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Routine analysis of amphetamine and methamphetamine in biological materials by gas chromatography–mass spectrometry and on-column derivatization

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Abstract

A simple determination method of amphetamine (AP) and methamphetamine (MA) in biological materials was developed using on-column derivatization and gas chromatography–mass spectrometry (GC–MS). AP and MA in biological materials were adsorbed on the surface of Extrelut and then extracted and derivatized simultaneously on the Extrelut column. AP and MA were derivatized to the *N*-propoxycarbonyl derivatives using propylchloroformate. Pentadeuterated MA was used as an internal standard. The recoveries of AP and MA from urine were 88.2 and 92.5%, and those from blood were 89.7 and 90.3%, respectively. The calibration curves showed linearity in the range of 12.5–2000 ng/ml (ng/g) for AP and MA in urine and blood, and 0.25–20 ng/mg in hair. When urine samples containing two different concentrations (200 and 1000 ng/ml) of AP and MA, blood samples containing two different concentrations (200 and 1000 ng/g) of AP and MA, hair samples containing two different concentrations (0.5 and 5.0 ng/mg) of AP and MA, the coefficients of variation of intra-day and inter-day were 0.68–3.60% in urine, 0.42–4.58% in blood, and 1.20–13.1% in hair. Furthermore, this proposed method was applied to a medico-legal case of MA intoxication.

Keywords: Amphetamine; Methamphetamine

1. Introduction

Methamphetamine (MA) and its metabolite amphetamine (AP) are powerful stimulants of the central nervous system and are abused in many countries. Deaths due to overdose have been reported after intake of MA and its related compounds. It is important to determine the concentration of stimulants in blood, in order to know the cause of death. In many cases, stimulants abuse is verified by detection of amphetamines and its metabolites in urine obtained from an abuser. Recently, hair analysis for abused and therapeutic drugs has been used as a tool for detection of drugs in forensic and clinical toxicology [1,2]. A simple and accurate method is needed for routine analysis and screening of stimulants in biological materials for forensic, judicial, and clinical purposes. Many chromatographic methods have been referred to determine amphetamines in biological materials [3,4], where extraction of the target drugs from sample matrices prior to chromatography was necessary. Free amines are difficult to extract from sample matrices and cannot be well separated, due to their high polarity and volatility. Also, the analysis of free amphetamines by GC or GC–MS is associ-

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ated with difficulties in sensitivity and reproducibility because of adsorption on and interaction with the column. This results in poor peak resolution. Therefore, derivatization is necessary to analyze these compounds by GC; thus sample preparation procedures become laborious, costly and time-consuming.

Our final goal is to develop a simple and accurate method for the determination of amphetamines in biological materials. Trifluoroacetylation is usually used for derivatization of amphetamines in many laboratories. However, there are some serious problems in the trifluoroacetylation of amphetamines; for example loss of the derivatives during derivatization or stability of the derivatives [5]. Recently, alkylchloroformates have been used for derivatization of amines in urine [6–12]. Therefore, we focused on these reagents for derivatization of amphetamines because the alkylchloroformates quickly react with primary and secondary amines in an alkali aqueous sample (Fig. 1) [13–15]. In this study, we examined derivatization of AP and MA on a granular support material (Extrelut). This proposed method was applied to a medico-legal case of MA intoxication.

2. Experimental

2.1. Materials

MA hydrochloride was purchased from Dainihon Pharmaceutical (Osaka, Japan). AP hydrosulfate and pentadeuterated MA (MA-d_5) hydrochloride were kindly donated by Dr. Hara of Fukuoka University. Propyl chloroformate was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Other reagents and solvents used were purchased at the highest commercial quality from Wako Pure Chemical (Osaka, Japan). Stock standard solutions (1.0 mg/ml) of MA and AP were dissolved in 0.1 *M* HCl and stored at 4 °C in a refrigerator. Extrelut material was purchased from Merck Japan (Tokyo, Japan), and washed with double the volume of diethyl ether and then dried at 60 °C for 1 h before use. A quantity (2 g) of washed Extrelut was then packed into a glass tube (10 mm I.D.×100 mm, Extrelut column).

Drug-free samples collected from a healthy adult male were used to make blank and spiked samples containing AP and MA. The drug-free samples and the samples collected from a medico-legal case were kept frozen at −20 °C until analyzed.

