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Metabolites of ephedrines in human urine after administration of a single therapeutic dose

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Abstract

Ephedrine (EPH), pseudoephedrine (PEPH), phenylpropanolamine (PPA), methylephedrine (MEPH) and cathine are sympathomimetic amines. These drugs are commonly found in over-the-counter (OTC) cold medicines and some dietary supplements. In Taiwan, the misuse of these drugs has resulted in an increase in athletic violations.

Excretion studies of the ephedrine-related drugs have been performed to better understand the metabolic yields of ephedrines in urine. After consuming a single clinical dose of each of these drugs, urine samples from volunteers ($n = 3$ for each drug) were subjected to *tert*-butyl-methyl-ether (TBME) extraction and trifluoroacetic acid (TFAA) derivatization before gas chromatography–mass spectrometry (GC–MS) analysis. Most ephedrines were excreted unchanged in urine, including EPH (40.9%), PEPH (72.2%), and PPA (59.3%). However, only a relatively small amount of MEPH (15.5%) was excreted unchanged in urine. In addition, a trace amount of PPA (1.6%) and cathine (0.7%) was found to be the metabolites of EPH and PEPH, respectively. Urinary EPH, PEPH, and PPA reached peaks at 2–6 h and disappeared in urine at approximately 24–48 h post-administration. For MEPH, the peaks of excretion extended from 4 to 12 h post-administration and were undetectable at approximately 48 h. A single clinical dose of EPH (25 mg) may exceed threshold level (10 μg/mL) in sport drug testing if the urine samples are tested within approximately 8 h post-administration. However, a single dose of MEPH (20 mg) never reached the threshold value (10 μg/mL).

Keywords: Doping control; Ephedrines; GC–MS; GC–NPD

1. Introduction

Ephedrine (EPH), pseudoephedrine (PEPH), phenylpropanolamine (PPA), methylephedrine (MEPH), and cathine are sympathomimetic amines. Their action and structure are closely related to amphetamine. Among the ephedrines, EPH, PEPH, and MEPH are the ephedra alkaloids found naturally in various *Ephedra* species [1,2], while cathine (norpseudoephedrine) occurs naturally in the khat plant. Ephedrines are commonly included in over-the-counter (OTC) cold medications for the treatment of nasal congestion, allergies, asthma, cough, fever and headache [1]. Ephedrines have also been the ingredients of several dietary supplements found in sports nutritional supplements for boosting energy during exercise or as weight reduction aids [2,3]. Adverse effects of ephedrine-related preparations

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include tremor, hypertension, cardiac arrhythmia, psychosis, seizure, myocardial infraction, intracranial hemorrhage and even death [4–7].

In sports, ephedrines are generally thought to have ergogenic effects, although their effects on performance enhancement of athletes are still unclear [8–10]. Nevertheless, widespread use of ephedrines in sports, such as body building, powerlifting, cycling and hockey, has been reported [11]. In Taiwan, stimulants, followed by anabolic steroids and diuretics, were found most frequently in drug testing in athletes (data not shown). In a previous study, we found that the majority of positive cases in sport drug testing in Taiwan were ephedrine-related stimulants, including PPA, PEPH, and EPH. In addition, approximately 80% of OTC cold medicines were found to contain banned ephedrines, in which MEPH was the most common drug listed [12]. In the total urine specimens tested in doping control from 1999 to 2001, approximately 2.8% samples contained banned ephedrines, in which 1.3% exceeded the IOC threshold levels. Among the urine specimens that exceeded the IOC threshold values, PEPH accounted for 44%, followed by EPH (28%), PPA (17%), and MEPH (11%) [13].

Currently, the World Anti-Doping Agency (WADA) lists EPH, MEPH and cathine as the prohibited substances and places PEPH and PPA on the 2005 Monitoring Program [14]. In the present study, we investigated the metabolic products in urine after the volunteers orally administered a single clinical dose of EPH, PEPH, PPA and MEPH. The present study describes the excreted metabolites of ephedrines in urine detected and confirmed by gas chromatography–mass spectrometry (GC–MS) and quantified by gas chromatography–nitrogen–phosphrous detector (GC–NPD).

