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Short communication

Rapid simultaneous determination of ephedrines, amphetamines, cocaine, cocaine metabolites, and opiates in human urine by GC–MS

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Abstract

This paper presents a simple and sensitive chromatographic procedure for the simultaneous determination and quantification of ephedrines, amphetamines, cocaine, cocaine metabolites, and opiates in human urine by gas chromatography–mass spectrometry. This method involves enzyme hydrolysis in the presence of a deuterated internal standard, liquid–liquid extraction, and derivatization with pentafluoropropionic anhydride and pentafluoropropanol. The recovery of each compound averaged at 65.8% or more. The limits of detection determined for each compound by using a 2-mL sample volume ranged from 5 to 50 ng/mL. The calibration curves were linear to 1000 ng/mL for all compounds when determined using methamphetamine-d_4 and MDMA-d_5 as internal standards. This method was successfully applied for the analysis of urine samples suspected to contain intoxicants such as methamphetamine and heroin.

Keywords: Ephedrines; Amphetamines; Cocaine; Opiates; Gas chromatography–mass spectrometry; Urine

1. Introduction

Although the use of methamphetamine (MA) continues to be a serious problem in Japan, there has been a rapid increase in the smuggling and use of 3,4-methylenedioxymethamphetamine (MDMA), which is also known as Ecstacy, and 3,4-methylenedioxyamphetamine (MDA). Recently, heroin abuse has often been observed in Japan.

Urine is generally used for the screening of these abused drugs. The traditional approach involves the screening of urine by performing an immunoassay and the subsequent confirmation of presumptive positive samples by gas chromatographic–mass spectrometric (GC–MS) analysis. Various commercial immunoassay kits are available worldwide for the detection of abused drugs in urine. In Japan, Triage® is the kit that is most frequently used for immunoassay screening of abused drugs in urine because it enables the simultaneous screening of eight types of abused drugs.

Amphetamine (AM) and opiate are often identified as false positive by Triage®. For example, both ephedrine and pseudoephedrine cross-react with AM due to structural similarities. Further, dihydrocodeine cross-reacts with opiate due to the same reason. The Chinese herb Ma Huang contains ephedrine and pseudoephedrine, and it is widely used as a bronchodilator and is marketed as an over-the-counter (OTC) medication in Japan. Dihydrocodeine is used as a cough remedy, and it is also available as an OTC medication.

Identification and quantitation of abused drugs in urine are important aspects of emergency and forensic toxicology because they may provide crucial information in determining the cause of impairment and/or death. Sometimes, in forensic cases, urine cannot easily be sampled due to small volume. Therefore, simultaneous analysis is desirable in such situations.

Although the simultaneous determination of AM, MA, MDA, and MDMA is now a common practice [1–3], to the best of our knowledge, no reports are available on the simultaneous detection of these drugs, including ephedra alkaloids and opiates, in human urine.

The purpose of this study was to develop a method that simultaneously determines amphetamines, ephedra alkaloids,

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cocaine, cocaine metabolites, and opiates in human urine by using GC–MS.