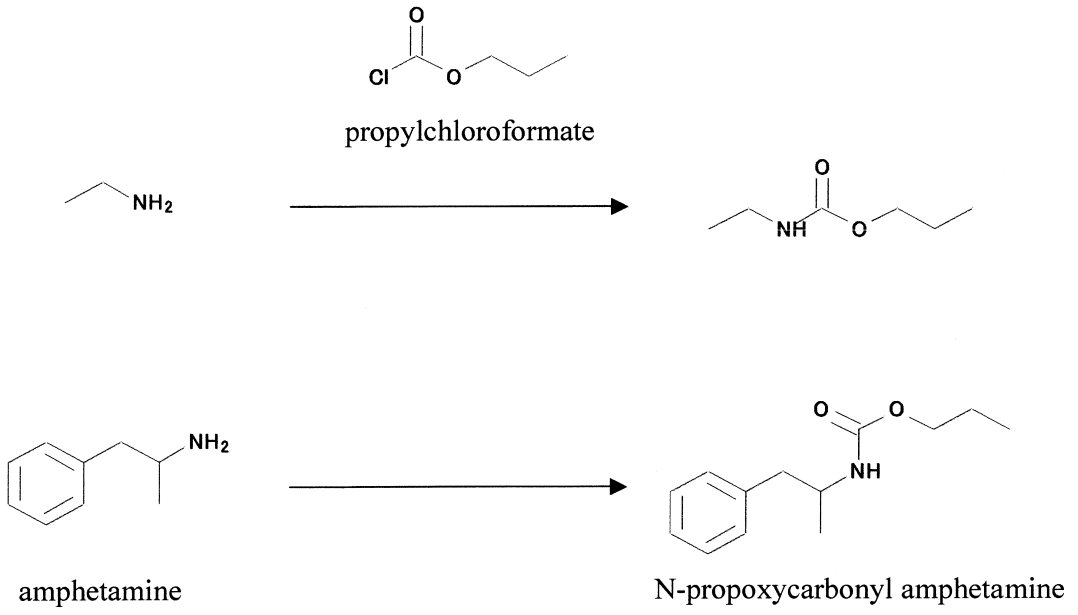

Fig. 1. Reaction scheme for the derivatization of amine with propylchloroformate.

2.2. Gas chromatography–mass spectrometry

The gas chromatograph–mass spectrometer (GC–MS) used was a Hewlett-Packard 5890 series II GC and 5971 A MSD, equipped with a 30 m×0.25 mm (I.D.) fused-silica capillary column (Hewlett-Packard, HP-5MS, film thickness 0.25 μm). The oven temperature was set at 100 °C for 1 min, and then programmed from 100 to 300 °C at 10 °C/min, and held at 300 °C for 3 min. The temperatures of the injection port and the detector were set at 250 and 280 °C, respectively. Splitless injection mode was used. The mass selective detector was operated in the electron impact (EI) mode at 70 eV of electron energy. Helium with a flow-rate of 0.8 ml/min was used as a carrier gas. Ions used for quantitation were m/z 130 for AP derivative, m/z 144 for MA derivative, m/z 148 for MA-d_5 (I.S.) derivative at selected ion monitoring (SIM). To confirm the mass fragment of the derivatives, data were obtained in a full scan mode with a scan range from m/z 50 to 550.

2.3. Extraction and derivatization procedure

2.3.1. Urine and blood

A urine sample (0.5 ml) and an internal standard (MA-d_5, 0.1 mg/ml, 5 μl) were mixed with borate buffer (50 m*M*, pH 10.5, 1.0 ml). The mixture was applied to an Extrelut column. After 20 min at room temperature, one ml of ethylacetate containing alkylchloroformate (10 μl/ml in ethylacetate) was introduced into the column. To react amphetamines and alkylchloroformate completely, the column was left for at least 10 min. The derivatives were then eluted with ethylacetate (3 ml). The eluent was evaporated in a vacuum using a centrifugal concentrator (CC-101, TOMY Seiko, Tokyo, Japan). The residue was then dissolved in 200 μl of ethylacetate and 1 μl of the sample was injected to a GC–MS.

In the case of blood, a blood sample (0.5 g) and an internal standard (MA-d_5, 0.1 mg/ml, 5 μl) were mixed with borate buffer (50 m*M*, pH 10.5, 1.0 ml). The following procedure was same in urine.

