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# Enantiospecific gas chromatographic–mass spectrometric analysis of urinary methylphenidate: Implications for phenotyping

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**Abstract**

A chiral derivatization gas chromatographic–mass spectrometric (GC–MS) method for urine methylphenidate (MPH) analysis was developed and validated to investigate preliminary findings regarding a novel MPH poor metabolizer (PM). Detection was by electron impact (EI) ionization-selected ion monitoring of the *N*-trifluoroacetylprolylpiperidinium fragments from MPH and the piperidine-deuterated MPH internal standard. The PM eliminated ~70 times more l-MPH in urine (9% of the dose over 0–10 h), and ~5 times more of the d-isomer (10% of the dose), than the mean values determined from 10 normal metabolizers of MPH. Only minor amounts of the metabolite *p*-hydroxy-MPH were found in the urine of both the PM and normal metabolizers, while the concentration of MPH lactam was not high enough to be detectable. The described method indirectly gauges the functional carboxylesterase-1 status of patients receiving MPH based on the evaluation of relative urine concentrations of d-MPH:l-MPH. Clinical implications concerning rational drug selection for an identified or suspected MPH PM are discussed.


*Keywords:* Methylphenidate; Esterase; Pharmacogenetics; Enantiomers; Urine

## 1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is among the most common childhood neuropsychiatric conditions diagnosed and treated worldwide. Additionally, it is increasingly recognized as being prevalent among adults [1]. Psychostimulant medications typically represent first line agents in the pharmacotherapy of ADHD, foremost of these being dl-methylphenidate (MPH; Fig. 1). Optimization of pharmacotherapy in ADHD generally requires some empiricism and approximately 20–30% of ADHD patients do not respond favorably to dl-MPH at any dose [2]. New medications for ADHD continue to be developed and include enantiopure d-MPH [3].

Following oral administration of dl-MPH, stereoselective first-pass metabolism typically limits the systemic exposure of the l-isomer to only 1–3% of the dose, while approximately 25% of d-MPH reaches the blood stream [4,5]. In effect, "in vivo resolution" of oral dl-MPH results in only the pharmacologically active d-isomer from appreciably accessing the central nervous system [6].

Most therapeutic drug monitoring studies of dl-MPH have utilized achiral analytical methods, and hence, were incapable of investigating any potential correlations between pharmacodynamics and MPH enantiomeric disposition. However, Jonkman et al. [7] reported in a pilot study that plasma concentrations of both l-MPH and d-MPH were significantly higher in ADHD children who did not respond favorably ($n=4$) to dl-MPH than in those who responded well ($n=8$). Mean plasma l-MPH concentrations were found to be approximately 50% that of d-MPH, regardless of response status. It is noted that such relatively high l-MPH plasma concentrations are unusual except following transdermal administration [8].

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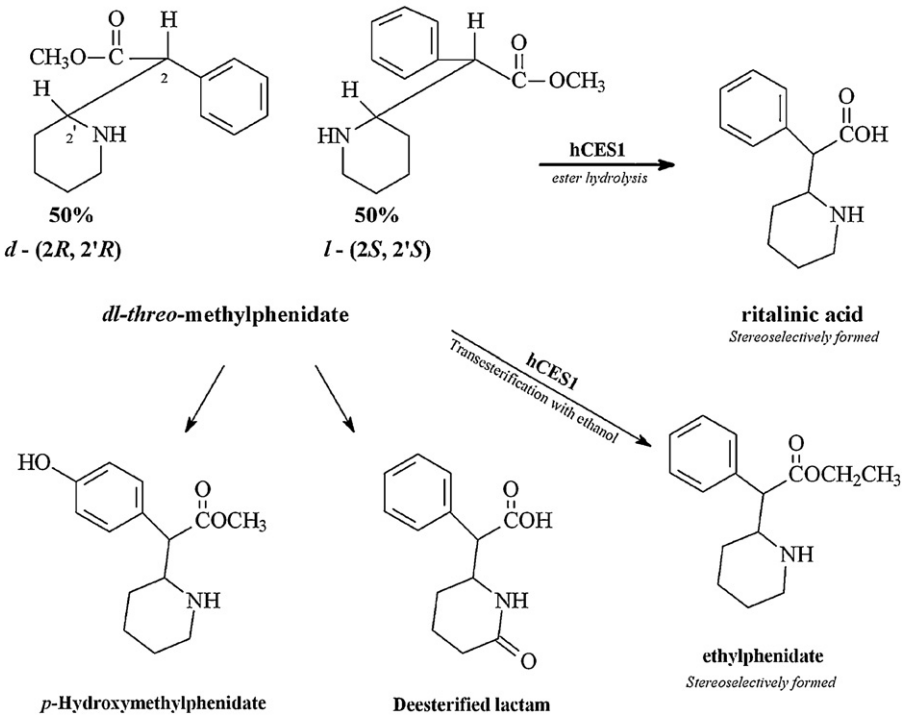