2. Materials and methods

Dihydrocodeine phosphate was purchased from Sankyo Co. Ltd. (Tokyo, Japan). Ephedrine hydrochloride and methamphetamine hydrochloride were purchased from Dai-Nippon Pharmaceutical Co. (Osaka, Japan). Methylephedrine hydrochloride was purchased from Maruishi Pharmaceutical Co. (Osaka, Japan). Norephedrine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Pseudoephedrine hydrochloride was extracted from Sudafed® (Pfizer, NJ). Amphetamine hydrosulfate was kindly donated by Dr. Yoshida, Showa University. The Parker method was used to synthesize MDA from 3,4-methylenedioxybenzaldehyde (i.e., Piperonal; Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) and nitroethane (Wako Pure Chemical Industries Ltd., Osaka, Japan) [4]. The Braun method was used to synthesize 3,4-methylenedioxyethylamphetamine (MDEA) from piperonyl methyl ketone (Tokyo Kasei Kogyo Co. Ltd.) and ethylamine hydrochloride (Tokyo Kasei Kogyo Co. Ltd.) by using sodium cyanoborohydride (Wako Pure Chemical Industries Ltd.) [5]. MDMA was prepared by the *N*-methylation of MDA according to Fitzgerald's method [6]. The deuterated compound methamphetamine-d_4 was synthesized from benzyl magnesium chloride and *N*-methylethyleneimide-d_4 by using the Nakahara method [7]. MDMA-d_5 was synthesized using lithium aluminum-d_3, similar to the synthesis of MDMA that has been described above. Cocaine hydrochloride was purchased from Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). Ecgonine was synthesized by refluxing cocaine and dilute hydrochloric acid, and ecgonine methyl ester was prepared by the methylation of ecgonine. Morphine hydrochloride was purchased from Dai-Nippon Sumitomo Pharmaceuticals Co. Ltd. (Osaka, Japan), and codeine phosphate was purchased from Sankyo Co. Ltd. Diacethylmorphine obtained by reacting morphine with acetic anhydride was used to synthesize 6-monoacetylmorphine. All synthesized compound's purity were checked by thin-layer, gas chromatography and GC–MS. A 6-monoacetylmorphine contained 0.5% of morphine but no other impurities were detected by these methods. All other solvents (Wako Pure Chemical Industries Ltd.) were of the analytical grade. Glucuronidase (type HP-2; Sigma) was used for the hydrolysis process. Pentafluoropropionic anhydride (PFPA; GL Sciences Inc., Tokyo, Japan) and 2,2,3,3,3-pentafluoro-1-propanol (PFPOH; Aldrich Milwaukee, WI) were used for the derivatization process.

2.1. Extraction

Urine (2.0 mL) was added to each silanized tube after the initial addition of 200 ng each of MA-d_4 and MDMA-d_5, which were used as internal standards. Subsequently, 0.1 mL of glucuronidase was added after each sample was adjusted to pH 5.0 by the addition of acetic acid. The tubes were capped, incubated for 2 h at 45 °C, cooled to room temperature, and then centrifuged at 3000 × *g* for 10 min. The supernatant was transferred to another tube and adjusted to pH 9.0 by the addition of 10% NH_4OH solution. After adding 5 mL of a diethyl ether:chloroform (4:1) mixture, the tubes were shaken and centrifuged. The upper organic layer was transferred to another tube, and 30 μL of acetic acid was added to it. The organic layer was evaporated under a stream of nitrogen.

2.2. Derivatization

The residue was derivatized at 70 °C for 40 min by using 100 μL of PFPA and 70 μL of PFPOH. The samples were cooled to room temperature and then evaporated to dryness under a stream of nitrogen. Finally, the samples were reconstituted with 30 μL of ethyl acetate, and a 1-μL aliquot was injected into the injection port.

2.3. GC–MS analysis

All the samples were analyzed on a Hewlett Packard model 5890 series II gas chromatograph equipped with a Hewlett Packard model 5971 mass selective detector. The chromatographic conditions were as follows: HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness); column temperature program (100 °C for 3 min with 20 °C/min ramp to a final temperature of 300 °C; 3 min hold). The temperatures of the injection port and mass selective detector interface were set at 250 and 280 °C, respectively.

2.4. Stability

Drug-free urine was spiked at three quality control concentrations (70, 500, and 900 ng/mL) without morphine at 500 and 900 ng/mL. Aliquots were prepared from each pool. Four aliquots of each pool were selected and tested by GC–MS. The remaining aliquots were stored at either room temperature, 4–5 °C, or −25 to −30 °C for 2 months. Four aliquots in each of the storage groups were removed and analyzed using the GC–MS method described previously.