2. Experimental

2.1. Chemicals and standards

All reagents were of analytical grade. Trifluoracetic anhydride (TFAA) and *tert*-butyl-methyl ether (TBME) were purchased from Riedel-de Haën (Wunstorfer Street, 40 Seelze, Germany). Ethyl acetate, potassium carbonate, sodium hydrogen carbonate, and phenazine were purchased from Mallinckrodt (MO, USA). 1*S*,2*R*(+)-Ephedrine and *R*,*R*(−)-pseudoephedrine were purchased from Cerilliant (Austin, TX, USA). (+,−)-Phenylpropanolamine, (1*R*,2*S*)-(−)-*n*-methylephedrine and (1*R*,2*R*)-(−)-norpseudoephedrine (cathine) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Instrumentation and conditions

A Hewlett-Packard HP 5890 GC interfaced with a 5972 mass selective detector (MSD) was used for confirmation of the ephedrines and a model of Hewlett-Packard HP 6890 gas chromatograph equipped with a nitrogen–phosphorous detector (GC–NPD) was applied for quantification purpose. Both GCs were equipped with HP-5MS crosslinked 5% diphenyl and 95% dimethylpolysiloxane capillary column (25 m × 0.25 mm × 0.33 μm film thickness). Helium was used as carrier gas with split flow rate of 1.1 mL/min. One microliter of sample was injected with the autosampler.

For the GC–MS, the injection port and the interface temperatures were set at 250 and 300 °C, respectively. The initial temperature was 90 °C followed by raising 15 °C/min to 240 °C and 10 °C/min to 300 °C (holding time 5 min). The analysis was carried out in a full scan mode with electron impact ionization at 70 eV and mass spectrum was obtained by scanning from *m/z* 50–550. For the GC–NPD, the injection port temperature was 250 °C and the initial temperature was 100 °C (holding time 1 min), followed by raising 10 °C/min to 200 °C and 20 °C/min to 300 °C (holding time 4 min). One microliter of sample was injected with autosampler.

2.3. Urinary samples

The human subject research review committee approved this study. Six adult volunteers (5 males and 1 female) took part in the excretion studies. Each volunteer consumed more than one drug, in which the second drug was administered 7 days after the end of previous excretion study. Urinary specimens were collected at 0, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h after the volunteers orally administered a single clinical dose of the following drugs: EPH (25 mg; $n = 3$), PEPH (30 mg; $n = 3$), PPA (40 mg; $n = 3$) and MEPH (20 mg; $n = 3$). The standard stock solutions of authentic EPH, PEPH, PPA, MEPH, and cathine were prepared in methanol and kept at −20 °C until used.

2.4. Extraction procedure for GC–NPD analysis

An aliquot of 1 mL urine was delivered into a 20-mL glass tube, followed by addition of 10 μL diphenylamine (internal standard; 0.4 mg/mL), 100 μL KOH (5N), 0.6 g NaCl, and 1 mL TBME. The mixture was shaken mechanically for 10 min and centrifuged at 2000 rpm for 8 min. The organic layer was transferred to a glass vial containing 100 mg sodium sulfate and then directly subjected to GC–NPD analysis.

2.5. Extraction and derivatization procedures for GC–MS analysis

To 1 mL urine sample aliquot, 50 μL phenazine (internal standard; 0.45 mg/mL), 1 g $NaHCO_3$:K_2CO_3 (3:2 w/w; pH 9–9.5) and 1 mL TBME:2-propanol (9:1 v/v) were added, followed by shaking and centrifugation at 2000 rpm for 8 min. The organic layer was transferred and evaporated to dryness under nitrogen. The sample extract was derivatized in 100 μL

TFAA and incubated at 70 °C for 20 min. Subsequently, the sample was evaporated to dryness and reconstituted with 500 μL ethyl acetate before injection onto the GC–MS.

