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Simultaneous supercritical fluid extraction and chemical derivatization for the gas chromatographic–isotope dilution mass spectrometric determination of amphetamine and methamphetamine in urine

Sheng-Meng Wang[a], Yun-Seng Giang[a,*], Yong-Chien Ling[b]

[a]*Department of Forensic Science, Central Police University, Kueishan, Taoyuan 33334, Taiwan*
[b]*Department of Chemistry, National Tsing Hua University, Hsinchu 30043, Taiwan*

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Abstract

An in-situ supercritical fluid extraction (SFE) and chemical derivatization (ChD) procedure followed by gas chromatography–isotope dilution mass spectrometry (GC–MS) for the determination of amphetamines in urine is described and evaluated. While using celite as the SFE wet-support, the one-pot sample pretreatment procedure also employs ammonium water to alkalize the urine matrix that contains protonated amphetamine (AP) and methamphetamine (MA). The mean recoveries achieved by simultaneous SFE–ChD, i.e., 95% (RSD=3.8%) for AP and 89% (RSD=4.0%) for MA, are significantly better than the corresponding overall recoveries obtained upon stepwise SFE–ChD, suggesting the unreacted trifluoroacetic anhydride (TFA) in the former procedure has strengthened the extracting power of CO_2 fluid as has been evidenced by a control test. As to GC–MS analysis, the optimal qualitative ions and quantitative ions of the respective analytes were determined via a rigorous evaluation process. Thus, the regression calibration curves for AP and MA in urine are linear within 100~50 000 ng/ml, with correlation coefficients typically exceeding 0.999. The limits of detection determined by two methods for AP and MA vary from 19 to 50 ng/ml, and limits of quantitation from 21 to 100 ng/ml. Precisions calculated for the triplicate analyses of AP and MA in a 500-ng/ml spiked control, two real-case samples and two quasi real-case samples, respectively, using regression calibration are typically below 10%. The method is simple and reliable. It may serve as an alternative to the existing confirmatory protocol for forensic urine drug testing.

Keywords: Supercritical fluid extraction; Chemical derivatization; Amphetamine; Methamphetamine

1. Introduction

Over the past decade there has been a tremendous increase in the use of supercritical fluid extraction (SFE) as a sample preparation method. The simplicity, speed (benefiting from the high diffusing ability and low viscosity of the fluid), high selectivity (through the adjustments of fluid's pressure and temperature), high efficiency, low cost, solvent saving, and being non-toxic have made this technique highly acclaimed for the recovery of drugs [1–10], explosives [11–

*Corresponding author.
E-mail address: ysgiang@sun4.cpu.edu.tw (Y.-S. Giang).

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14], pesticides [15–17], polychlorobiphenyls and dioxins [18–26], caffeine [27], fire accelerants [28–33], and many other organic and organomettallic compounds [34–38] from a variety of matrices, both biological and non-biological, and both solid and liquid.

While there are several reports in the literature on the simultaneous processing of SFE and chemical derivatization (ChD) [39,40,27], none of them deal with the analysis of abused drugs such as amphetamine (AP) and methamphetamine (MA), and none of them explore liquid samples such as urine. Moreover, unlike most of the ordinary organic analysis where the analytes are in their free-molecule form, urinary AP and MA, however, may exist as protonated ionic form. From the viewpoint of academic or basic research, we wish to make a little modification on the operation procedure of the existing simultaneous SFE–ChD approach so as to broaden its spectrum of application. On the other hand, our previous unpublished work on the analysis of aqueous hair samples has shown simultaneous SFE–ChD followed by gas chromatography–isotope dilution mass spectrometry (GC–MS) using selective ion monitoring (SIM) to be suitable for the recovery and analysis of trace amount of amphetamines. It was that experience that further prompted us to conduct the present study so that a promising alternative to the currently prevalent protocol, i.e., sequential solid-phase extraction (SPE) and ChD followed by GC–MS, for forensic urine drug testing may be provided.