2.5. Application

A 28-year-old man was found dead in his home, lying in a living room in which a syringe was discovered near the body. Heroin was detected in this syringe. He was not known to be an MA and/or Heroin addict. Moreover, the exact intake was not documented in police report. At the autopsy, several needle marks were noted. And, no particular morphological changes were noted. AM and opiate were detected by Triage® using urine. According to the Police report, about 24 h elapsed between the supposed moment of heroin inject and specimen collection. Autopsy consent got from his family.

3. Results and discussion

Conditions for enzymatic hydrolysis were optimized using a urine sample obtained from a healthy volunteer who was taking

dihydrocodeine. Absolute recoveries could not be determined due to the unavailability of dihydrocodeine–glucuronide and 6-acetylmorphine–glucuronide conjugate standards. The following experimental parameters were optimized: enzyme activity, hydrolysis pH, hydrolysis temperature, and incubation time. In the enzyme activity experiments, 2-mL aliquots of urine were adjusted to pH 5.0 and incubated at 45 °C for 2 h with increasing amounts of β-glucuronidase (0, 2500, 5000, 7500, and 10000 U). Optimal results were obtained on the addition of 5000 U β-glucuronidase, and this activity was maintained at a constant value during all the subsequent experiments. In the hydrolysis pH experiments, the urine sample was buffered to pH 4.5, 5.0, and 5.5 by using acetic acid. Enzymatic hydrolysis was performed for 2 h at 20 °C (room temperature), 37 and 45 °C. Optimal recovery was obtained at pH 5.0, and the optimum temperature was observed to be 45 °C. The samples were incubated for various time intervals, namely, 0.5, 1, 2, 4, 6, 8, and 16 h at pH 5.0 and 45 °C. Optimal recovery was obtained after 2 h of incubation. Varying the pH of the urine sample significantly influenced the recovery of abused drugs, even under alkaline conditions. The optimum extraction pH was found to be 9.0.

We then evaluated the applicability of several reported liquid–liquid extraction procedures for the extraction of amphetamines, ephedra alkaloids, cocaine, cocaine metabolites, and opiates from urine. The procedure involving the use of ethyl acetate extracts was not clean and was time-consuming with respect to solvent evaporation. Although extraction with the diethyl ether:chloroform (4:1) mixture yielded a low recovery of all the compounds than extraction with ethyl acetate, the process exhibited rapid evaporation and clean derivatization.

Table 1
Retention times and selected ions for GC–MS identification and quantitation

Compound	Retention time (min)	*m/z*[a]
MEP	6.75	72, **134**, 162
AM	6.77	91, 118, **190**
NEP	7.16	119, **190**, 280
EP	7.67	119, 160, **204**
MA-d_4	7.67	122, 160, **208**
MA	7.75	118, 160, **204**
PEP	8.12	119, 160, **204**
EME	8.17	82, **182**, 345
MDA	9.28	**135**, 162, 325
MDMA-d_5	10.02	163, **208**, 344
MDMA	10.05	162, **204**, 339
MDEA	10.33	162, **218**, 353
COC	12.87	82, **182**, 303
MOR	13.10	119, 207, **414**
DHCD	13.34	185, 284, **447**
COD	13.36	178, 445, **284**
6-MAM	13.72	204, 473, **414**

Nonstandard abbreviations: MEP, methylephedrine; AM, amphetamine; NEP, norephedrine, EP, ephedrine; MA-d_4, methamphetamine-d_4; MA, methamphetamine; PEP, pseudoephedrine; EME, ecgonine methylester; MDA, 3,4-methylenedioxyamphetamine; MDMA-d_5, 3,4-methylenedioxymethamphetamine-d_5; MDMA, 3,4-methylenedioxymethamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; COC, cocaine; MOR, morphine; DHCD, dihydrocodeine; COD, codeine; 6-MAM, 6-monoacetylmorphine.

[a] Ions that were selected for quantitation have been represented in boldface.