2.6. Calibration curves

When MEPH was analyzed by GC–MS, not only was its LOD relatively high, but also had poor chromatogram (Fig. 1). Consequently, all the quantifications of compounds in this study were carried out by GC–NPD. The calibration solutions were spiked in triplicates with appropriate amounts of authentic reference standards to the drug-free urine. One set (six different concentrations) of standards, including 2.5, 5, 10, 20, 40, and 80 μg/mL, was used for constructing EPH, PEPH, PPA and cathine calibration curves. Another set of standards, including 0.25, 0.5, 1, 2, 4 and 8 μg/mL, was prepared for constructing a MEPH calibration curve. The concentration of the internal standard (IS; phenazine) was 100 μg/mL. The calibration curve for linear regression analysis of each analyte was constructed by plotting the peak area ratios of the reference standard and the internal standard versus the various concentrations of the analyte.

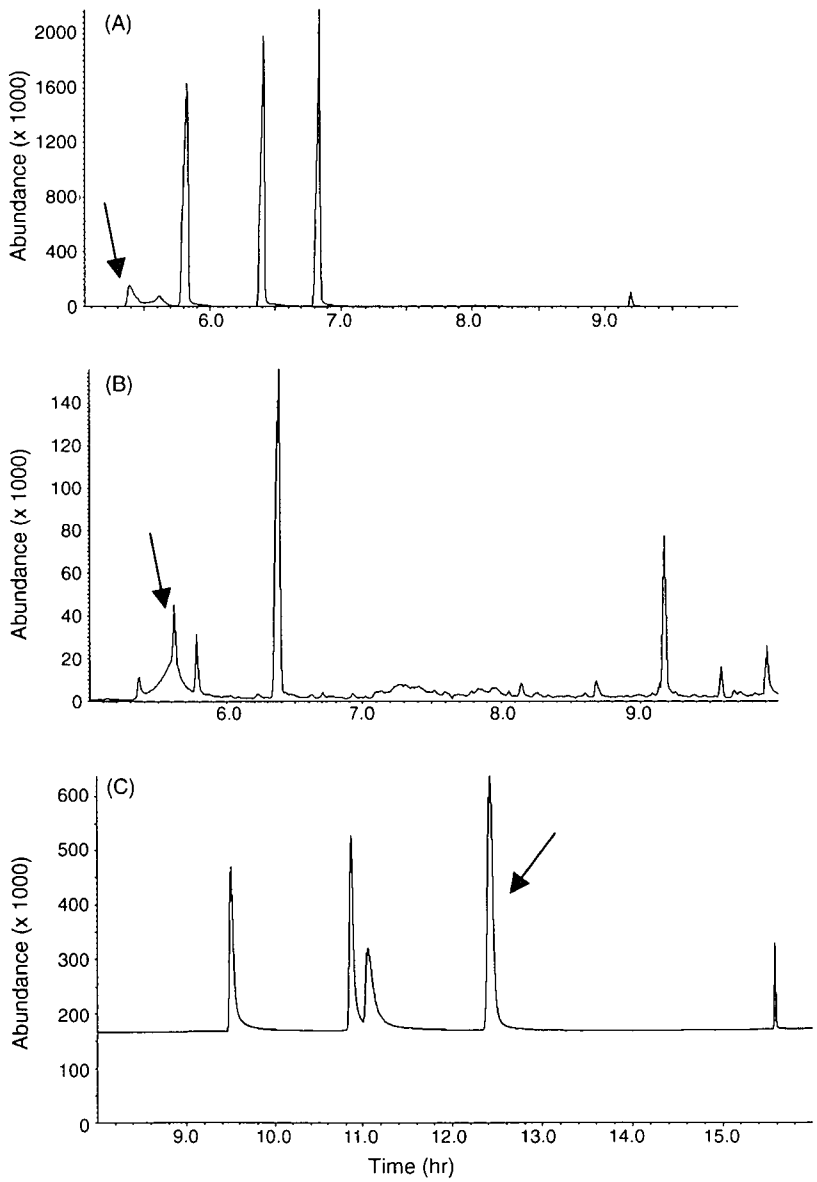

Fig. 1. Chromatograms of MEPH analyzed by GC–MS and GC–NPD. MEPH detected by GC–MS in spiked (100 μg/mL) urine (A) and in excreted urine (B). MEPH was spiked in urine and detected by GC–NPD (C). Arrows denote MEPH peaks.