Fig. 1. Structures of MPH enantiomers and corresponding human metabolites. Figure adapted from Patrick et al. [16].

Patrick et al. [9] applied chiral liquid chromatography–mass spectrometry (LC–MS) to a single dose pharmacokinetic study of 20 normal adult subjects orally dosed with immediate-release dl-MPH·HCl (0.3 mg/kg) with or without ethanol. In that study, one individual was found to have l-MPH plasma concentrations 1–2 orders of magnitude greater than typical l-MPH values, and d-MPH plasma concentrations were also well above expected values (Fig. 2). Further, this individual, who we will refer to hereafter as a poor metabolizer (PM) of MPH, did not form any detectable ethylphenidate which is the esterase mediated transesterification product of concomitant MPH and alcohol. Whether this MPH PM status represents a distinct pharmacogenetic polymorphism, i.e., ≥1% of the population, or only a rare metabolic defect, remains to be established. Pharmacogenomic studies are in progress [10].

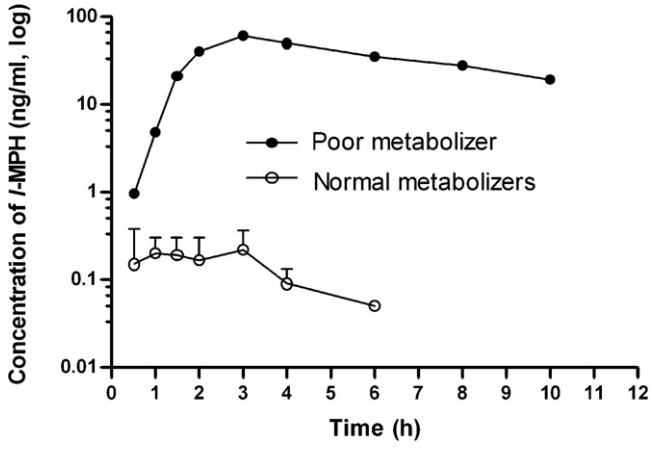


Fig. 2. l-MPH plasma concentration over time for the MPH PM (●) compared to the mean values for 19 normal metabolizers (◯). Subjects received MPH·HCl (0.3 mg/kg).

The present investigation extends the plasma MPH isomeric disposition findings for this PM to urine analysis, which in turn was compared to urine MPH concentrations from 10 study peers (5 male and 5 female) previously found to exhibit normal MPH pharmacokinetics [9]. Urine sampling offers some advantages over plasma sampling for exploring MPH PM population prevalence: (a) MPH concentrates in urine, allowing more routine and less sensitive analytical instrumentation than chiral column LC–MS/MS [9]; (b) urine sampling is less invasive and less expensive than plasma sampling; (c) urine does not carry the risk of blood-born disease transmission.

The following describes a practical gas chromatographic–mass spectrometric-electron impact (GC–MS-EI) method to establish normal versus PM MPH status based on relative urinary l-MPH versus d-MPH concentrations. GC separable MPH diastereomers were formed using commercial (*S*)-*N*-trifluoroacetylprolyl chloride (TFP-Cl) and piperidine-deuterated dl-MPH was utilized for analytical control. Earlier methods for enantiospecific urinary MPH analysis have used GC-nitrogen phosphorous detection [11] and GC-electron capture detection [12], and synthetic (*S*)-*N*-heptafluorobutyrylprolyl chloride with adamantanemethylamine as the internal standard.