The retention times and monitored ions for particular compounds are listed in Table 1. The chromatogram of the GC–MS is presented in Fig. 1. The total ion current (TIC) and extracted ions in the GC–MS chromatograms were obtained by the single

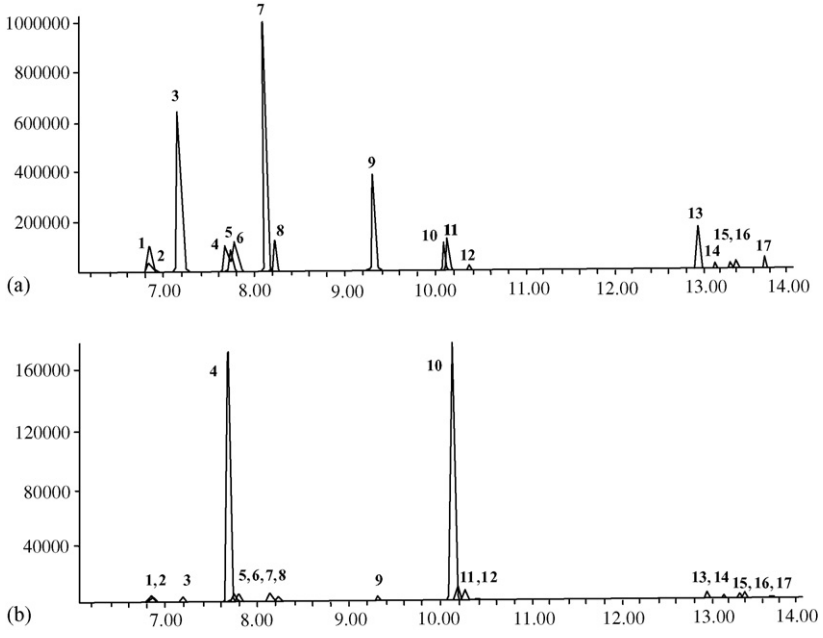

Fig. 1. (a) The GC–MS–SIM chromatogram for the PFA derivatization of the following compounds: (1) methylephedrine (1 μg/mL), (2) amphetamine (1 μg/mL), (3) norephedrine (1 μg/mL), (4) methamphetamine-d_4 (100 ng/mL), (5) ephedrine (1 μg/mL), (6) methamphetamine (1 μg/mL), (7) pseudoephedrine (1 μg/mL), (8) ecgoninemethylester (1 μg/mL), (9) 3,4-methylenedioxyamphetamine (1 μg/mL), (10) 3,4-methylenedioxymethamphetamine-d_5 (100 ng/mL), (11) 3,4-methylenedioxymethamphetamine (1 μg/mL), (12) 3,4-methylenedioxyethylamphetamine (1 μg/mL), (13) cocaine (1 μg/mL), (14) morphine (1 μg/mL), (15) dihydrocodeine (1 μg/mL), (16) codeine (1 μg/mL), and (17) 6-monoacetylmorphine (1 μg/mL). (b) The GC–MS–SIM chromatogram for the PFA derivatization of all compounds at LOQ concentrations without IS (100 ng/mL).