Table 1
Limit of detection (LOD) and linearity of each compound analyzed by GC–NPD

Drug	LOD (μg/mL)	Concentration range (μg/mL)	Equation	r^2
EPH	0.11	2.5, 5, 10, 20, 40, 80	$y = 0.3544x - 0.6029$	0.9996
PEPH	0.08	2.5, 5, 10, 20, 40, 80	$y = 0.2195x + 0.1657$	0.9987
PPA	0.21	2.5, 5, 10, 20, 40, 80	$y = 0.3772x + 0.4305$	0.9993
MEPH	0.02	0.25, 0.5, 1, 2, 4, 8	$y = 0.1023x + 0.0249$	0.9916
Cathine	0.25	2.5, 5, 10, 20, 40, 80	$y = 0.2889x - 0.8356$	0.9989

Table 2
Recovery of ephedrines analyzed by GC–NPD

Amount (μg/mL)	Percentage recovery (%CV)				
	EPH	PEPH	PPA	MEPH	Cathine
GC–NPD					
5	85.7 (7.3)	66.6 (6.3)	105.0 (13.3)	85.3 (6.3)	74.8 (3.1)
25	94.4 (2.7)	91.9 (2.8)	86.3 (3.2)	83.7 (2.5)	68.5 (6.4)
100	103.5 (1.6)	91.9 (2.8)	85.8 (1.5)	81.4 (1.3)	[a]

[a], No analysis was carried out.

3. Results and discussion

3.1. Analytical parameters

3.1.1. Limit of detection (LOD) and linearity

The LOD is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte [15]. Accordingly, the LODs of the ephedrines, measured by GC–NPD, were obtained from the results after analyzing seven aliquots of a known amount of each analyte as described in Ref. [15]. The LODs obtained for EPH, PEPH, PPA, MEPH and cathine were 0.11, 0.08, 0.21, 0.02 and 0.25 μg/mL, respectively.

Linear calibration curves were established for the ephedrines by the procedure described in Section 2. The calibration curves were linear from 2.5 to 80 μg/mL for EPH, PEPH, PPA and cathine and from 0.25 to 8 μg/mL for MEPH (Table 1).

3.1.2. Recovery

For the recovery studies, three different target concentrations, i.e. 5, 25 and 100 μg/mL, were applied to EPH, PEPH, PPA and MEPH, and two to cathine. Five replicates for each concentration were analyzed by GC–NPD to determine extraction efficiency of these substances during the sample preparation procedures. The recoveries for 5, 25, and 100 μg/mL ranged from 85.7 to 103.5% for EPH; 66.6–91.9% for PEPH; 85.8–105% for PPA; and 81.4–85.3% for MEPH. For cathine, the recoveries for two target concentrations, 5 and 25 μg/mL, were found to be 74.8 and 68.5%, respectively (Table 2).

3.1.3. Accuracy and precision

The intra-assay accuracy and precision of EPH, PEPH, PPA and MEPH were determined in a single analytical batch of six replicates and four target concentrations by GC–NPD (Table 3). Four target concentrations at 7.5, 15, 30 and 60 μg/mL were applied for EPH, PEPH and PPA; and at 2.5, 5, 20 and 40 μg/mL were used for MEPH. Over the range of target concentrations analyzed, the overall accuracy of the compounds ranged from 87.0 to 126.7% with levels of precision were well within an acceptable range, i.e. from 0.5

Table 3
Accuracy and precision for the determination of MEPH, EPH, PEPH and PPA by GC–NPD

	Amount (μg/mL)	Intra-assay		Interassay	
		Target (%)	%CV	Target (%)	%CV
EPH	7.5	87.6	0.5	80.1	4.8
	15	88.8	1.5	86.6	5.6
	30	90.9	1.8	88.9	4.4
	60	94.2	0.6	91.5	3.1
PEPH	7.5	102.8	2.3	91.6	7.3
	15	87.0	1.2	85.8	7.4
	30	87.1	1.2	84.3	5.4
	60	94.1	0.7	90.3	
PPA	7.5	109.8	3.2	105.7	6.6
	15	113.0	2.3	111.2	5.0
	30	110.7	3.5	110.6	3.9
	60	111.9	1.1	112.1	2.2
MEPH	2.5	126.7	1.6	126.8	8.9
	5	105.5	1.9	114.6	6.6
	20	99.9	1.9	99.9	3.5
	40	105.2	1.1	105.1	2.3