2.3.2. Hair

The washing and degradation conditions of hair were referred the published procedure [16]. In brief, the hair sample was washed with 0.1% sodium dodecylsulfate and deionized water under sonication. The washed hair (10 mg), an internal standard (MA-d_5, 0.01 mg/ml, 5 μl) and NaOH (1 *M*, 0.5 ml) was heated at 75 °C for 20 min. The mixture was then applied to an Extrelut column. The extraction procedure was the same as in urine samples.

2.4. Method validation

To construct calibration curves, urine and blood samples spiked with AP and MA at concentrations ranging from 5.0 to 2000 ng/ml (ng/g) were prepared and analyzed using the above procedure. In the case of hair, AP and MA were spiked at concentrations ranging from 0.25 to 10 ng/mg, and analyzed using the above procedure. The calibration curves were obtained by plotting the peak area ratio between the derivatives of AP and MA and that of MA-d_5 (I.S.). Reproducibility was evaluated by analyzing samples containing two different concentrations (200 and 1000 ng/ml in urine, 200 and 1000 ng/g in blood, 0.5 and 5.0 ng/mg in hair) of AP and MA on the same day in five replicates (intra-day reproducibility) and over five consecutive days in duplicates (inter-day reproducibility).

3. Results and discussion

3.1. Optimization of extraction parameters

In order to obtain acceptable precision and sensitivity for the determination of MA and AP in blood, extraction parameters optimized in our previous report were utilized [17]. The recoveries of the derivatives from blood in pH 10.5 were higher than other pH and the coefficients of variation were smaller. The extracted amounts of the derivatives were dependent on the added amount of propylchloroformate and were maximized at a concentration of 10 μl/ml. The recoveries of AP and MA from the spiked blood (1000 ng/g) in this method were 89.7 and 90.3% and the coefficients of variation were 2.69 and 1.90%, respectively.

Typical chromatograms extracted from urine are shown in Fig. 2, where no interference from endogenous substances was observed. The recoveries of AP and MA from the spiked urine (1000 ng/ml) in

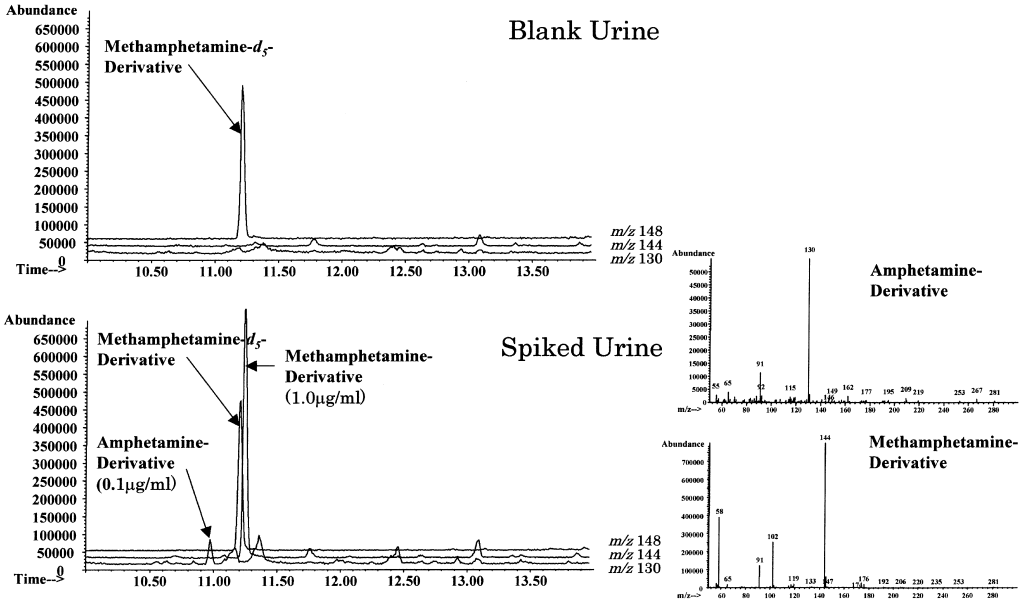

Fig. 2. Typical SIM chromatograms and mass spectra of the derivatives extracted from spiked and blank urines.

this method were 88.2 and 92.5% and the coefficients of variation were 1.84 and 1.28%, respectively.

