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Simultaneous determination of amphetamine and methamphetamine enantiomers in urine by simultaneous liquid–liquid extraction and diastereomeric derivatization followed by gas chromatographic–isotope dilution mass spectrometry

Sheng-Meng Wang[a,*], Ting-Cheng Wang[b], Yun-Seng Giang[a]

[a] *Department of Forensic Science, Central Police University, Kueishan, Taoyuan 33334, Taiwan, ROC*
[b] *Forensic Science Center, Kaohsiung Municipal Police Headquarters, Kaohsiung 800, Taiwan, ROC*

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Abstract

A simple, rapid, reliable, and economic analytical scheme starting with in situ liquid–liquid extraction and asymmetric (or diastereomeric) chemical derivatization (ChD) followed by gas chromatography (GC)-isotope dilution mass spectrometry (MS) is described for the simultaneous determination of *d*- and *l*-amphetamine (AP) and methamphetamine (MA) in urine which could have resulted from the administration of various forms of questioned amphetamines or amphetamines-generating drugs. By using *l*-*N*-trifluoroacetyl-1-prolyl chloride (*l*-TPC) as chiral derivatizing agent, resolutions of 2.2 and 2.0 were achieved for the separation of AP and MA enantiomeric pairs, respectively, on an ordinary HP-5MS capillary column. The GC–MS quantitation was carried out in the selected ion monitoring (SIM) mode using *m*/*z* 237 and 251 as the quantifier ions for the respective diastereomeric pairs of AP-*l*-TPC and MA-*l*-TPC. The calibration curves plotted for the two pairs of analytes stretch with good linearity down to 45 ng/mL, and the limits of detection and quantitation determined were as low as 40 and 45 ng/mL, respectively. Also, a comparative study using 10 real-case urine specimens previously screened as positive for MA administration showed mostly tolerable biases between the two sums (of concentration) of *d*- and *l*-MA obtained via an asymmetric *l*-TPC-ChD approach and via an ordinary pentafluoropropionylation (PFPA-ChD) approach, respectively, as well as between the two sums of *d*- and *l*-AP obtained thereupon, thus validating the proposed analytical scheme as a promising forensic protocol for the detailed analysis of enantiomeric amphetamines in urine.

Keywords: Amphetamine; Methamphetamine; Derivatization, GC; Enantiomer separation

1. Introduction

Over the years forensic toxicologists have been involved in such difficult yet inevitable issues as (1) the enantioselective data interpretation of amphetamines-generating drugs (also referred to as "precursor drugs to amphetamines") as well as (2) the differentiation between illegal consumption of abused amphetamines-generating drugs (or amphetamines themselves) and legitimate administration of prescribed amphetamines-generating drugs. Various and questionable amounts of AP and/or methamphetamine (MA) have often been detected in urine following the administration of such prescription drugs or controlled substances as amphetaminil, benzphetamine, clobenzorex, deprenyl (selegiline), dimethylamphetamine, ethylamphetamine, famprofazone, fencamine, fenethylline, fenproporex, furfenorex, mefenorex, mesocarb, prenylamin, etc. [1–20] (these substances along with their brand names, stereoisomerisms, medical or illegal status, important metabolites, and expected urine levels are summarized in Table 1 [13,21–32]). The many possibilities of initially ingesting the suspect drug of various optical purities [33–39] or using Vicks nasal inhalers [3,40] further complicate both the chemical analysis and the data interpretation.

* Corresponding author. Fax: +886 3 3275907.
E-mail address: wang531088@sun4.cpu.edu.tw (S.-M. Wang).

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Table 1
A summary of some common (a) amphetamine- and (b) methamphetamine-generating drugs

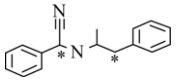
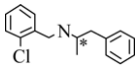
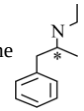
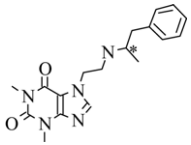
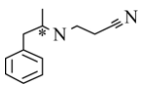
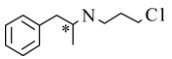
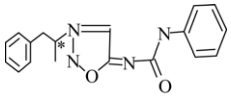
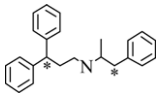
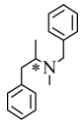
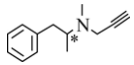
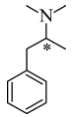
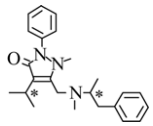
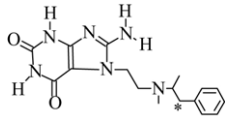
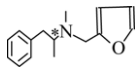
Brand name	IUPAC name	Chemical structure[a]	Medical or illegal status	Important metabolites	Percent of dose excreted		Sample for study	Reference
					AP	MA		
(a)								
Amphetaminil	α-[(1-Methyl-2-phenylethyl)amino] benzeneacetonitrile		Psychotropic drug	AP	3.3	NA[b]	Human urine	[21]
Clobenzorex	*N*-[(2-Chlorophenyl)methyl]-α-methyl-benzeneethanamine		Treatment of obesity	AP; 4-Hydroxyamphetamine; 4-Hydroxyclobenzorex	1365[c]	NA[b]	Human urine	[22]
Ethylamphetamine	*N*-Ethyl-α-methylbenzeneethanamine		Schedule I drug in USA; no recognized medical use	AP; 4-Hydroxyethylamphetamine	9.0–14.7	NA[b]	Human urine	[23]
Fenethylline	3,7-Dihydro-1,3-dimethyl-7-[2-[(1-methyl-2-phenylethyl)amino]ethyl]-1H-purine-2,6-dione		Schedule I drug in USA; treatment of narcolepsy and children with attention deficit disorder	AP; Theophylline; Hippuric acid	24.5	NA[b]	Human urine	[24]
Fenproporex	3-[(1-Methyl-2-phenylethyl)amino]-propanenitrile		Treatment of obesity	AP	27–31	NA[b]	Human urine	[25]
Mefenorex	*N*-(-3-Chloropropyl)-α-methyl- benzeneethanamine		Treatment of obesity	AP; 4-Hydroxymefenorex	15	NA[b]	Human urine	[26]
Mesocarb	3-(1-Methyl-2-phenylethyl)-*N*-(phenylaminocarbonyl)-sydnoneimine		A stimulant; treatment of phantom pain syndrome	AP; Hydroxymesocarb; Dihydroxymesocarb	4	NA[b]	Rat urine[d]	[27]
Prenylamine	*N*-(1-Methyl-2-phenylethyl)-γ-phenyl-benzenepropanamine		A coronary vasodilator; treatment of angina	AP; Norephedrine; Diphenylpropylamine	0.14	NA[b]	Human urine	[28]