Criteria adopted for conclusive drug identification and quantification require the appearance of the monitored ions at correct retention times with acceptable intensity ratios among these ions. The retention times and ion intensity ratios observed in the test sample are compared with those established by an (or a set of) authentic calibration standard(s) incorporated in the same analytical batch [41]. Considering the fact that the respective analytes and internal standards (I.S.s) will all go through the proposed chemical processes and undertake the isotope dilution SIM GC–MS analysis, the quality of the results relies on a sound sample pretreatment (including the simultaneous SFE–ChD), an effective GC–MS analytical methodology, and a critical data evaluation process. The important roles of the I.S.s, ChD agent, and selected monitoring ions in the overall analytical process cannot be over-emphasized. Although some related studies on various I.S.s, ChD agents and sample preparation procedures have previously been reported for the urinalyses of amphetamines [41], it is still worthwhile to conduct another detailed evaluation on the effectiveness of the newly proposed I.S.s, ChD agent and selected ions so that another quality drug urinalysis may be achieved.

2. Experimental

2.1. Materials

Racemic *d,l*-methamphetamine (MA; in free-base form) in methanol, *d,l*-amphetamine (AP; in free-base form) in methanol, *d,l*-methamphetamine-d_8 (MA-d_8; in free-base form) in methanol and *d,l*-amphetamine-d_8 (AP-d_8; in free-base form) in methanol were purchased from Radian (USA); ammonium hydroxide (28.0–30.0%) from J.T. Baker (USA); trifluoroacetic anhydride (TFA) from Ferak Berlin (Germany); ethyl acetate (EA) from Fisher (USA); liquid carbon dioxide from Scott Specialty Gases (USA); celite from ISCO (USA). All of the above agents were directly used without further purification.

Trifluoroacetylamphetamine (AP-TFA) and trifluoroacetylmethamphetamine (MA-TFA) were used as the I.S.s for the calculation of: (1) ChD yields of MA and AP, respectively; (2) SFE recoveries of MA-TFA and AP-TFA, respectively; and (3) simultaneous SFE–ChD recoveries of MA and AP, respectively. AP-TFA was prepared as follows: To a reaction vial containing 0.5 g of *d,l*-AP (neat liquid in free-base form; Sigma) was added 3 ml of TFA. The reaction mixture in the sealed vial was incubated at 80°C for 18 h and then cooled to room temperature. The crude TFA derivative was purified by purging with dry nitrogen followed by vacuuming to dryness. The resulting residue was identified as AP-TFA of 99.43% purity based on its GC–MS total ion chromatogram. Likewise, MA-TFA of 99.98% purity was prepared using 0.5 g of *d,l*-MA (neat liquid in free-base form; Sigma).

2.2. Sample preparation

For establishing the calibration curves, free-base amphetamines were used instead of their salts (AP·H_2SO_4 and MA·HCl). So doing would not affect the effectiveness of the salt-oriented procedure described below or the final results. An appropriate amount (1, 5, 10, 50 μl for 100 μg/ml solution; 10, 50 μl for 1000 μg/ml solution) of AP–MA binary standard solution and 20 μl of 100 μg/ml AP-d_8–MA-d_8 binary I.S. solution were added to 1 ml of blank urine. This would make the corresponding spikes contain 100, 500, 1000, 5000, 10 000 and 50 000 ng, respectively, of both AP and MA, and 2000 ng of both AP-d_8 and MA-d_8. For the analyses of real-case samples, 20 μl of the AP-d_8–MA-d_8 solution was added to 1 ml of test urine. A ca. 200-μl portion of ammonium hydroxide was then added to alkalize the urine (pH 10–12). The resulting solution was transferred to a 2.5-ml extraction cell that had previously been filled with celite. The cell was subjected to vacuum to remove methanol (the solvent used for the standard solutions), water (from urine), and other volatiles or semi-volatiles (from urine). This drying step took about 3 h. To save time, it is advisable to run a number of samples at a time.

To each dry sample was added 100 μl of TFA. The mixture was subjected to simultaneous SFE and ChD. This step utilized an ISCO SFX 220 SFE system equipped with an SFX 220 extractor, an SFX 200 controller and a 260D syringe pump, and was performed at two stages. Stage 1: simultaneous static SFE and ChD under 4000 p.s.i. at 90°C for 5 min (1 p.s.i.=6894.76 Pa). Stage 2: dynamic elution with 12.5 ml of supercritical carbon dioxide using a variable restrictor to keep the flow-rate at 1 ml/min. The eluent was trapped with 5 ml of EA and concentrated to 200 μl by purging with nitrogen gas. A 1-μl aliquot of this solution was injected for each GC–electron impact (EI) MS analysis.