Table 4
Mean concentrations of unchanged parent compound in urine

Time (h)	Mean ± S.D. (μg/mL)			
	EPH	PEPH	PPA	MEPH
0	0	0	0	0
2	**22.3 ± 16.1** (2.2)	50.6 ± 52.6	67.7 ± 77.8	1.0 ± 1.8
4	**26.9 ± 29.2** (2.7)	76.6 ± 53.0	62.5 ± 38.2	8.3 ± 7.7
6	**24.1 ± 21.0** (2.4)	115.5 ± 143.8	37.7 ± 27.9	3.0 ± 1.4
8	**12.5 ± 6.2** (1.3)	47.0 ± 4.9	25.9 ± 12.6	4.0 ± 3.8
12	9.3 ± 4.6	26.5 ± 4.9	13.0 ± 6.8	6.1 ± 6.0
24	3.7 ± 1.1	6.4 ± 4.9	5.8 ± 2.0	0.2 ± 0.2
48	0	0.4 ± 0.6	0	0.2 ± 0.4
72	0	0	0	0
96	0	0	0	0

Note: Bold numbers denote the concentrations in each drug that exceeded threshold values, i.e. 10 μg/mL for EPH and MEPH; number in parenthesis denotes threshold ratio of measured value and threshold value.

to 3.5%. The interassay accuracy and precision were determined from six separate analytical runs, using the same concentrations as used in the intra-assay studies and here, too, the results for accuracy were comparable to the intra-assay analyses with the precision varied slightly higher. However, the interassay accuracy measured MEPH concentrations ranged slightly higher, from 99.9 to 126.8% with precision ranging from 2.3 to 8.9%.

3.2. Urinary metabolic characteristics

3.2.1. Urinary metabolic products of ephedrines

After the volunteers orally received EPH (25 mg), PEPH (30 mg), PPA (40 mg) and MEPH (20 mg), urine samples collected from 0 to 96 h were quantified by GC–NPD (Table 4). The unchanged compounds of EPH, PEPH, and PPA in urine reached peaks at approximately 2–6 h post-administration and almost completely eliminated from the body system at approximately 24–48 h. For MEPH, however, the peaks of concentration reached between 4 and 12 h post-administration and completely eliminated in approximately 48 h.

A metabolic scheme of ephedrines is proposed in Fig. 2. The total unchanged parent compounds of EPH, PEPH, PPA and MEPH in urine were found to be 40.9% (10.2 mg), 72.2% (21.7 mg), 59.3% (23.7 mg) and 15.5% (3.1 mg), respectively (see Fig. 2). These results illustrated that a wide range of the excreted amount of these unchanged compounds were present in urine. The small recovery of MEPH in urine was likely to be attributed to metabolism itself rather than analytical factors, since the recovery studies showed comparable results between MEPH and the other ephedrines (see Table 2). Low excretion of MEPH in urine was also reported by other studies, in which 33–40% unchanged parent compound were found [1,16].

In addition to the unchanged parent compounds were present in urine in excretion studies, relatively minute metabolic products were noted, including PPA (1.6%), EPH (1.6%) and cathine (0.7%) which were metabolites of EPH, MEPH and PEPH, respectively. These results were basically in agreement with studies done by others [17,18]. However, no any other metabolic product, except the unchanged parent compound, was detected in urine when PPA was consumed. These results support the finding from a study done by Heimlich et al. [19]. It was reported that the minute amounts of metabolites were undergone *N*-demethylation before being excreted in urine [1,20]. Our results showed that most of the ephedrines left body system at early hours (2–6 h) post-administration and completely eliminated at approximately 24–48 h. This finding is in agreement with that of the other's [20]. Although concentration of