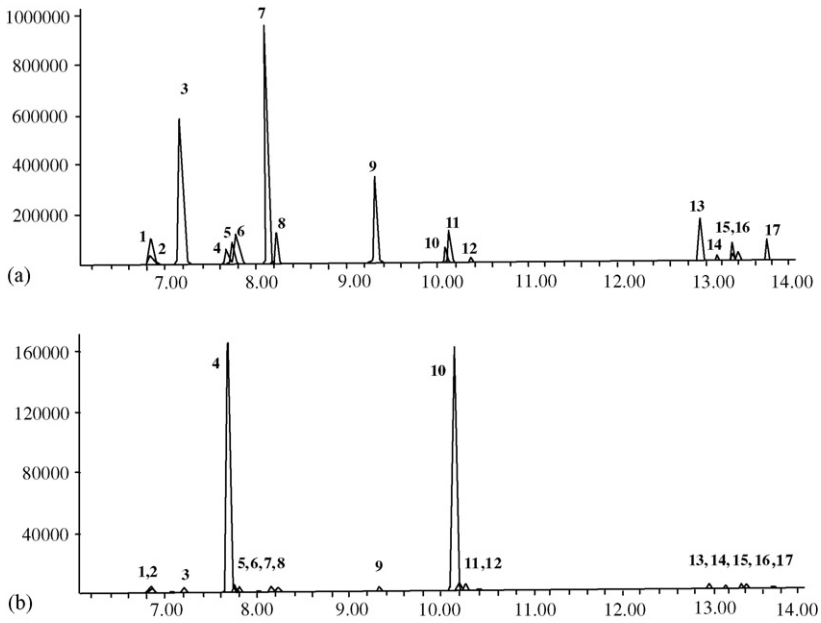

Fig. 2. (a) The GC–MS–SIM chromatogram for a blank urine sample spiked with the standard solution of each compound and IS. Blank urine (2.0 mL) spiked with a solution of 1 μg/mL (urine concentration; 500 ng sample/mL of urine) was extracted using the method described in the text. (b) The extracted GC–MS–SIM chromatogram for a blank urine sample spiked with all compounds at LOQ levels without IS (100 ng/mL).

ion monitoring (SIM) of a spiked urine reference sample that showed sharp peaks and good chromatographic separation of all compounds, except methylephedrine and AM. The identification was based on the base peak ions, fragment ions, and retention times. Since both methylephedrine and AM showed different base peak ion values (*m/z* 134 and 190, respectively) on monitoring, chromatographic separation achieved by GC–MS did not present any problems in identification (Fig. 2).

Determination of all drugs in urine was validated by GC–MS, and the following results were obtained.

3.1. Validation results

Calibration by internal standardization was performed using linear regression employing 1/*x* weighting. Peak area ratios of target compounds and their respective internal standards were calculated for each standard curve. At least six analyte concentrations were used for each standard curve.

The limit of detection (LOD) and limit of quantitation (LOQ) of the method were determined by analyzing three levels of samples in triplicate and spiked with each drug, except morphine, at concentrations ranging from 2.5 to 1000 ng/mL. The calibration curves were prepared by adding each compound (2.5, 12.5, 25, 50, 75, 100, 250, 600, and 1000 ng/mL) to a blank urine sample. LOD was determined as the lowest concentration at which all replicates produced results for the qualifying ion ratios of all compounds within acceptable limits (±20% of the calibrator ratios). LOQ was determined as the lowest concentration at which the qualifying ion ratios of the analyte were within the 20% limit established by calibration, and the determined con-

Table 2
Validation parameters for the target drugs

Compound	Internal standard	LOD (ng/mL)	LOQ (ng/mL)	Linearity	Regression line
MEP	MA-d_4	25	50	50–1000	$y = 0.0003x - 0.0211$
AM	MA-d_4	12.5	50	50–1000	$y = 0.0015x + 0.0531$
NEP	MA-d_4	5	12.5	12.5–1000	$Y = 0.0018x - 0.1845$
EP	MA-d_4	5	12.5	12.5–1000	$y = 0.0019x - 0.0132$
MA	MA-d_4	12.5	25	25–1000	$y = 0.0066x - 0.05$
PEP	MA-d_4	12.5	12.5	12.5–1000	$y = 0.0127x - 0.7334$
EME	MA-d_4	12.5	50	50–1000	$Y = 0.0003x - 0.0011$
MDA	MDMA-d_5	12.5	50	50–1000	$y = 0.0012x - 0.0256$
MDMA	MDMA-d_5	50	100	100–1000	$y = 0.0048x - 0.0745$
MDEA	MDMA-d_5	50	100	100–1000	$y = 0.0062x - 0.0696$
COC	MDMA-d_5	50	100	100–1000	$y = 0.0011x - 0.0359$
MOR	MDMA-d_5	250	250	250–1000	$y = 0.0002x - 0.005$
DHCD	MDMA-d_5	50	75	75–1000	$y = 0.0001x - 0.0038$
COD	MDMA-d_5	50	100	100–1000	$y = 0.0001x - 0.0048$
6-MAM	MDMA-d_5	50	100	100–1000	$y = 0.0001x - 0.0048$