2.3. Gas chromatography–mass spectrometry

GC–MS analyses were carried out using a Hewlett-Packard HP-5890 Series II gas chromatograph coupled to an HP-5971 Series mass-selective detector. The column used was a DB-5 capillary column (30 m×0.25 mm I.D., 0.33 μm film thickness). The GC system was operated in the splitless mode (i.e., purge off) while performing injection, but 1 min later the purge valve was turned on. The injector temperature was 250°C. The column temperature was programmed from 100 to 280°C at 10°C/min, with the final temperature held for 12 min. Helium was used as the carrier gas at a flow-rate of 1 ml/min. Effluents from the GC column was transferred via a transfer line held at 280°C to a 70 eV EI ionization source held at 180°C.

Having conducted a detailed preliminary study as that described by Liu for the quantitative determination of pentobarbital [41], we performed formal GC–MS analyses of the present analytes using the SIM mode accompanied by extracted ion chromatograms (EICs). The calibration curves were produced by plotting the peak-area ratio (analyte:I.S.) against the concentration of the appropriate analyte in the fortified samples. The peak-area ratio used was the mean of triplicate analyses.

3. Results and discussion

3.1. Selection of qualitative and quantitative ions

Anhydrous TFA was chosen as the ChD agent throughout this study for three reasons [41–45]: (1) it is reportedly reactive and convenient towards amphetamines; (2) TFA derivatized analytes will yield more abundant fragment ions, provide more structural information, and offer stronger GC–MS signals than their underivatized counterparts benefiting from their higher electron affinities; and (3) the shifting of characteristic peaks such as the base peak from otherwise non-discriminating low-mass region to higher but known masses in the MS spectra will facilitate the discrimination of the analytes. On the other hand, it has generally been recognized that GC resolution between AP (or MA) and its deuterium-labeled analog will not be satisfactory unless the analog is labeled with more than nine deuterium atoms. Being more available, however, d_8-labeled I.S.s instead of d_9-I.S.s were used throughout the present study. This along with the demand of rapid analysis in practice led to inadequate GC separation

between AP-d_8-TFA and AP-TFA and between MA-d_8-TFA and MA-TFA even if in the absence of other analytes or impurities. To secure the accuracy and precision of the analysis, it is therefore necessary to properly select the so-called ''qualitative ions'' and hence the ''quantitative ions'' by minimizing their cross-interference or so-called ''cross-contribution''. This evaluation process was performed according to that described by Liu for the quantitative determination of pentobarbital [41]. Thus, the three qualitative ions for AP-d_8-TFA were determined to be m/z 143, 126 and 96; those for AP-TFA, m/z 140, 118 and 91; those for MA-d_8-TFA, m/z 161, 122 and 113; those for MA-TFA, m/z 154, 118 and 110. The quantitative ions for AP-d_8-TFA, AP-TFA, MA-d_8-TFA and MA-TFA were m/z 143, 140, 161 and 154, respectively. These selected ions were all in agreement with those previously predicted through the full-scan mass spectra analyses; that is, AP, AP-d_8, MA, MA-d_8, and their TFA derivatives all underwent the same, yet regiospecific, type of major fragmentation, i.e., β-cleavage.

3.2. efficiency of simultaneous supercritical fluid extraction and chemical derivatization

In order to achieve an immunity to a wide variety of chemical and physical interferences and to improve the quantitative analytical quality, isotope-dilution method was employed in this study. As is described in Section 2.2, known amounts of d_8-labeled I.S.s were routinely added to the urine sample prior to performing the simultaneous SFE–ChD. Nevertheless, it is informative to have insight into the actual efficiency of the combined SFE–ChD. This, in turn, necessitated that the present study be started with the optimization of the experimental conditions of simultaneous SFE–ChD, which was most easily realized by employing Taguchi's method [46]. Based on our previous experience in related work and those experimental conditions reported by other researchers for separate SFE and ChD [1–40], we chose pressure, temperature, time given for static SFE, and amount of TFA added as the four factors to be investigated. The three levels set for the pressure were 3000, 4000 and 5000 p.s.i.; those for the temperature, 70, 80 and 90°C; those for the time-length of static SFE, 5, 12.5 and 20 min; those for the amount of TFA, 5, 100 and 200 μl. The three-level L_9 (3^4) orthogonal table used for the optimization process and the corresponding recoveries of AP-TFA and MA-TFA obtained under the nine candidate conditions are displayed in Table 1. Apparently, entry VI tops all the other eight entries with regard to the recoveries of the TFA derivatives, thus leading to the selection of entry VI's levels as the optimal conditions for routine use (as are described in Section 2.2). This manner of selection of optimal