Typical chromatograms extracted from hair are shown in Fig. 3, where no interference from endogenous substances was observed. The recoveries of

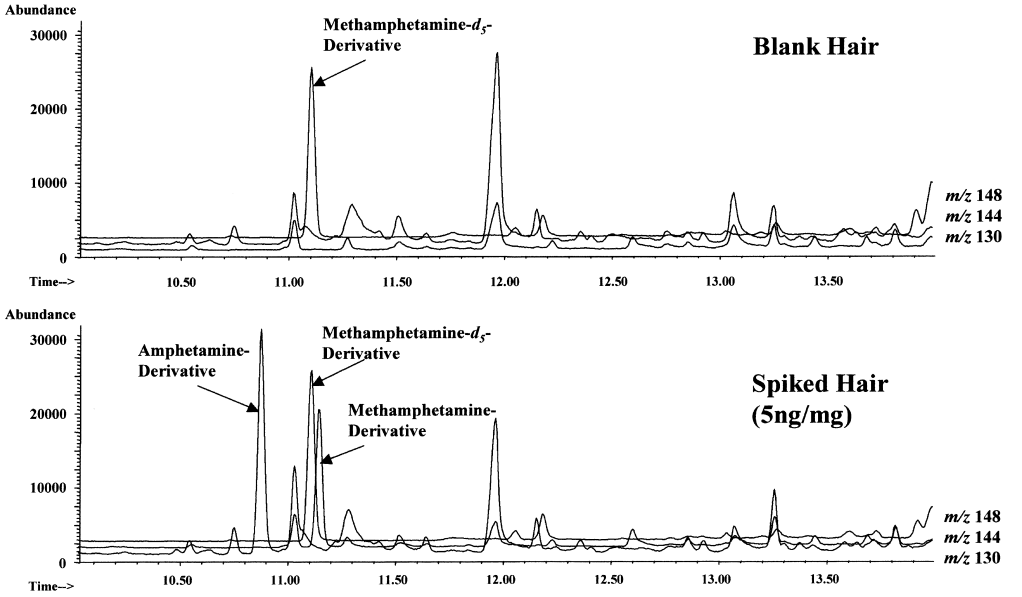

Fig. 3. Typical SIM chromatograms of the derivatives extracted from spiked and blank hairs.

AP and MA from the spiked hair (5 ng/mg) in this method were 89.7 and 90.3% and the coefficients of variation were 3.73 to 2.60%, respectively.

3.2. Calibration curves and reproducibility

The calibration curves showed linearity in the range of 12.5–2000 ng/ml (urine), 12.5–2000 ng/g (blood), and 0.25–10 ng/mg (hair) for AP and MA, respectively. The correlation coefficients of the calibration curves of AP and MA were 0.999 and 0.999, respectively. The limit of detection in urine, blood, and hair was 0.5 ng/ml, 0.5 ng/g, and 0.25 ng/mg, respectively (Table 1). This proposed method gave a substantial amount for detection of therapeutic levels. The intra-day and inter-day coefficients of variation for two different concentrations concentrations (200 and 1000 ng/ml in urine, 200 and 1000 ng/g in blood, 0.5 and 5.0 ng/mg in hair) were from 0.42 to 13.1%, respectively (Table 2). Considering the intra-day and inter-day coefficients of variation with the liquid–liquid or solid-phase extraction methods, the proposed method is acceptable.

3.3. Application of a medico-legal case

A 34-year-old man naked to the waist was found struggling on the back seat of a car at around 02:00 h

Table 2
Accuracy, inta- an inter-day precision for analysis of amphetamines

Sample	Intraday ($n=5$)[a] mean±SD	CV (%)	Interday ($n=10$)[b] mean±SD	CV (%)
Blood (ng/g)				
Amphetamine				
200	210±1.72	0.82	179±8.20	4.58
1000	1099±29.6	2.69	954±14.4	1.51
Methamphetamine				
200	211±0.89	0.42	191±1.72	0.90
1000	1105±21.0	1.90	988±11.8	1.20
Urine (ng/ml)				
Amphetamine				
200	202±1.37	0.68	204±2.75	1.35
1000	1188±21.9	1.84	1202±13.3	1.11
Methamphetamine				
200	222±8.00	3.60	216±4.00	1.85
1000	1075±13.8	1.28	1015±29.5	2.91
Hair (ng/mg)				
Amphetamine				
0.5	0.59±0.032	5.42	0.62±0.053	8.40
5.0	4.73±0.176	3.73	5.13±0.062	1.20
Methamphetamine				
0.5	0.52±0.022	4.27	0.56±0.073	13.1
5.0	5.04±0.131	2.60	4.92±0.184	3.73

[a] Intra-day precision analysis was performed on a single day of analysis ($n=5$).
[b] Inter-day precision analysis was performed over five consecutive days in duplicates ($n=10$).