Table 3
Extraction recovery, intermediate precision, and accuracy

Analyte	Relative recovery (%)			Intermediate precision (R.S.D.%)			Accuracy (bias%)		
	70 ng/mL	500 ng/mL	900 ng/mL	70 ng/mL	500 ng/mL	900 ng/mL	70 ng/mL	500 ng/mL	900 ng/mL
MEP	89.6	92.3	88.5	6.3	7.2	7.9	6.2	3.2	−3.4
AM	97.6	95.3	93.2	2.4	3.9	5.0	6.8	3.6	−2.3
NEP	87.5	88.2	90.7	5.6	8.3	7.0	5.7	0.3	7.1
EP	79.4	85.2	90.2	11.3	10.6	8.1	3.5	0.4	−7.5
MA	93.4	92.8	93.8	6.1	5.6	4.2	2.4	0.8	5.9
PEP	94.6	95.1	97.8	4.8	5.5	6.9	3.5	1.7	5.6
EME	84.3	87.6	85.4	7.9	6.6	10.0	3.5	4.6	12.3
MDA	85.9	88.3	92.8	4.3	7.9	7.8	4.2	−3.3	−8.8
MDMA	88.4	92.2	95.1	3.4	5.2	6.6	5.6	−6.1	6.3
MDEA	90.3	88.4	92.5	5.6	4.7	10.3	4.4	3.1	9.4
COC	88.6	89.7	91.2	5.2	8.4	9.8	2.7	3.5	6.7
MOR		71.9	80.3		7.1	8.7		2.5	−5.2
DHCD	74.7	77.2	80.3	8.3	7.7	8.9	4.6	3.7	7.2
COD	70.5	69.1	73.6	9.2	8.4	11.7	2.8	5.3	12.6
6-MAM	65.8	73.2	75.3	8.4	7.0	12.5	−14.1	8.4	−5.3

Relative recovery was determined by comparing the results of urine samples spiked with each drug and extracted using the method described with those of total samples that were prepared by adding drugs directly to the extraction solvent. Intermediate precision was determined by analyzing individually prepared spiked samples for 5 consecutive days. All the results were validated using urine samples that were individually spiked with each compound.

centration was within ±20% of the expected concentration. The determined LOD and LOQ are listed in Table 2.

The limit of linearity was established by analyzing increasing concentrations of the target compounds until one or more of the qualifying ion ratios exceeded the 20% limit or the determined concentration was greater than ±20% of the expected concentration. The calibration curves were linear to 1000 ng/mL for all compounds (Table 2), and the mean results were within 20% of the expected concentration. The correlation coefficient (r^2) was >0.998 for all compounds.

The relative recoveries were determined by comparing the peak areas of the extracted urine sample with the peak areas of an unextracted solution at the same concentration. The analysis was carried out in six replicates at three quality control concentrations without morphine. A majority of drugs exhibited relative recoveries between 65.8 and 97.8 (Table 3). The method showed good accuracy throughout the calibration range for each quality control concentration.

Relative standard deviation (R.S.D.%), time-different intermediate precision (R.S.D.%), and accuracy (bias%) are listed in Table 3. The repeatability of this method was determined at three calibrator concentrations by analyzing six replicate samples of spiked blank urine that were spiked at three calibrator concentrations. In all cases, the R.S.D. was below 15%, and the accuracy, which was measured against the relative error, was well within the acceptable limits.

The stability of study samples was analyzed by GC–MS at 1 and 2 weeks after preparation. A significant loss of 6-monoacetylmorphine was observed in the short-term temperature stability experiment at room temperature. Other stability experiments indicated almost stable the storage temperature to 4–5 °C or the freezer temperature. Moreover, calibrator and quality control samples were stable for at least 6 months at −25 to −30 °C.