Table 1 (*Continued*)

Brand name	IUPAC name	Chemical structure[a]	Medical or illegal status	Important metabolites	Percent of dose excreted		Sample for study	Reference
					AP	MA		
(b)								
Benzphetamine	*N*,α-Dimethyl-*N*-(phenylmethyl)-benzeneethanamine		Treatment of obesity	AP; MA; 1-(4-Hydroxyphenyl)-2-(*N*-methyl-*N*-benzylamino)propane	7.6–8.9	2.2–3.1	Human urine	[29]
Deprenyl	*N*,α-Dimethyl-*N*-2-propenyl-benzeneethanamine		Treatment of Parkinson's disease	MA; AP; Desmethyldeprenyl	5–7	11–16	Human urine	[13]
Dimethylamphetamine	*N*,*N*-α-Trimethyl-benzeneethanamine		No recognized medical use; an illicit drug	MA; AP; Dimethylamphetamine-*N*-oxide	1.3	11.3	Human urine	[30]
Famprofazone	4-Isopropyl-2-methyl-3-[*N*-methyl-*N*-(α-methyl-phenylethyl)-aminomethyl]-1-phenyl-3-pyrazolin-5-one		Antipyretic & analgesic	MA; AP; 3-Hydroxymethylpyrazolone	Not quantified	6.2–18.7	Human urine[e]	[31]
Fencamine	*N*-Methyl-*N*-(1-methyl-2-phenylethyl)-*N*′-3,7-dihydro-1,3,7-trimethyl-8-[[2-[methyl(1-methyl-2-phenylethyl)amino]ethyl]amino]-1H-purine-2,6-dione		Treatment of depression	MA; AP	Not quantified	Not quantified	Human urine	[32]
Furfenorex	*N*-Methyl-*N*-(1-methyl-2-phenylethyl)-2-furanmethanamine		Treatment of obesity	AP; MA; 1-Phenyl-2-(*N*-methyl-*N*-γ-valerolactonylamino) propane	6.1–8.5	3.3–4.4	Human urine	[29]

[a] The asterisks designate the asymmetric carbons and imply the possible stereoisomerism.
[b] NA: not applicable. Drug is not metabolized to MA.
[c] The maximum concentration in ng/mL.
[d] Measured for 24 h.
[e] Measured for 72 h.

In other words, putting aside the technical aspect, the relative amounts detected for the stereoisomeric excretions do have a crucial bearing on the form and source of the drug to be traced. In this context, some commonly encountered facts are explanative. For instance, all the legal Vicks inhalers use merely *l*-MA, and a dose of 50 mg suffices the urine collected thereupon to test positive for unchanged MA and its major metabolite, AP, in *l*-form exclusively. Coincidentally, some prescribed amphetamines-generating drugs present in the beginning only the *l*-isomers (e.g., *R*-*l*-deprenyl) and generate MA and AP later also in *l*-form exclusively. In contrast, specimens involving prescribed racemic amphetamines-generating drugs or illegal racemic MA itself have often analyzed higher proportions of *l*-MA relative to *d*-MA and lower yet various proportions of *l*-AP relative to *d*-AP. This is because under a normal pH value *d*-MA metabolizes faster than *l*-MA [41,42]. If racemic MA is actually administered, *l*-MA excreted in urine will predominate over *d*-MA with increasing *l*-MA/*d*-MA ratio during most of the course of metabolization. On the other hand, there is not much doubt of illegal use of a controlled substance if merely *d*-MA and/or *d*-AP are/is found in the specimen. Thus, the importance of correctly determining and interpreting the stereoisomerism of MA/AP in urine so as to unambiguously differentiate the form and source of the originally administered drugs cannot be over-emphasized.

A number of instrumental methods have been utilized for the analysis of enantiomeric amphetamines, including the more prevalent gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), high performance liquid chromatography (HPLC), and LC–MS [43–55]. Among them GC–MS employing chiral-phase columns or preceded by asymmetric (or diastereomeric) chemical derivatization (ChD) of the analyte is the most extensively used and the only method adopted by most of the workplace drug testing programs. So far as the asymmetric ChD and resolution of enantiomeric amphetamines is concerned, (*S*)-(−)-*N*-trifluoroacetyl-1-prolyl chloride (*l*-TPC) is the most often used chiral ChD agent [53–55]. The experimental conditions and procedure are simple. The ChD reactions for the enantiomeric pair are equally rapid and complete [40]. Prompted by the works of Fitzgerald et al. and Cody et al. [4,40,53], we wish to perform a few qualitative/quantitative analytical experiments to further investigate the feasibility of combining liquid–liquid extraction (LLE) with asymmetric ChD in one pot and evaluate the efficacies of using this combined LLE-ChD procedure with the previously established GC–isotope dilution MS methodology.

2. Experimental

2.1. Materials

Racemic *d*,*l*-MA [1 mg (i.e., 0.5 mg *d*-MA + 0.5 mg *l*-MA)/mL in methanol], *d*,*l*-AP (1 mg/mL in methanol), *d*,*l*-MA-d_8 (0.1 mg/mL in methanol) and *d*,*l*-AP-d_8 (0.1 mg/mL in methanol) were purchased from Cerilliant Co., USA. The MA/AP binary working solution and MA-d_8/AP-d_8 binary internal standards (ISs) working solution were both prepared as 10 μg/mL in D.I. water with respect to each authentic compound.

The derivatizing agents, *l*-TPC and pentafluoropropionic anhydride (PFPA), were purchased from Aldrich Chemical Co., USA. Ethyl acetate (EA), anhydrous potassium carbonate, anhydrous sodium hydroxide, concentrated hydrochloric acid, and *n*-hexane were from Fisher Scientific, USA. All of the above agents and solvents were in analytical or reagent grade and were directly used without further purification.