As a secondary component to the present investigation, the prospect that the MPH PM may excrete elevated levels of normally minor MPH metabolites was explored. The metabolic fate of MPH in humans is well characterized. Rapid methyl ester hydrolysis usually limits the half-life to 2–3 h

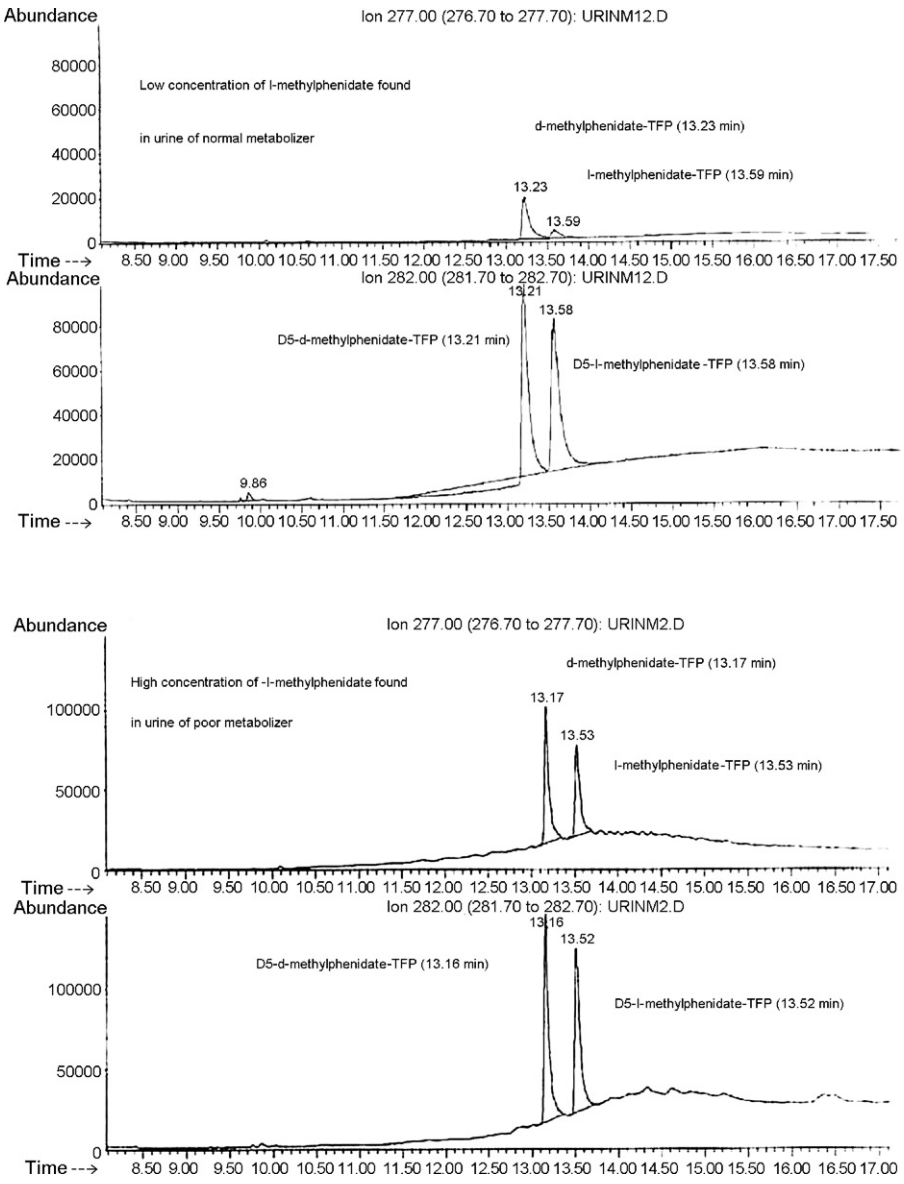


Fig. 3. GC–MS-EI ionization of urine extracts from a MPH normal metabolizer (top two ion profiles) and from the MPH PM (bottom two ion profiles). Detection was by SIM of the TFP-piperidinium fragment *m/z* 277. Chiral derivatization generated separable diasteriomers and the piperidine-deuterated MPH provided analytical control ($d_5$-chromatograms). The urinary concentration of both l-MPH and d-MPH was greatly elevated in the PM.

