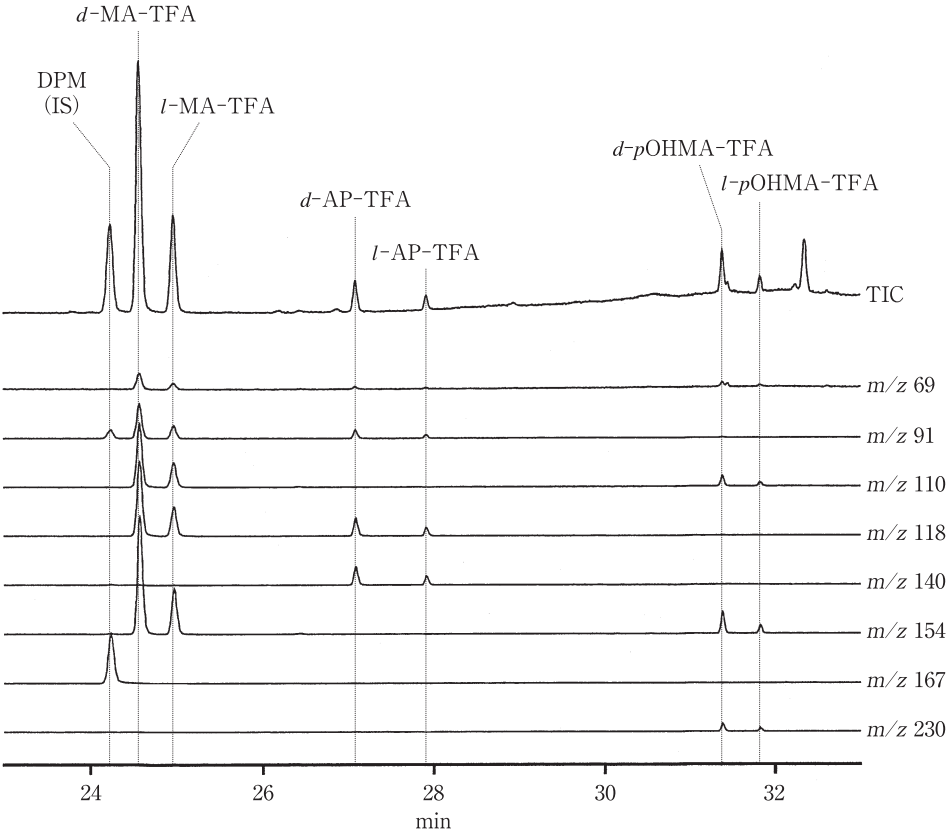

**Chiral analysis of *d*-/*l*-isomers of MA, AP and *p*OHMA in urine of an MA abuser and a selegiline user by capillary electrophoresis (CE).**

**Figure 1.10**




**Detection of TFA-derivatives of MA, AP and *p*OHMA in urine of an MA abuser by the usual GC/MS.**

**Figure 1.11**




**Chiral analysis of the TFA- derivatives of *d*-/*l*-isomers of MA,AP and *p*OHMA in urine of an MA abuser by chiral GC/MS.**

The analysis of amphetamines by GC/MS after TFA derivatization (*Figure 1.10)* is used most commonly and shows high reliability.

The chiral GC/MS analysis of amphetamines (*Figure 1.11)* suffers from its instability of the chiral column especially caused by the coexistence of water; care should be taken to dehydrate the organic extract completely.

The usual pretreatment-HPLC analysis (*Figure 1.5)* was made using the same type of HPLC as the one, which had been distributed to every critical care medical center by the Ministry of Health, Labor and Walfare of Japan in 1998.

The Simon's reaction-HPLC analysis (*Figure 1.6)* gives the sensitivity about 100 times higher than that of TLC[c], although the system of the former is complicated.