2.2. Sample preparation

2.2.1. Simultaneous LLE and l-TPC-ChD

To 1 mL of fortified or real-case urine sample in a screw-cap topped test tube were added 50 μL of the ISs solution, 0.5 mL of saturated potassium carbonate, 4 mL of *n*-hexane and 50 μL of *l*-TPC. After 10 min of shaking, the mixture was subjected to centrifugation at 3000 rpm for 5 min. The upper layer was transferred to a concentration tube and purged at 45 °C with nitrogen gas to dryness. More EA was added to make up a 200 μL solution. A 1 μL aliquot of this solution was injected for the GC–MS analysis.

2.2.2. Stepwise LLE and PFPA-ChD

To 1 mL of fortified or real-case urine sample in a screw-cap topped test tube were added 50 μL of ISs solution, 1 mL of 2 N sodium hydroxide and 8 mL of EA. The mixture was shaken for 5 min followed by centrifugation at 3000 rpm for 5 min. The supernatant was transferred to another screw-cap topped test tube, to which was added 2 mL of 0.5 N hydrochloric acid. After 5 min of shaking and 5 min of centrifugation, the upper layer was transferred to still another screw-cap topped test tube and alkalized to pH 12–13 with 2 N sodium hydroxide. A 4 mL portion of EA was added followed by 5 min of shaking and 5 min of centrifugation. The upper layer was transferred to a screw-cap topped derivatizing tube and purged at 50 °C with nitrogen gas to dryness. The residue was re-dissolved with 50 μL of EA, and to this solution 50 μL of PFPA was added. The mixture was incubated at 80 °C for 20 min, allowed to cool down to ambient temperature, transferred to a concentration tube, and purged at 50 °C with nitrogen gas to dryness. More EA was added to make up a 200 μL solution. A 1 μL aliquot of this solution was injected for the GC–MS analysis.

2.3. GC–MS analysis

The 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 (MSD). The GC column used was a HP-5 MS capillary column (30 m × 0.2 mm i.d., 0.33 μm film thickness). The GC was operated in the

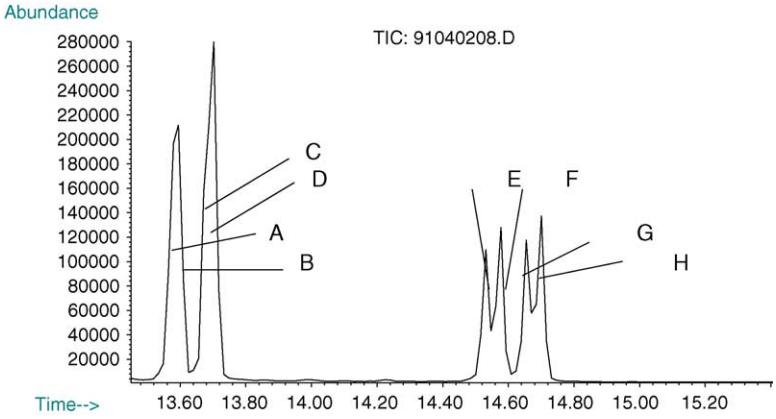


Fig. 1. The GC-isotope dilution EIMS SIM chromatogram obtained for the simultaneous LLE/*l*-TPC-ChD of a urinary spike containing 250 ng/mL each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP. Peaks assignments (RT in min): (A) *l*-TP-AP-d_8 (13.58); (B) *l*-TP-AP (13.61); (C) *d*-TP-AP-d_8 (13.69); (D) *d*-TP-AP (13.71), (E) *l*-TP-MA-d_8 (14.54); (F) *l*-TP-MA (14.59); (G) *d*-TP-MA-d_8 (14.67); (H) *d*-TP-MA (14.71).

splitless mode (i.e., purge off) when performing injection with the aid of an HP-7673 autosampler, but 1 min later the purge valve was turned on. The injector temperature was 250 °C. For the analysis of *l*-TPC derivatized amphetamines, the column temperature was programmed from 60 to 250 °C at 25 °C/min, with the initial temperature held for 5 min and final temperature 5.4 min. For the analysis of PFPA derivatized amphetamines, the column temperature was programmed from 100 to 250 °C at 25 °C/min, with the initial and final temperatures both held for 5 min. Helium of 99.999% purity 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 electron impact (EI) ionization source held at 180 °C. The GC–MS instrument was operated in the selected ion monitoring (SIM) mode accompanied by extracted ion chromatograms (EIC). The calibration curves were produced by plotting the quantifier-ion-abundance ratios (analyte: IS) obtained from the SIM measurements against the concentrations of the appropriate analytes in the fortified samples. The quantifier-ion-abundance ratio used was the mean of triplicate analyses.

3. Results and discussion

3.1. Mass chromatography

Shown in Fig. 1 is the GC-EIMS SIM chromatogram obtained for the simultaneous LLE and *l*-TPC-ChD of a urinary spike containing 250 ng/mL each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP. The retention times (RTs) of the respective *l*-TPC derivatized analytes and ISs are also listed. If we look solely at the relevant RT differences, then GC-EIMS SIM using an ordinary GC column can already afford the complete separation of the four *l*-TPC derivatized *l*-amphetamines (i.e., *l*-TP-AP-d_8, *l*-TP-AP, *l*-TP-MA-d_8, and *l*-TP-MA) from their corresponding diastereomers, i.e., the four *l*-TPC derivatized *d*-amphetamines (i.e., *d*-TP-AP-d_8, *d*-TP-AP, *d*-TP-MA-d_8, and *d*-TP-MA), although the four *l*-TPC derivatized analytes (i.e., *l*-TP-AP, *d*-TP-AP, *l*-TP-MA and *d*-TP-MA) can only be partially separated from their IS counterparts. In contrast, shown in Fig. 2 is the GC-EIMS SIM chromatogram obtained for the previously developed stepwise LLE/PFPA-ChD of another spike of the same composition. This chromatogram