Table 1
The L_9 (3^4) orthogonal table used for the optimization of the experimental conditions of simultaneous SFE–ChD (the left five columns) and the respective recoveries[a,b] of AP-TFA and MA-TFA resulting from the nine candidate conditions

Entry	Pressure (p.s.i.)	Temperature (°C)	Time for static SFE (min)	Amount of TFA (μl)	Recovery (%)					
					AP-TFA			MA-TFA		
					Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
I	3000	70	5	5	1.69	2.17	1.93	0.25	0.23	0.68
II	3000	80	12.5	100	40.07	25.83	25.10	9.20	14.79	16.08
III	3000	90	20	200	44.65	56.72	43.45	22.71	21.52	24.55
VI	4000	70	12.5	200	42.48	58.17	46.34	12.49	26.69	54.43
V	4000	80	20	5	2.41	1.69	2.90	0.20	0.38	0.27
VI	4000	90	5	100	92.93	95.10	93.17	99.74	91.40	82.86
VII	5000	70	20	100	65.84	78.36	83.78	14.73	17.34	6.80
VIII	5000	80	5	200	64.69	55.52	58.41	57.19	54.34	48.28
IX	5000	90	12.5	5	0.60	0.50	0.52	0.33	0.18	0.34

[a] All recoveries were calculated based on using 10 μl of 1000-μg/ml sample solution and 10 μl of 100-μg/ml I.S. solution.
[b] Trifluoroacetylamphetamine (AP-TFA) was used as the I.S. for the recovery calculation of MA-TFA, and trifluoroacetylmethamphetamine (MA-TFA) as that of AP-TFA.

conditions and hence the achievement of optimal ''quality'' of recovery data was also justified by two preliminary processes. First, when the ANOVA (i.e., analysis of variance) was applied to the data, the *F*-test at 95% level of confidence indicated that the four factors stated above were all prominent; that is, there were always significant differences among the nine means of recoveries. The most influential factor was the amount of TFA added, with the *F* values being 268.5 for AP-TFA and 39.3 for MA-TFA, compared to $F_{0.05}=3.55$. Furthermore, by ''optimal quality of recovery data'' we meant to maximize the appropriate recoveries. When the four factors were set at their respective optimal levels, it was found that each of the factors did result in the maximized sum of ''iso-level'' recoveries. Taking AP-TFA as an example, the four maximized sums are 435.19% for 4000 p.s.i., 427.64% for 90°C, 465.61% for 5 min, and 600.18% for 100 μl; all are far larger than their second largest counterparts. Here, once again, the amount of TFA added appears to be the most influential factor. As the second preliminary procedure to justify the selection of the above four optimal levels, the so-called ''response tables'' in terms of signal-to-noise ratios, i.e., the η values, were set up in an effort to maximize the appropriate η values and hence to minimize the variances of recovery data. It turns out that all the factors and levels showed rather minor effect on the variances of recoveries. Therefore, it should still be acceptable if any of the final choices of the optimal levels gives only the second largest or even the smallest η value.

At the optimal levels of the four factors, the effectiveness of the combined SFE–ChD was further validated by performing additional triplicate measurements and comparing the resulting recoveries and precisions with those obtained from two stepwise procedures (Table 2). Since the overall efficiency of stepwise ChD–SFE is equal to the yield of the ChD step times the recovery of the SFE step and our actually achieved TFA-derivatization yields were always nearly 100% (i.e., greater than 99%), it follows that simultaneous ChD–SFE (i.e., procedure A) typically gave higher recoveries than stepwise ChD–SFE (i.e., procedure B preceded by TFA-derivatization). The superiority of simultaneous ChD–SFE to stepwise ChD–SFE in efficiency is attributed to the unreacted TFA in the former procedure that considerably strengthens the extracting power of CO_2 fluid as was evidenced by the also better recoveries resulting from procedure C where another 5 μl of TFA (functioning as ''modifier'') was purposely added to the CO_2 fluid with other conditions being the same as those of procedure B. As to the precisions calculated for the recoveries of AP-TFA and MA-TFA after simultaneous SFE–ChD, the two relative standard deviations (RSDs) listed in Table 2 for procedure A based upon triplicate analyses, 3.8% and 4.0%, are both indicative of the reliability of the proposed method.