[5], with the exception being the newly identified PM [9]. This deesterification pathway yields the inactive [13] metabolite ritalinic acid (Fig. 1), which accounts for most of a MPH dose in the urine [14,15]. The highly elevated plasma l-MPH concentrations observed in the MPH PM presented the possibility that compensatory increases in usually minor metabolic pathways might prevail given the apparent impairment in the enzymatic hydrolysis rate. Accordingly, the pathways of *para*-hydroxylation to the active metabolite HO-MPH [13] and/or 6′-oxidation [14] to the inactive [15] MPH lactam were also examined. Evidence of enantioselective hydroxylation of l-MPH in rats [16] and baboons [6], as well as the possible accumulation of l-HO-MPH in brain [6], further compelled the investigation of alternative oxidative biotransformation pathways.

## 2. Experimental

dl-MPH·HCl in methanol (1 mg/mL calculated as free base; Cerilliant, Round Rock, TX), TFP-Cl in dichloromethane (1 M; Aldrich, St. Louis, MO), sodium carbonate (Fischer Scientific, Fair Lawn, NJ), acetonitrile (Mallinekrodt Inc., Paris, KT), *n*-butyl chloride (Burdick & Jackson, Muskegon, MI), and glusulase (glucuronidase/arylsulfatase; Roche Applied Sciences, Indianapolis, IN) were used. dl-HO-MPH·HCl [13,17,18] and piperidine-deuterated dl-MPH·HCl [9,17–19] were synthesized in house and contained approximately 25% of the $D_5$-isotopolog and no detectable $D_0$. It is noted that piperidine-deuterated $D_9$-MPH·HCl has recently become commercially available (Cerilliant). The MPH lactam was donated by Ciba-Geigy Corp.