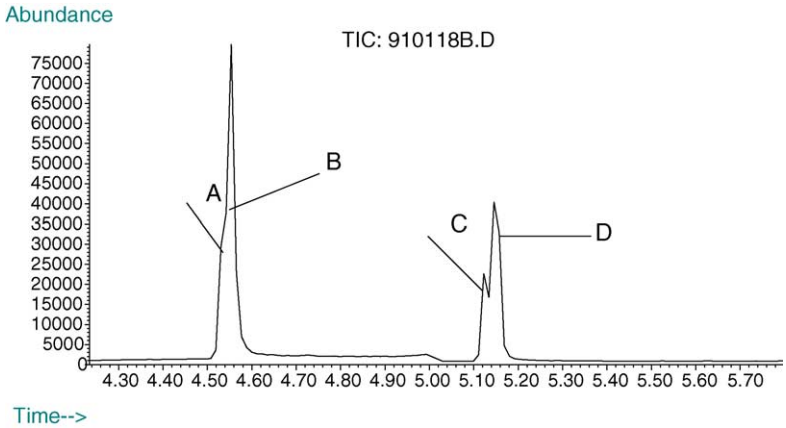


Fig. 2. The GC-isotope dilution EIMS SIM chromatogram obtained for the stepwise LLE/PFPA-ChD of a urinary spike containing 500 ng/mL each of racemic *d*,*l*-MA and racemic *d*,*l*-AP. Peaks assignments (RT in min): (A) PFP-AP-d_8 (4.54); (B) PFP-AP (4.56); (C) PFP-MA-d_8 (5.13); (D) PFP-MA (5.16).

indicates that, using the same GC–MS methodology, stepwise LLE/PFPA-ChD can neither afford any resolution of the racemic analytes nor complete separation of the analyte from the IS. Remarkably, with the aid of the highly specific qualifier- and quantifier-ions monitoring, both simultaneous LLE/*l*-TPC-ChD and stepwise LLE/PFPA-ChD are able to achieve satisfactory "effective" GC-EIMS SIM resolution (as opposed to the poor "superficial" GC resolution based merely on the RT difference) between the derivatized analyte and the derivatized IS and hence the accuracy and precision for the title GC–MS analysis of the analyte. Having gone through the same evaluation process in the light of "minimized analyte-IS ion cross contribution" as that for trifluoroacetic anhydride (TFA) derivatized amphetamines [56,57], the qualifier/quantifier ions selected for the four diastereomeric pairs of TP-amphetamines and four enantiomeric pairs of PFP-amphetamines are displayed in Table 2. To contrast the resolving power of *l*-TPC-ChD with that of PFPA-ChD, the GC-EIMS SIM resolutions calculated for the relevant stereomeric pairs are also shown. Thus, the four *l*-TP-amphetamines are all well resolved with their diastereomeric *d*-counterparts, with all the resolutions exceeding 2.0, far better than 1.5, a widely recognized leveling for well-resolving two neighboring chromatographic peaks. By the way, the mass spectra obtained upon the GC-EIMS full scan of the above mentioned TPC-amphetamines and PFP-amphetamines are shown in Figs. 3 and 4.

Table 2
Qualifier and quantifier ions selected for (a) the four diastereomeric pairs of TP-amphetamines, and (b) the four enantiomeric pairs of PFP-amphetamines

l-TPC derivatized analyte or IS	Qualifier ions (*m*/*z*)	Quantifier ions (*m*/*z*)	Resolution
(a)			
l-TP-AP-d_8	240, 126, 96	240	2.2
d-TP-AP-d_8	240, 126, 96	240	
l-TP-AP	237, 118, 91	237	2.2
d-TP-AP	237, 118, 91	237	
l-TP-MA-d_8	258, 122, 92	258	2.6
d-TP-MA-d_8	258, 122, 92	258	
l-TP-MA	251, 118, 91	251	2.0
d-TP-MA	251, 118, 91	251	
PFPA derivatized analyte or IS	Qualifier ions (*m*/*z*)	Quantifier ions (*m*/*z*)	Resolution
(b)			
l-PFP-AP-d_8	193,[a] 126, 96	193	0
d-PFP-AP-d_8	193,[a] 126, 96	193	
l-PFP-AP	190,[a] 118, 91	190	0
d-PFP-AP	190,[a] 118, 91	190	
l-PFP-MA-d_8	211,[a] 163, 122	211	0
d-PFP-MA-d_8	211,[a] 163, 122	211	
l-PFP-MA	204,[a] 160, 118	204	0
d-PFP-MA	204,[a] 160, 118	204	

[a] Base peak.

3.2. Quantitation

Since the accuracy of the proposed method relies largely on the accuracy of the calibration standards, it was desirable to assess in advance the purities and concentrations of the authentic primary solutions used for the preparation of working solutions. In 1981, Liu et al., using a chiral-column GC–MS, determined the contamination of *d*-TPC in the commercial *l*-TPC reagent to be 5.19%, which was close to the 6% reported by the manufacturer [54]. For the present study, however, the manufacturer of the *l*-TPC reagent did not report the percent contamination of *d*-TPC, implying a high optical purity of *l*-TPC. Indeed, as are presented in Table 3, our calculated (based on the relevant observed quantifier-ion abundances) enantiomeric impurities in the commercial *d*- and *d*,*l*-amphetamine and methamphetamine standards are typically below 4.1%.

The method calibration curves of the amphetamines in urine were plotted basically using five calibrators containing, respectively, 50, 100, 250, 500, and 1000 ng/mL each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP (i.e., 10, 20, 50, 100, 200 μL, respectively, of the 10 μg/mL racemic MA/AP working solution in 1 mL of blank urine) and 250 ng/mL each of *d*-MA-d_8, *l*-MA-d_8, *d*-AP-d_8, and *l*-AP-d_8 (i.e., 50 μL of the 10-μg/mL racemic ISs working solution in 1 mL of blank urine). The method limit of quantitation [(M)LOQ] was determined by a definition currently prevailing in the forensic practice [58].

Table 3
Calculation of enantiomeric impurities in commercial *d*- and *d*,*l*-amphetamine and methamphetamine standards based on the observed quantifier-ion abundances

Tested standard (250 ng/mL)	Peak	Run 1	Run 2	Run 3	Optical purity
d-AP	*l*-AP	9364	7484	9258	Mean percent *l*-AP as impurity in *d*-AP = 4.1%
	d-AP	245326	193466	201568	
	l-AP/*d*-AP	0.0382	0.0387	0.0459	
d-MA	*l*-MA	7959	5087	6153	Mean percent *l*-MA as impurity in *d*-MA = 3.2%
	d-MA	227261	184945	185988	
	l-MA/*d*-MA	0.0350	0.0275	0.0330	
d,*l*-AP	*l*-AP	98218	77249	88776	Mean *l*-AP/*d*-AP = 0.9796
	d-AP	98247	80852	90238	
	l-AP/*d*-AP	0.9997	0.9554	0.9838	
d,*l*-MA	*l*-MA	74984	75728	76737	Mean *l*-MA/*d*-MA = 1.0446
	d-MA	68127	74271	75689	
	l-MA/*d*-MA	1.1006	1.0196	1.0138	