3.3. One-point calibration vs. regression calibration

The multiple-point (regression) calibration curves plotted for AP and MA in urine (equations: $y=0.64x+0.28$ for AP; $y=0.39x+0.21$ for MA) are both linear within 100~50 000 ng/ml, with correlation coefficients typically exceeding 0.999. Precisions calculated for the analyses of 500 ng/ml (the cutoffs for reporting ''positive'') AP and MA controls are ca. 1.2% and 1.0%, respectively, based upon triplicate analyses. As to the accuracy, regression calibration approach within its linearity range gives errors typically within ±20%, the tolerance limits for workplace drug urinalysis. The uncertainty is attributed partially to the sample preparation error and partially to the injection error even if an auto-sampler was employed.

Probably for the reasons of timing and economy, one-point calibration method [41] has often been used in practice as an alternative to regression calibration method. Therefore, it is of primary importance to have a general understanding of the analytical accuracy resulting from the one-point calibration approach for the analyses of AP and MA in urine by the proposed scheme including the sample preparation procedure and the GC–MS profiling. Since the cutoff concentrations for reporting positive AP and MA urinalyses are both 500 ng/ml, we simply chose 500 ng/ml as the one-point calibration standard concentration. As is shown in Table 3, one-point calibration approach yields acceptably small quantitative errors (ca. −1%) only in the vicinity of the calibrator's concentrations, 500 ng/ml. Negative errors appear exclusively at concen-

Table 2
The recoveries[a,b] of AP-TFA and MA-TFA and the relevant precisions achieved by following three different procedures

Experimental procedure[c]	No. of run	AP-TFA (%)	MA-TFA (%)
Procedure A[d]	Run 1[e]	92	85
	Run 2[e]	99	92
	Run 3[e]	94	90
		$\bar{X}=95$	$\bar{X}=89$
		SD=3.6	SD=3.6
		RSD=3.8%	RSD=4.0%
Procedure B[f]	Run 1	78	83
	Run 2	90	82
	Run 3	87	83
		$\bar{X}=85$	$\bar{X}=83$
		SD=6.2	SD=0.6
		RSD=7.3%	RSD=0.7%
Procedure C[g]	Run 1	92	85
	Run 2	94	83
	Run 3	94	84
		$\bar{X}=93$	$\bar{X}=84$
		SD=1.2	SD=1.0
		RSD=1.2%	RSD=1.2%

[a] All recoveries were calculated based on using 10 μl of 1000-μg/ml sample solution and 10 μl of 100-μg/ml I.S. solution.

[b] Trifluoroacetylamphetamine (AP-TFA) was used as the I.S. for the recovery calculation of MA-TFA, and trifluoroacetylmethamphetamine (MA-TFA) as that of AP-TFA.

[c] Only key conditions are described.

[d] Procedure A (the proposed procedure): (1) add 100 μl of TFA to underivatized sample and perform simultaneous static SFE–ChD under 4000 p.s.i. at 90°C for 5 min; (2) add I.S.[a] prior to GC–MS analysis.

[e] These three runs were performed in addition to those three denoted ''entry VI'' in Table 1 and served as a confirmatory test, all six runs being under the optimal experimental conditions.

[f] Procedure B: (1) perform static SFE on ready-made AP-TFA and MA-TFA under 4000 p.s.i. at 90°C for 5 min; (2) Add I.S.[a] prior to GC–MS analysis.

[g] Procedure C: (1) perform static SFE on ready-made AP-TFA and MA-TFA under 4000 p.s.i. at 90°C for 5 min with another 5 μl of TFA added; (2) add I.S.[a] prior to GC–MS analysis.