If heroin abuse is suspected, 2 mL urine is ideal for the analysis of 6-monoacetylmorphine by using this method. However, in the case of AM analysis or a highly concentrated sample, a lesser volume of urine is required.

In order to evaluate the method developed in this study by using real samples collected from forensic autopsy and emergency critical care cases involving the use of AMP and/or opiate, we analyzed the urine samples that tested positive on using the Triage® kit in the past 6 months. No analytical or chromatographic errors were encountered, thereby demonstrating the

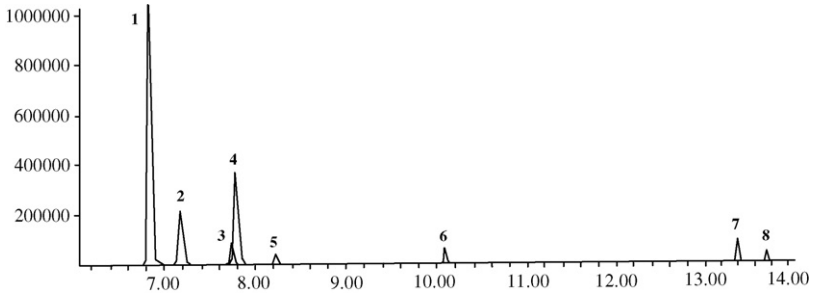

Fig. 3. The GC–MS–SIM chromatogram of a urine extract. Peak identification: (1) amphetamine (6.1 μg/mL, quantitated by diluted urine (1:10)); (2) norephedrine (314 ng/mL); (3) methamphetamine-d_4 (IS); (4) methamphetamine (3.2 μg/mL, quantitated by diluted urine (1:10)); (5) pseudoephedrine (406 ng/mL); 6, 3,4-methylenedioxymethamphetamine-d_5 (internal standard); (7) codeine (745 ng/mL); (8) 6-monoacetylmorphine (860 ng/mL).

robustness of this procedure. Currently, this method is being routinely used in our laboratory for the confirmation of presumable AMP- and/or opiate-positive urine samples.

3.2. Applicability

The present method has been used to quantify the concentration of AM, norephedrine, MA, pseudoephedrine, codeine, and 6-monoacetylmorphine in urine (Fig. 3). In the light of high concentration of AM, he mainly abused MA. Probably, following injection of heroin, 6-MA and codeine concentrations increased until death. These findings demonstrated the usefulness of the simultaneous analysis of urine from MA and heroin abusers. Most cases do not require quantitative analysis of urine sample, if urine drug screening was judged only immunoassay using cut-off concentration. However, causative drug should be identified for positive results by instrument. Moreover, important information will be acquired particularly pharmacokinetics by quantitative analysis. The concentration of metabolite has a close relationship with the intake of parent compound. However, the relationship of parent compound and metabolites concentration in urine has yet been clearly defined. Quantitative analysis of urine sample, although absolutely, are definitely worthwhile.

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References

[1] P.R. Stout, C.K. Horn, K.L. Klette, J. Anal. Toxicol. 26 (2002) 253–261.
[2] B.D. Paul, J. Jemionek, D. Lesser, A. Jacobs, D.A. Searles, J. Anal. Toxicol. 28 (2004) 449–455.
[3] Z. Huang, S. Zhang, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 792 (2003) 241–247.
[4] M.A. Parker, D. Marona-Lewicka, D. Kurrasch, A.T. Shulgin, D.E. Nichols, J. Med. Chem. 41 (1998) 1001–1005.
[5] U. Braun, A.T. Shulgin, G. Braun, J. Pharm. Sci. 69 (1980) 192–195.
[6] R.L. Fitzgerald, R.V. Blanke, R.A. Glennon, M.Y. Yousif, J.A. Rosecrans, A. Poklis, J. Chromatogr. 490 (1989) 59–69.
[7] Y. Nakahara, K. Takahashi, M. Shimamine, Y. Takeda, J. Forensic. Sci. 36 (1991) 70–78.