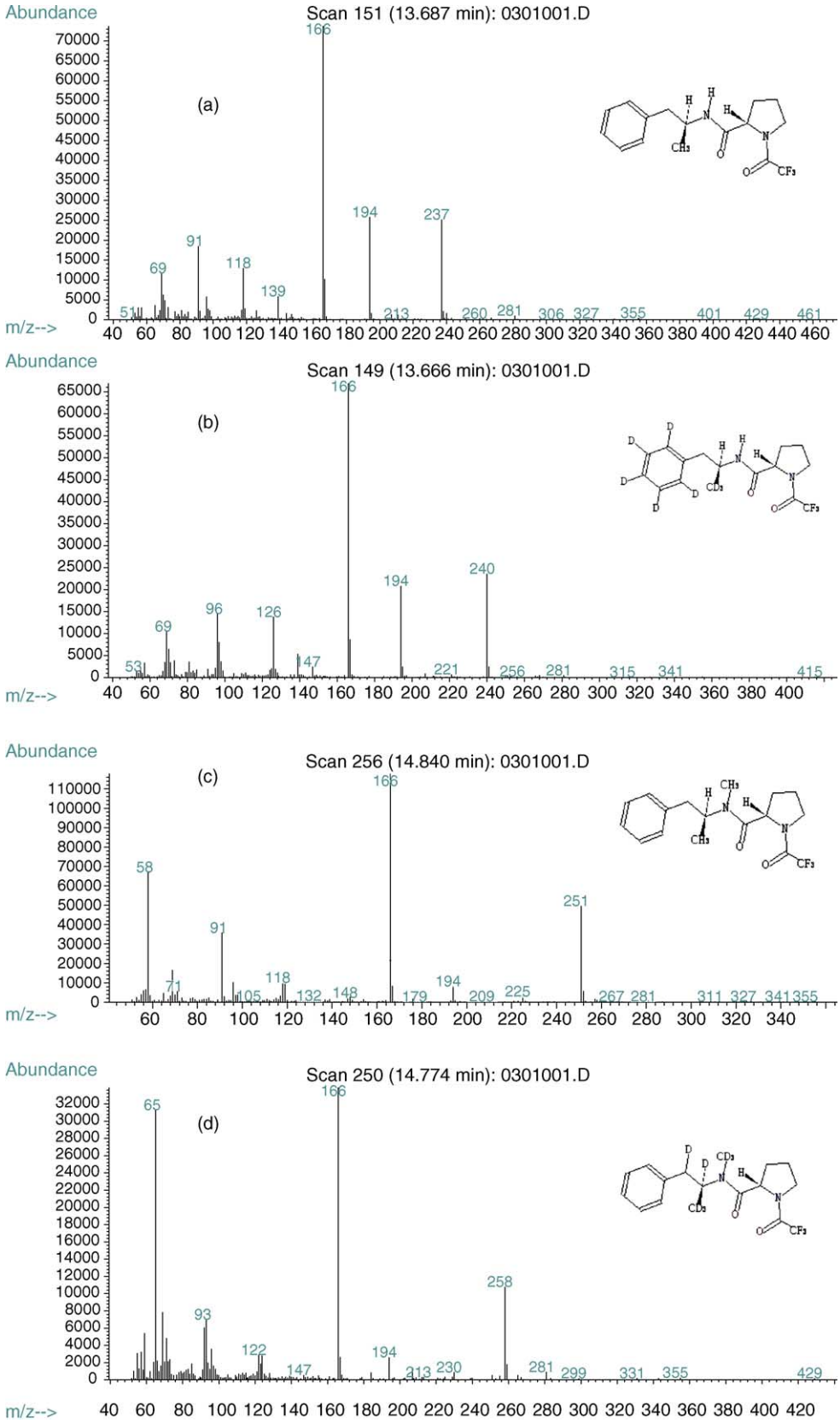


Fig. 3. The mass spectra of (a) *l*-TP-AP and/or *d*-TP-AP, (b) *l*-TP-AP-d_8 and/or *d*-TP-AP-d_8, (c) *l*-TP-MA and/or *d*-TP-MA, and (d) *l*-TP-MA-d_8 and/or *d*-TP-MA-d_8.