Table 3
Quantitative errors resulting from one-point calibration for the analyses of AP and MA in urine by the proposed method (calibrator: 500 ng/ml)

Actual concentration of spike (ng/ml)	AP (ng/ml) (error, %); ratio=0.61[a]	MA (ng/ml) (error, %); ratio=0.57[a]
100	290 (+190)	220 (+120)
500	495 (−1)	495 (−1)
1000	860 (−14)	530 (−47)
5000	2580 (−48)	1900 (−62)
10 000	5370 (−46)	2750 (−72)
25 000	12 550 (−50)	9040 (−64)
50 000	26 300 (−47)	17 610 (−65)

[a] The ratio [(selected ion intensity)$_{\text{analyte}}$/(selected ion intensity)$_{\text{internal standard}}$] of the one-point calibration standard.

trations above 500 ng/ml whereas positive errors at below 500 ng/ml. Based on these results, it is suggested that all submitted amphetamine-allied samples be quantitated via the regression calibration approach unless most of the submitted samples have been anticipated or known to have analytes around the one-point calibration standard concentration, 500 ng/ml.

3.4. Limits of detection (LODs) and limits of quantitation (LOQs)

The LOD, and LOQ, were determined in this study by two methods [47]. Method A is currently more prevalent in the forensic practice. After serial analyses of urinary spikes containing 5000, 4000, 3000, 2000, 1000, 500, 250, 100, 50, 25 and 10 ng/ml of AP and MA, the respective lowest concentrations of the two analytes that analyzed accurately within ±30% of their respective target concentrations were designated as the respective LODs of the two analytes, the string, in Taiwan, being that two ion ratios of each TFA derivative (i.e., $I_{m/z\ 118}/I_{m/z\ 140}$ and $I_{m/z\ 91}/I_{m/z\ 140}$ for AP-TFA; $I_{m/z\ 118}/I_{m/z\ 154}$ and $I_{m/z\ 110}/I_{m/z\ 154}$ for MA-TFA) matched within ±20% of those of the calibrators. In turn, the LOQs were the respective lowest concentrations of the two analytes that quantitated within ±20% of their respective target concentrations, the string being that the above stated two ion ratios of each TFA derivative also matched within ±20% of those of the calibrators. Thus, listed on the left of Table 4 are the LODs and LOQs resulting from the serial analyses of AP and MA, the two LODs being both 50 ng/ml and the two LOQs both 100 ng/ml. In contrast, method B is somewhat academic. Nevertheless, its relevant data are presented in support of the practicability of the title drug-testing scheme. Here the two limits are defined as the analyte concentrations giving peaks in the EIC with heights equal to the mean+$n\times$standard deviation, where $n=3$ for the LOD and $n=10$ for the LOQ [48]. The mean is the measured average of noises taken from a baseline region located far away from the analyte peak using a fortified sample. Accordingly, the standard deviation is the measured fluctuations of the noises. Our fortified sample was made by adding 50 μl of 100 μg/ml AP-MA binary standard solution and 20 μl of 100 μg/ml AP-d_8–MA-d_8 binary I.S. solution to 1 ml of blank urine, and was pretreated and analyzed according to the above described procedure. Shown on the right of Table 4 are the LODs and LOQs calculated for m/z 143 (AP-d_8), 140 (AP), 161 (MA-d_8) and 154 (MA) using an HP MS Chemstation software. The LODs of the four compounds calculated through their TFA derivatives vary from 19 to 24 ng/ml, and the LOQs from 21 to 27 ng/ml. Overall, those low limits sufficiently meet the requirements of most of the workplace drug testing programs and even the criminal cases in Taiwan, where amphetamines ''may not be detected'', i.e., zero tolerance.

Table 4
Limits of detection (LODs) and limits of quantitation (LOQs) for the analysis of AP, AP-d_8, MA and MA-d_8

Analyte	Method A[a]		Method B[a]	
	LOD (ng/ml)	LOQ (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)
AP	50	100	19	23
AP-d_8	–	–	22	25
MA	50	100	24	27
MA-d_8	–	–	19	21

[a] Methods A and B and their criteria are given in the text.

3.5. Case study

The analytical scheme proposed in this report as a choice of confirmatory protocol for forensic urine drug testing was applied to the determination of AP and MA in a number of real urinary samples that had previously been screened by a fluorescence polarization immunoassay (FPIA) as positive for both AP and MA. Some of the samples were even denoted ''HIGH'' with respect to MA and/or AP. After getting through the above stated analytical procedure and regression calibration, all the results were in good agreement with those reported for the FPIA preliminary test. Those that were denoted ''HIGH'' in the preliminary test were also proven to be at high concentrations by the proposed confirmatory test. Shown in the upper half of Table 5 are the representative results obtained upon the triplicate analyses of two real-case samples both definitely involving MA administration. Although in real-case sample 2