Table 1
Quantitation limit and linearity of the method

Sample	Detection limit	Range of linearity	Linearity[a]	Correlation coefficient
Blood (ng/g)				
Amphetamine	5.0	12.5–2000	$y = 0.654x - 0.006$	0.999
Methamphetamine	5.0	12.5–2000	$y = 0.887x - 0.003$	0.999
Urine (ng/ml)				
Amphetamine	5.0	12.5–2000	$y = 0.856x - 0.013$	0.999
Methamphetamine	5.0	12.5–2000	$y = 0.882x - 0.008$	0.999
Hair (ng/mg)				
Amphetamine	0.25	0.25–10.0	$y = 0.018x - 0.008$	0.999
Methamphetamine	0.25	0.25–10.0	$y = 0.019x - 0.004$	0.999

[a] x are amounts of anaytes (ng/g, ng/ml, ng/mg), and y ar peak area ratios.

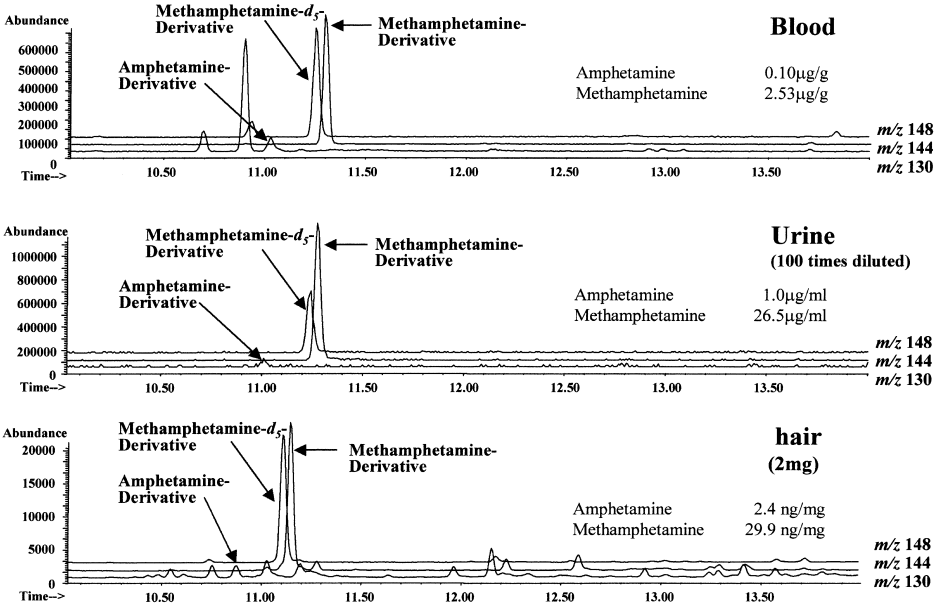

Fig. 4. SIM chromatograms of the derivatives extracted from blood, urine and hair of a medico-legal case.

on 23 August, and an ambulance was called. According to the rescue party, he had hyperpyrexia and his breath did not smell of drink. He was sent to a hospital and arrived at 03:10 h. On admission, his rectal temperature was 42 °C and he died at 05:35 h. The doctor suspected the cause of death was thermoplegia. In order to decide the cause of death, an autopsy of the deceased, with his father's consent, was performed at 13:50 h on the same day.

This proposed method was applied to blood, urine and hair samples of this medico-legal case. Typical EI-SIM chromatograms are shown in Fig. 4. A sharp and symmetrical peak of amphetamines was obtained without disturbance of endogenous interferences. Methamphetamine and its metabolite amphetamine were detected in the all samples collected in this medico-legal case. The concentrations of amphetamine and methamphetamine were 0.10 and 2.53 μg/g (blood), 1.0 and 26.5 μg/ml (urine), 2.4 and 29.9 ng/mg (hair), respectively.

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