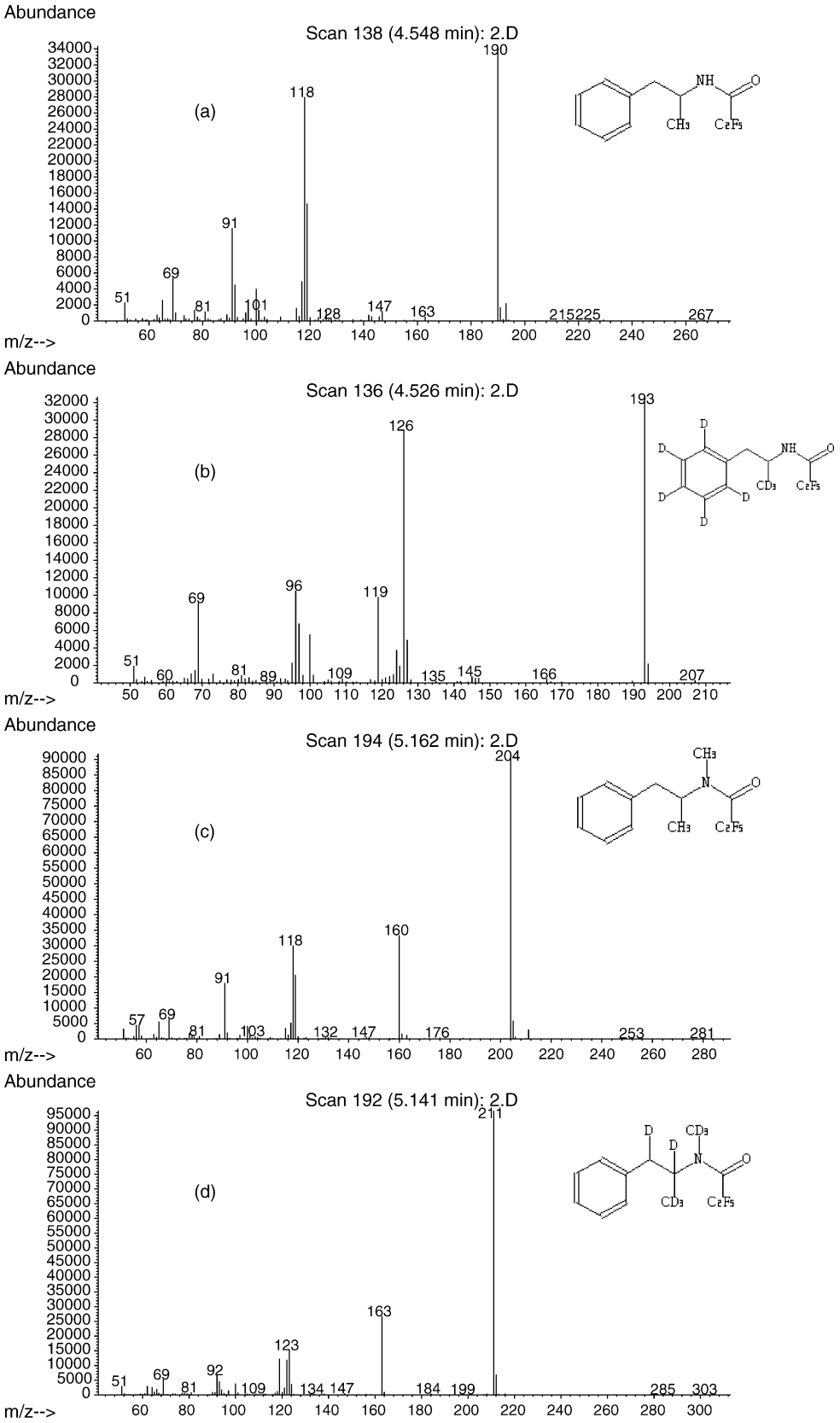


Fig. 4. The mass spectra of (a) *d,l*-PFP-AP, (b) *d,l*-PFP-AP-d_8, (c) *d,l*-PFP-MA, and (d) *d,l*-PFP-MA-d_8.

Table 4
Calibration equations, linearity ranges, linear correlation coefficients (r^2), and LODs/LOQs for the analyses of amphetamines in urine by (a) simultaneous LLE/*l*-TPC-ChD followed by GC-EIMS SIM, and (b) stepwise LLE/PFPA-ChD followed by GC-EIMS SIM

Analyte	Calibration equation	Linearity ranges (ng/mL)	Linear correlation coefficients (r^2)	LOQ (ng/mL)	LOD (ng/mL)
(a)					
l-MA	$y = 0.0043x + 0.0668$	45–1000	0.9953	45	40
d-MA	$y = 0.0045x + 0.0801$	45–1000	0.9978	45	40
l-AP	$y = 0.0042x - 0.0176$	45–1000	0.9995	45	40
d-AP	$y = 0.0043x - 0.0663$	45–1000	0.9958	45	40
(b)					
d,*l*-MA	$y = 0.0021x + 0.008$	45–2000	0.9993	45	40
d,*l*-AP	$y = 0.0021x + 0.0466$	45–2000	0.9994	45	40

After serial total-analyses of urinary spikes containing lower and lower concentrations of the relevant analytes (i.e., 1000, 500, 250, 100, 50, 45, 40, 35 ng/mL, respectively, each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP), the respective lowest concentrations of the analytes that analyzed accurately within ±20% of the respective target concentrations were designated as the respective LOQs of the analytes, the string being that the two qualifier-ion-abundance ratios of each derivatized AP or MA (e.g., $Abund_{m/z\ 118}/Abund_{m/z\ 237}$ and $Abund_{m/z\ 91}/Abund_{m/z\ 237}$ for TP-AP; $Abund_{m/z\ 118}/Abund_{m/z\ 251}$ and $Abund_{m/z\ 91}/Abund_{m/z\ 251}$ for TP-MA) matched within ±20% of those of the calibrators. Due to practical feasibility, the method limit of detection [(M)LOD] was, however, simply defined as the lowest concentration of the analyte that gives the two qualifier-ion-abundance ratios matching within ±20% of those of the calibrators. Thus, presented in Table 4a are the regressed calibration equations, linear working ranges (each stretching down to the LOQ), linear correlation coefficients (r^2), and LODs/LOQs for the analyses of *d*-MA, *l*-MA, *d*-AP, and *l*-AP in urine by simultaneous LLE/*l*-TPC-ChD and GC-EIMS SIM. The respective enantiomeric pairs of amphetamines turn out to be well resolved and analyzed with the same LOD (40 ng/mL) and the same LOQ (45 ng/mL). All of these features significantly surpass the criteria adopted by most of the workplace urine drug testing programs (in Taiwan the cut-offs for MA ingestion: both 500 ng MA and 200 ng AP found in 1 mL urine), and even meet the requirements of the criminal cases in Taiwan, ROC, where amphetamines must not be detected, namely, zero tolerance (in the ROC criminal practice, this zero-tolerance policy has been implemented by using the respective LOQs as cutoffs.) It should be addressed that, for the present comparative study between the *l*-TPC and PFPA approaches, the latter approach also offers comparable linear working ranges (90–2000 ng/mL for a pair of enantiomers), linear correlation coefficients (typically above 0.995), and LODs/LOQs as is shown in Table 4b.

3.3. Comparative analyses between l-TPC and PFPA approaches

To cross-examine the quantitative-analytical accuracy of asymmetric *l*-TPC and ordinary PFPA approaches, comparisons were made between the two sums (of concentration) of *d*- and *l*-MA as well as between the two sums of *d*- and *l*-AP obtained thereupon. Of the 10 real-case urine specimens previously screened as positive for MA administration, only sample 6 produced a bias (*l*-TPC relative to PFPA) as large as +20% for the MA comparison (Table 5a), and only samples 8 and 9 resulted in biases (+37.7% and +20.5%, respectively) larger than 20% for the AP comparison (Table 5b). Although biases or suchlike indicators are not fit for the comparison of AP/MA ratios, the three sets of AP/MA ratios in Table 5c resulting from the two approaches do correlate considerably with one another. That is, eight out of 10 samples produced a *d*,*l*-AP/*d*,*l*-MA ratio (via PFPA approach) standing between the *l*-AP/*l*-MA and the *d*-AP/*d*-MA (via *l*-TPC approach). Only samples 2 and 10 each gave a *d*,*l*-AP/*d*,*l*-MA ratio from the PFPA (0.2 and 0.17, respectively) slightly larger than the larger of the *l*-AP/*l*-MA and *d*-AP/*d*-MA from the *l*-TPC (0.17 and 0.15, respectively).