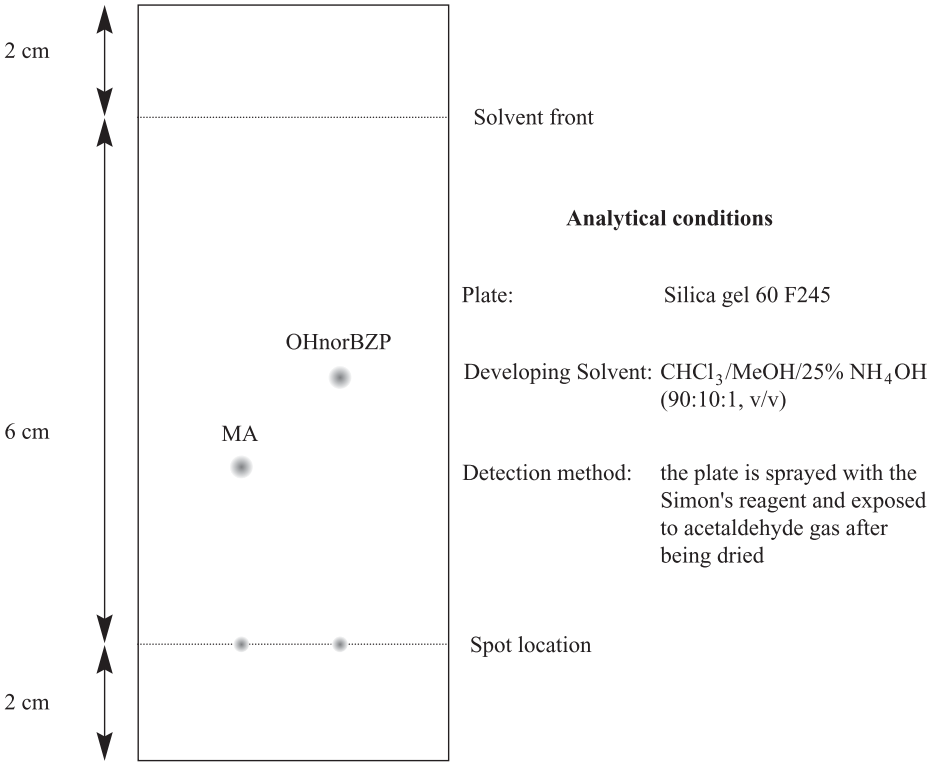
Although the instrument to be used for the chiral LC/MS analysis and the corresponding column are expensive, it is advantageous in that no derivatization is required before analysis. There is a possibility of leakage of cyclodextrin from the packing material of the analytical (separation) column, resulting in contamination of the ion source and thus rapid decrease in sensitivity of the instrument.

The chiral analysis by CE (*Figure 1.9*) does also not need derivatization. This is the newest instrument; there is no concern about contamination due to carry-over (a phenomenon of contamination by a residual compound which had been used in the previous measurement) with this instrument.

## Detection limits[d] of MA obtained by various methods

GC analysis with an FID and NPD (*Figure 1.4*): 0.5 and 0.05 ng/1 μL, respectively; the usual GC/MS analysis (*Figure 1.10*): 0.05 ng/1 μL; the chiral GC/MS analysis (*Figure 1.11*): 0.01 ng/1 μL; the usual pretreatment-HPLC analysis (*Figure 1.5*): 5 ng/10 μL; the Simon's reaction-HPLC analysis (*Figure 1.6*): 1 ng/20 μL; the usual LC/MS analysis (*Figure 1.7*): 1 ng/10 μL; the chiral LC/MS analysis (*Figure 1.8*): 0.5 ng/10 μL; the CE analysis (*Figure 1.9*): 100 ng/mL; the Simon's reaction TLC (*Figure 1.12*): 100 ng/spot.

**Figure 1.12** TLC plate




**Detection of MA and OHnorBZP by the Simon's reaction TLC.**

## Toxic and fatal concentrations

Although there are no reports dealing with the relationship between urinary concentrations of MA and poisoning symptoms (toxic and fatal), those between the blood MA concentrations and the symptoms were reported as follows [14–16]: 30–40 μg (all values expressed as not in the salt form but in the free form)/mL, fatal; 5–7 μg/mL, severe (poisoning death possible); about 1.5 μg/mL, highly symptomatic (abnormal excitement and high derangement); about 0.8 μg/mL, intermediately symptomatic (excitement, hallucination and strange behavior); about 0.15 μg/mL, slightly symptomatic (the levels of habituals); less than 0.15 μg/mL, no symptoms (therapeutic levels). There is also a report insisting no relationship between blood MA concentrations and psychic symptoms [17].