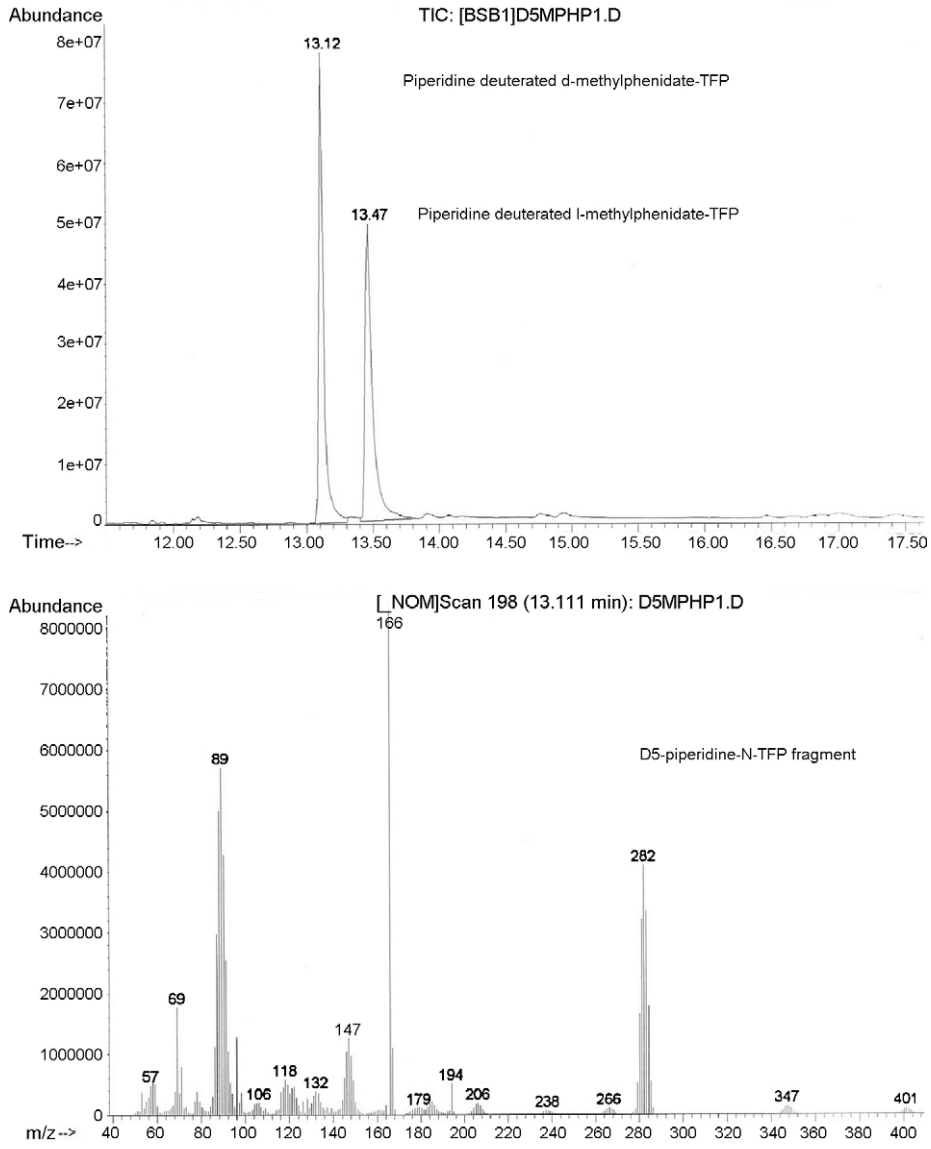


Fig. 4. GC–MS-EI total ion monitoring of a $d_2$–$d_8$ deuterated MPH isotopolog mixture. Selected monitoring of $d_5$-MPH (TFP-piperidinium fragment *m/z* 282) served as the internal standard.

### 2.1. Research subjects

The study was conducted in the General Clinical Research Center at the Medical University of South Carolina. The Office of Research Integrity approved the written informed consent provided by each subject. Normal adult volunteers were used, aged 23–40 years and deemed healthy based on medical history, physical examination, 12-lead electrocardiogram, and routine laboratory tests including complete blood count, serum electrolytes, blood glucose, urinalysis, urine drug screen and liver function indices. All subjects were also non-smokers within 15% of ideal body weight.

### 2.2. Drug administration and sampling

The study required subjects to arrive the evening prior to the test session, begin fasting, and stay overnight. Subjects were given 15 min to eat an entire standard breakfast and then received an oral dose of immediate-release dl-MPH·HCl (Ritalin®; 0.3 mg/kg) 1 h later using 5 and 10 mg tablets with the 5 mg tablets cut to the nearest 2.5 mg. A standardized lunch was also provided during the course of the study and consumed within 30 min. All urine was collected over 10 h from the time of MPH dosing, total volumes were recorded, and samples stored at 2–5 °C throughout the day to minimize any potential post-sampling hydrolysis [20]. Three 10 mL aliquots from the total collected urine were taken and frozen at −70 °C until analysis. Blank urine for the calibrators was collected from a volunteer not exposed to dl-MPH·HCl.

### 2.3. Instrumentation

All analyses were conducted with an Agilent Model 6890 GC-5973N MS with ChemStation. GC separations were on a 30 m × 0.32 mm, 0.25 μm film thickness (DB-5 J & W Scientific, Folsom, CA) 5% phenylmethylpolysiloxane fused-silica

column. Pulsed-splitless injections (2 μL) were used. The injector port was operated at 250 °C and the helium carrier gas linear velocity was 50 cm/s. EI ionization (70 eV) was used for MPH and the MPH lactam analyses. Negative ion chemical ionization (NICI; methane) was used to maximize sensitivity in HO-MPH analyses.

### 2.4. Method validation and MPH sample analysis

The lower limit of quantification (LLOQ) was estimated when the accuracy was within 80–120%, and the relative standard deviation (%R.S.D.) of precision studies was within 20%. The accuracy and precision of the assay were determined from the analysis of blank human urine samples spiked with MPH isomers at three different concentrations (20, 150 and 500 ng/mL) on three successive days. The extraction recovery of MPH (40, 300 and 1000 ng/mL) and piperidine-deuterated dl-MPH (1000 ng/mL) was performed by comparing the peak areas of extracted samples to that of extracted blank urine spiked after work-up with MPH and piperidine-deuterated dl-MPH that represent 100% recovery. Five determinations of each concentration have been applied to all these validation studies [21].

For human sample analysis, blank urine aliquots (2 mL) in 15 mL screw-cap test tubes were fortified with dl-MPH to provide 0, 100, 250, 1000, 5000 and 10,000 ng/mL of each MPH isomer, generating two separate enantiomeric calibration curves. Subject urines were thawed and 2 mL aliquots were run in parallel with the calibrators. Piperidine-deuterated dl-MPH (10 μg) was added as an internal standard to the calibrators and unknowns. The pH was adjusted to approximately 9.5 using 1.2 M sodium carbonate and extracted with 3 mL of *n*-butyl chloride:acetonitrile (4:1) by vortexing for 1 min. The organic phases were transferred into 4 mL screw-cap silanized vials and the solvent was evaporated to dryness under nitrogen. Chiral derivatization used TFP-Cl (200 μL: 1 M in dichloromethane chloride) with Teflon® capped vials heated at 58 °C for 35 min. The samples were diluted with dichloromethane to a total volume of 0.5 mL and transferred to silanized GC autosampler vials for GC–MS analysis.