The diastereomeric *l*-TPC-ChD is absolutely superior to the enantiomeric PFPA-ChD in differentiating the source of the sample. Judging from the relatively high ratios of both *l*-MA/*d*-MA (5.20) and *l*-AP/*d*-AP (4.74) in Table 3a and b, respectively, and considering the fact that *d*-MA normally metabolizes faster than *l*-MA, sample 4 must have involved the administration of optically impure *l*-MA. Based on a similar reasoning that the *l*-MA/*d*-MA ratios of samples 9–11 are 0.69, 0.85 and 1.06, respectively (Table 5a), and that *d*-MA normally metabolizes faster than *l*-MA, these three samples should have involved the ingestion of racemic MA. The other nine samples analyzed *l*-MA/*d*-MA ratios ranging mostly 0.12–0.15 (except that sample 5 analyzed 0.35) and *l*-AP/*d*-AP ratios 0.17–0.39, strongly suggesting the previous ingestion of optically impure *d*-MA.

3.4. Between-sample and within-sample precisions

The accuracy and precision of the whole proposed analytical scheme, i.e., simultaneous LLE/*l*-TPC-ChD followed by GC-isotope dilution EIMS SIM, were further validated by a series of experimental evaluations using three urinary control samples, A, B, and C, each containing 250 ng/mL each of *d*-MA, *l*-MA, *d*-AP, *l*-AP, *d*-MA-d_8, *l*-MA-d_8, *d*-AP-d_8, and *l*-AP-d_8. As shown in Table 6, the between-sample RSDs calculated for the determined concentrations of the four an-

Table 5
Comparative analyses of real-case urine specimens via *l*-TPC and PFPA approaches: (a) comparison of MA data; (b) comparison of AP data; (c) comparison of AP/MA ratio data

Sample number	Via *l*-TPC approach				Via PFPA approach	Bias in *l* + *d* (*l*-TPC relative to PFPA)	
	l-MA (ng/mL)	*d*-MA (ng/mL)	*l* + *d* (ng/mL)	*l*/*d*	*d*,*l*-MA (ng/mL)	(ng/mL)	(%)
(a)							
1	1896	10460	12356	0.18	13316	−960	−7.2
2	1067	4576	5643	0.23	5221	+422	+8.1
3	2673	514	3187	5.20	3601	−414	−11.5
4	4125	12371	16496	0.33	14021	+2475	+17.6
5	6349	16231	22581	0.39	21498	+1083	+5.0
6	7011	19246	26258	0.36	21880	+4378	+20.0
7	7911	11456	19367	0.69	18010	+1357	+7.5
8	2393	2801	5149	0.85	4614	+535	+11.6
9	3727	3494	7221	1.06	6485	+736	+11.3
10	1327	5332	6659	0.24	5915	+744	+12.6

Sample number	Via *l*-TPC approach				Via PFPA approach	Bias in *l*+ *d* (*l*-TPC relative to PFPA)	
	l-AP (ng/mL)	*d*-AP (ng/mL)	*l* + *d* (ng/mL)	*l*/*d*	*d*,*l*-AP (ng/mL)	(ng/mL)	(%)
(b)							
1	171	1406	1577	0.12	1605	−28	−1.7
2	123	819	942	0.15	1061	−119	−11.2
3	313	66	379	4.74	372	+7	+1.9
4	217	1426	1643	0.15	1571	+72	+4.6
5	665	4385	5051	0.15	4542	+509	+11.2
6	542	4105	4647	0.13	3976	+671	+16.9
7	446	1176	1622	0.37	1409	+213	+15.1
8	199	509	708	0.39	514	+194	+37.7
9	296	585	881	0.50	730	+151	+20.5
10	129	817	946	0.15	1017	−71	−7.0

Sample number	Via *l*-TPC approach		Via PFPA approach
	l-AP/*l*-MA (%)	*d*-AP/*d*-MA (%)	*d*,*l*-AP/*d*,*l*-MA (%)
(c)			
1	0.09	0.13	0.12
2	0.12	0.17	0.20
3	0.12	0.13	0.10
4	0.05	0.12	0.11
5	0.10	0.27	0.21
6	0.08	0.21	0.18
7	0.06	0.10	0.08
8	0.08	0.18	0.11
9	0.08	0.17	0.11
10	0.10	0.15	0.17

Table 6
Between-sample precisions and accuracies calculated for the analyses of three 250 ng/mL control samples by simultaneous LLE/*l*-TPC-ChD and GC-EIMS SIM

	l-MA		*d*-MA		*l*-AP		*d*-AP	
	l-MA found (ng/mL)	Peak-abundance ratio to IS	*d*-MA found (ng/mL)	Peak-abundance ratio to IS	*l*-AP found (ng/mL)	Peak-abundance ratio to IS	*d*-AP found (ng/mL)	Peak-abundance ratio to IS
Control A[a]	250	1.15	232	1.04	235	1.11	231	1.03
Control B[a]	233	1.07	245	1.10	236	1.11	244	1.09
Control C[a]	250	1.14	249	1.20	254	1.05	268	1.09
Mean	244	1.12	242	1.11	242	1.09	248	1.07
S.D.	9.82	0.044	8.89	0.081	10.69	0.035	18.77	0.035
R.S.D. (%)	4.0	3.9	3.7	7.3	4.4	3.2	7.6	3.3
Accuracy (%)	−2.4		−3.2		−3.2		−0.8	

[a] Each control sample contains 250 ng/mL each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP as the analyte, and also 250 ng/mL each of *d*-MA-d_8, *l*-MA-d_8, *d*-AP-d_8, and *l*-AP-d_8 as the IS in 1 mL of blank urine.