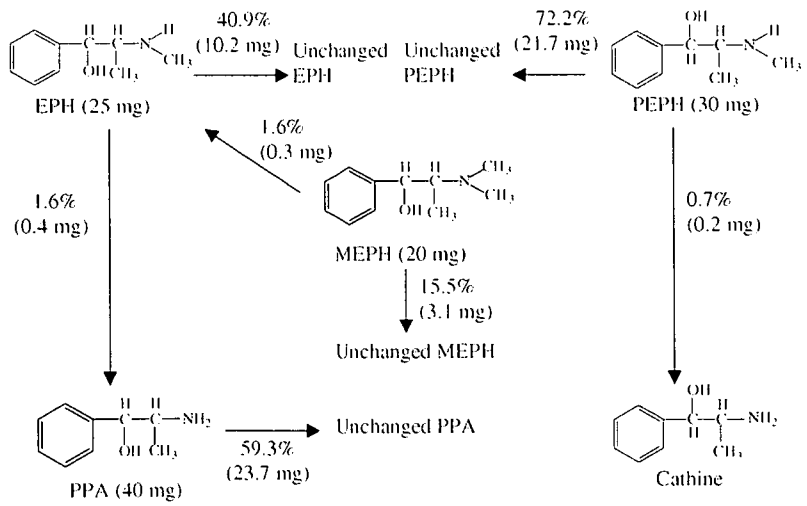

Fig. 2. Scheme for metabolism of ephedrines in urine. Total amount of each metabolite found in urine is shown in parenthesis and its corresponding percent of the dose administered.

MEPH in urine was low, it remained in body system longer than the other compounds analyzed.

3.3. Analytical approach and implications of analytical findings

3.3.1. Analytical approach

When GC–MS was used for analyzing ephedrines, two problems existed: (1) a poor chromatographic quality displayed in MEPH analysis and (2) co-elution of PPA and cathine diasteroisomers shown in the chromatogram. In our routine procedure for the detection of ephedrines and other stimulants, GC–NPD is not only used to screen for volatile nitrogen-containing and excrete free compounds [21], but also enable us to separate two pairs of diasteroisomers (EPH/PEPH and PPA/cathine) [13]. Consequently, the quantification of the ephedrines was carried out by GC–NPD. Recently, a study showed that EPH/PEPH and PPA/cathine two pairs of diasteroisomers were separable by GC–MS when derivatization reagents *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), trimethylsilyl-imidazole (TSIM), and *N*-methyl-bis-trifluoroacetamide (MBTFA) were used [22]. Another study reported that these two pairs of diasteroisomers could be separated by derivatizing with carbon disulfide [23].

3.3.2. Implications of analytical findings in sports drug testing

According to the current (2005) World Anti-Doping Code, only EPH, MEPH and cathine are listed as prohibited substances and their cut-off levels are 10 μg/mL for EPH and MEPH, and 5 μg/mL for cathine [14]. In the present study, we found that the average concentrations of EPH exceeded the cut-off value after a single clinical dose (25 mg) was taken. The EPH exceeded cut-off value as early as 2 h post-administration and prolonged until approximately 8 h. However, the concentration levels of MEPH never reached the threshold value with the single dose (20 mg) administered in this study (Table 4). The low yield of MEPH in urine may be attributed to the nature of its overall metabolism as mentioned previously. This result may also explain our previous findings that although MEPH had the highest occurrence rate among ephedrines in the ingredient lists (52%) of the OTC cold medicines tested, it had the lowest positive rate (11%) in our drug testing in sports [13].

When a ratio of EPH concentration detected at each time point of urine collection was determined by the cut-off level, the value could reach 2.7-folds over the cut-off concentration (Table 4). In other words, one single dose (25 mg) of ephedrine may cause doping violation if an athlete administered 25 mg EPH within 8 h being tested. For MEPH, however, the ratios at any time points of post-administration were all below 1.0 suggesting an adverse result was not likely unless a larger dose (>20 mg) was administered. Because in our laboratory, cathine was not available for carrying out an excretion study, data on its metabolic products are, therefore, not shown.

4. Conclusions

A wide range of unchanged ephedrines was found present in urine after oral administration. The excretion rate of metabolites could reach peak as early as 2 h post-administration. If an athlete orally administers a single clinical dose (25 mg) of EPH and is tested within 8 h post-administration, he/she is likely to be sanctioned according to the current doping control rules. However, for MEPH to exceed the threshold value in urine, a dose higher than 20 mg is necessary.

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