Table 5
Results obtained using regression calibration for the analyses of two real-case samples, both definitely involving MA administration, and their tenfold diluted spikes

		AP (ng/ml)	MA (ng/ml)
Real-case sample 1	Run 1	7420	61 970[a]
	Run 2	4760	58 320[a]
	Run 3	4910	66 320[a]
		$\bar{X}$=5697	$\bar{X}$=62 203
		RSD=26.2%	RSD=6.4%
Real-case sample 2	Run 1	32 980	1390
	Run 2	36 240	1260
	Run 3	31 020	1440
		$\bar{X}$=33 413	$\bar{X}$=1363
		RSD=7.9%	RSD=6.8%
Quasi real-case sample 1[b]	Run 1	440	5910
	Run 2	420	6430
	Run 3	430	6040
		$\bar{X}$=430	$\bar{X}$=6126
		RSD=2.3%	RSD=4.4%
Quasi real-case sample 2[c]	Run 1	3120	130
	Run 2	3370	120
	Run 3	3260	120
		$\bar{X}$=3250	$\bar{X}$=123
		RSD=3.9%	RSD=4.7%

[a] Obtained by extrapolation.
[b] Prepared by diluting real-case sample 1 tenfold with blank urine.
[c] Prepared by diluting real-case sample 2 tenfold with blank urine.

most of the MA had been metabolized into AP, the concentrations of both AP and MA in both of the real-case samples were still found to be much higher than the cutoffs (i.e., 500 ng/ml MA plus 200 ng/ml AP for reporting positive of MA). To make the amounts of the analytes more challenging to the assay, two quasi real-case samples were prepared by diluting the above real-case samples tenfold with blank urine and were subjected to the assay. The results are shown to be satisfactory in the lower half of Table 5. It is noteworthy that except for the analysis of AP in real-case sample 1, where the RSD is somewhat large (26.2%), the other seven analyses of AP or MA showed acceptable to fair precisions (2.3–7.9%). As mentioned above, the uncertainty is attributed partially to the sample preparation error and partially to the injection error even if an autosampler was employed.

4. Conclusions

The results presented in this report demonstrated that simultaneous SFE and ChD followed by isotope dilution GC–MS is a sound analytical scheme for the determination of AP and MA in urine and meets the criteria adopted by the US HHS and DoD (Department of Defense) drug testing programs. Under the optimal experimental conditions and with the optimal qualitative and quantitative ions, the regression calibration curves for AP and MA in urine are linear within 100~50 000 ng/ml, with correlation coefficients typically exceeding 0.999. The LODs determined by two methods for AP, AP-d_8, MA, and MA-d_8 vary from 19 to 50 ng/ml, and LOQs from 21 to 100 ng/ml. The RSDs calculated for the triplicate analyses of AP and MA in a 500-ng/ml spiked control, two real-case samples and two quasi

real-case samples, respectively, using regression calibration are typically below 10%. In summary, the use of TFA derivatization and d_8-labeled I.S.s not only conformed well to the proposed sample pretreatment procedure and GC–MS methodology, but also assist the identification and quantification of the targeted analytes.

Compared to the sample pretreatment procedure of the existing protocol, i.e., sequential SPE–ChD, the only possible drawback of the proposed one lies in the lengthy (3 h; yet simple) vacuum-drying step prior to the simultaneous SFE–ChD. To save time, it is advisable to run a number of samples at a time while processing other works, just like using so-called ''Manifold system'' in SPE. Also, for the proposed method to be even more efficient, specific and realistic, it would need to be challenged with other similar drugs (e.g., phentermine, propylhexidrine) and tested against more specimens with multiple types of drugs to determine the effects of possible interference from other drugs. Nevertheless, from the viewpoint of academic or basic research, the authors of this report wish to position the proposed analytical scheme in an alternative to the existing confirmatory protocol for forensic urine drug testing. Optimistically speaking, the success of the present study may pave a new way for drug analysis involving more viscous and/or interfering liquid matrices with which traditional liquid–liquid extraction (LLE) inevitably suffers from lengthy and complicated operation procedure, excessive use of harmful organic solvents, high background, and low level of automation while conventional SPE may suffer from cartridge clogging. The proposed method is particularly promising when matrix solid-phase dispersion technique (MSPD) [49–51] is hybridized with SFE.

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