Table 7
Within-sample precisions calculated for the analyses of three 250 ng/mL control samples by simultaneous LLE/*l*-TPC-ChD and GC-EIMS SIM

Sample and GC–MS run[a,b]	*l*-MA		*d*-MA		*l*-AP		*d*-AP	
	l-MA found (ng/mL)	Peak abundance	*d*-MA found (ng/mL)	Peak abundance	*l*-AP found (ng/mL)	Peak abundance	*d*-AP found (ng/mL)	Peak abundance
A_1	233	138747	245	80925	236	104749	244	140597
A_2	247	132445	279	79273	244	113394	276	138341
A_3	238	129709	259	71867	241	104401	279	136731
Mean	239	133633	261	77355	240	107514	266	138550
S.D.	7.1	4635	17.1	4824	4.0	5094	19.4	1933
R.S.D. (%)	3.0	3.5	6.5	6.2	1.7	4.7	7.3	1.2
$A_{12\,h}$	232	131473	233	70272	247	103038	269	135624
$A_{24\,h}$	240	156077	266	84877	240	122081	251	151356
$A_{48\,h}$	236	122576	253	61518	252	95218	275	127543
Mean	236	136708	251	72222	246	106779	265	138174
S.D.	4.0	17353	16.6	11801	6.0	13816	12.5	12109
R.S.D. (%)	1.7	12.7	6.6	16.3	2.4	12.9	4.7	8.8
B_1	243	143392	262	77490	237	108934	243	139919
B_2	238	140024	265	81491	232	119180	244	135619
B_3	252	138104	279	78526	250	111499	279	139211
Mean	244	140507	269	79169	240	113204	255	138249
S.D.	7.1	2677	9.1	2076	9.3	5331	20.5	2306
R.S.D. (%)	2.9	1.9	3.4	2.6	3.9	4.7	8.0	1.7
$B_{12\,h}$	257	142044	265	73730	247	106286	249	135888
$B_{24\,h}$	246	153574	293	85508	241	113476	247	142317
$B_{48\,h}$	252	155369	298	89775	248	119706	249	141946
Mean	252	150329	285	83004	245	113156	248	140050
S.D.	5.5	7231	17.8	8310	3.8	6715	1.2	3609
R.S.D. (%)	2.2	4.8	6.2	10.0	1.5	5.9	0.5	2.6
C_1	253	114144	290	62991	240	95884	272	127622
C_2	248	123134	248	64892	237	99599	268	125296
C_3	250	118704	281	65078	256	106946	267	133887
Mean	250	118661	273	64320	244	100809	269	128935
S.D.	2.5	4495	22.1	1155	10.2	5629	2.6	4443
R.S.D. (%)	0.4	3.8	8.1	1.8	4.2	5.6	1.0	3.4
$C_{12\,h}$	252	123393	258	62513	245	101241	262	123204
$C_{24\,h}$	249	119684	276	56471	247	100641	283	133007
$C_{48\,h}$	251	131020	259	63738	248	109393	238	123357
Mean	251	124699	264	60907	247	103758	261	126522
S.D.	1.5	5779	10.1	3890	1.5	4889	22.5	5616
R.S.D. (%)	0.6	4.6	3.8	6.4	0.6	4.7	8.6	4.4

[a] Each control sample contains 250 ng/mL each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP as the analyte, and also 250 ng/mL each of *d*-MA-d_8, *l*-MA-d_8, *d*-AP-d_8, and *l*-AP-d_8 as the IS in 1 mL of blank urine.

[b] Denotations (taking control A as an example): A_1, A_2, A_3: control A subjected to triplicate GC–MS analyses immediately after simultaneous LLE//*l*-TPC-ChD; $A_{12\,h}$, $A_{24\,h}$, $A_{48\,h}$: control A subjected to GC–MS analysis 12, 24, and 48 h (one run at a time) after simultaneous LLE//*l*-TPC-ChD.

alytes are all below 7.6%, and those calculated for the four analyte-to-IS peak-abundance ratios are all below 7.3%. As to the accuracy, the deviations calculated for the four means of the determined concentrations from their theoretical value, i.e., 250 ng/mL, are typically within −3.2%. Thus, the whole analytical scheme has proved to a certain extent reliable and robust.

Displayed in Table 7 are the relevant within-sample precisions calculated for two fashions of triplicate GC-EIMS SIM analyses of the three control samples. For the conventional triplicate GC–MS analyses (i.e., performing the triplicate GC–MS analyses at a time immediately after simultaneous LLE//*l*-TPC-ChD), the twelve within-sample RSDs calculated for the determined concentrations of the four analytes times three samples are at most 8.0%, and the twelve within-sample R.S.D.s calculated for the peak-abundance readings are at most 6.2%. For the "delayed" triplicate GC–MS analyses [i.e., performing the GC–MS analysis 12, 24, and 48 h (one run at a time) after simultaneous LLE//*l*-TPC-ChD], the twelve within-sample R.S.D.s calculated for the determined concentrations of the four analytes times three samples are at most 8.6%, and the twelve

within-sample R.S.D.s calculated for the peak-abundance readings are mostly below 10.0% except for control A giving three readings in the range of 12.7–16.3%. It follows that generally a good precision holds for the proposed GC-EIMS SIM analysis of *l*-TPC derivatized amphetamines until 48 h after the proposed simultaneous LLE/*l*-TPC-ChD.

4. Conclusions

The results presented in this report demonstrated that simultaneous LLE and *l*-TPC-ChD followed by isotope dilution GC-EIMS SIM is a sound analytical scheme for the complete resolution and evidential determination of AP and MA enantiomers in urine, and should meet the requirements of most of the workplace urine drug testing programs and even the criminal cases in Taiwan, ROC, where amphetamines are of zero tolerance.

While the forensic toxicologist has long and largely engaged in the analysis of amphetamines-generating drugs as well as the differentiation between the uses of illegal and legitimate forms of amphetamines-generating drugs or amphetamines themselves, most of the countries in the world so far have not incorporated the determination of amphetamines enantiomers into their key drug testing programs. The proposed analytical scheme is simple, rapid, effective, reliable, economic (it uses an ordinary column instead of a chiral-phase column, and uses screw-cap topped test tubes instead of SPE cartridges) and robust. It may serve as a confirmatory protocol for forensic urine drug testing. In addition, based on our previous experience in drug analysis, the proposed method with little or minor modification is also well suited for dealing with such sample matrices as blood and homogenized tissues. In this regard, LLE is superior to SPE in that for viscous liquid matrices conventional SPE cartridges may suffer from clogging. On the other hand, LLE using screw-cap topped test tubes, while free from clogging, is readily subjected to mechanical shaking and centrifugation in batches. Most important: LLE allows for simultaneous extraction and *l*-TPC-ChD whereas SPE cannot.

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