## Notes

a) Since the metabolite of MA, *p*OHMA, is largely excreted into urine in the form of the glucuronate conjugate (*p*OHMA-glucuronide), it is occasionally not detected without any hydrolytic treatment of urine with β-glucuronidase or acid.
b) The material compounds for chemical synthesis of MA or AP are neither metabolized into MA, AP nor *p*OHMA, except DMA and deprenyl.
c) The data of the Simon's reaction TLC using a fluorescent plate (Merck, Darmstadt, Germany) with silica gel thickness of 0.25 mm are shown in *Figure 1.12* (an example of analysis of the authentic MA and OHnorBZP). Under the conditions, the $R_f$ values were 0.35 and 0.50, respectively.
d) All detection limits obtained using MS are those measured in the scan mode. When selected ion monitoring (SIM) is used for quantitation, the sensitivity 10–100 times higher can be achieved.

## References

1) The Pharmaceutical Society of Japan (ed) (1992) Standard Methods of Chemical Analysis in Poisoning – With Commentary. Nanzando, Tokyo, pp 283–329 (in Japanese)
2) The National Police Agency (ed) (2000) Police White Paper 2000, Department of Printing of the Ministry of Finance, Tokyo, p 38 (in Japanese)
3) The National Police Agency (ed) (2000) DRUG 2000. Public Relations Department of the National Police Agency, Tokyo, p 15 (in Japanese)
4) Inoue T, Suzuki S (1986) Studies on metabolism of methamphetamine analogs 6. Analysis of dimethylamphetamine and its metabolites. Rep Natl Res Inst Police Sci 39:228–234 (in Japanese with an English abstract)
5) Inoue T, Suzuki S (1987) The metabolism of dimethylamphetamine in rat and man. Xenobiotica 17:965–971
6) Inoue T, Suzuki S (1986) The metabolism of benzphetamine and furfenorex in man. Xenobiotica 16:691–698
7) Chinaka S, Tanaka S, Takayama N et al. (2000) Determination of methamphetamine in human urine by HPLC using post-column Simon's reaction. J Health Sci 46:16–20
8) Nagai To, Matsushima K, Nagai Ta et al. (2000) Interpretation and enantiomer analysis of methamphetamine abusers, urine and illegally brewed methamphetamine. J Anal Toxicol 24:140–145
9) Hasegawa M, Matsubara K, Fukushima S et al. (1999) Stereoselective analyses of selegiline metabolites. Forensic Sci Int 101:95–106
10) Blackburn D, Burghard G (1965) Radiosynthesis of dextramphethamine-C14 sulfate. J Pharm Sci 54:1586–1588

11) Niwaguchi T, Inoue T, Suzuki S (1982) The metabolism of 1-phenyl-2-(N-methyl-N-benzylamino)propane (benzphetamine) in vivo in the rat. Xenobiotica 12:617–625
12) Amemiya M, Nagai T (2000) Study on the screening test for urine of stimulant abusers using Triage®. Jpn J Sci Tech Iden 4:95–98 (in Japanese with an English abstract)
13) Inoue T, Niwaguchi T (1981) Effect of diuretic furosemide on the excretion of methamphetamine and its metabolite into rat urine. Rep Natl Res Inst Police Sci 34:142–146 (in Japanese with an English abstract)
14) Nagata T, Hara K, Kageura M et al. (1980) Significance of blood and tissue levels of methamphetamine. Jpn J Legal Med 34:354 (in Japanese)
15) Nagata T (1986) Toxicological examination from the medico-legal aspects. Jpn J Legal Med 40:455–464 (in Japanese with an English abstract)
16) Inoue T, Tanaka K (1999) Stimulants Q&A: A Chemical Guide for Investigators. Reibunsha, Tokyo, pp 21–22 (in Japanese)
17) Miyazaki T (1986) Relationship between methamphetamine and amphetamine concentrations in the blood and symptoms in methamphetamine abusers. Jpn J Legal Med 40:132–139 (in Japanese with an English abstract)