MPH isomers were detected by EI selected ion monitoring (SIM) of the TFP-piperidyl fragment *m/z* 277 for the analyte and *m/z* 282 for the pentadeuterated isotopolog. The GC was held at 70 °C for 1.5 min, then ramped to 315 °C at 15 °C/min and held for 4 min leading to a total run time of 18 min.

### 2.5. HO-MPH and dl-MPH lactam reference standards

The standard of dl-HO-MPH (1 μg) was treated with TFP-Cl (200 μl; 1 M in dichloromethane) at 58 °C for 35 min to yield *N*,*O*-$(TFP)_2$ diastereomeric derivatives of d- and l-HO-MPH suitable for chiral analysis. NICI (methane) was used for urine analysis, with detection by SIM of the base peak ion *m/z* 441 [M-TFP; *m/z* 635 M (10%)]. The GC was held at 120 °C for 1.5 min, then ramped to 280 °C at 15 °C/min and held for 6 min. It was then ramped at 20 °C/min to 315 °C and held for 11 min for a total run time of 31 min. The dl-MPH-lactam, being incapable of chiral derivatization, was used directly as a methanolic solution (~50 μg in 150 μL) for GC–MS-EI. The GC was held at 70 °C for 1.5 min, then ramped at 20 °C/min to 315 °C and held for 16 min: total run time 30 min.

### 2.6. Screening for HO-MPH and MPH lactam in urine

Urine (2 mL) was added to screw-cap test tubes (15 mL) and the pH adjusted to ~5.5 with 2 M sodium acetate. Glusulase (50 μL β-glucuronidase/arylsulfatase) was added and then incubated for 2.5 h at 37 °C. The pH was adjusted to ~9.5 with 1.2 M sodium carbonate and the samples were extracted with *n*-butyl chloride:acetonitrile (3 mL; 4:1) by vortexing for 1 min. The organic phases were transferred to 4 mL screw-cap silanized vials and the solvent was evaporated to dryness under nitrogen. The residues were treated with TFP-Cl (200 μL: 1 M in dichloromethane) as above. The cooled solutions were transferred into GC autosampler vials and brought to 0.5 mL with dichloromethane for analysis. The MPH lactam was not detectable by GC–MS-EI-total ion monitoring of an extracted urine sample from the MPH PM, nor from that of a normal MPH metabolizer. Further, extracted base peak 6′-oxopiperidinium ion profiling was used to enhance the potential for detection.

## 3. Results

### 3.1. Method validation

An enantiospecific GC–MS-EI method for analyzing MPH was developed, validated (Table 1) and used to distinguish the unique MPH urinary excretion profile of a MPH PM relative to normal individuals (Table 2; Fig. 3). Detection was by SIM of the high abundance (74%) *m/z* 277 TFP-piperidine fragment of MPH [piperidinium *m/z* 84 (100%)]. Chiral derivatization and a GC temperature program allowed baseline separation of d-TFP-MPH ($t_R$ = 13.2 min) and l-TFP-MPH ($t_R$ = 13.5 min). The isomer elution order was established as previously described [22,23]. Piperidine-deuterated MPH provided for analytical control in quantitative determinations (Fig. 4). This internal standard was composed of all deuterium isotopologs from $d_2$ to $d_8$, with $d_5$ in the highest percentage, i.e., approximately 25% [17,18]. Accordingly, the $d_5$-TFP-MPH fragment *m/z* 282 was monitored. Urine calibrators fortified with racemic MPH generated calibration plots for 0–1000 ng/mL d-MPH ($R^2$ = 0.998) and l-MPH ($R^2$ = 0.998). These calibrators were run in parallel with the unknowns to permit quantitation by linear regression. Urine calibrators at 5000 and 10,000 ng/mL were also analyzed with the calibators in the event that such high MPH concentrations may be relevant to the PM. However, with these two highest concentrations being several-fold higher than those found in the urine of any subject in the present 0.3 mg/kg dl-MPH oral dose study, these two highest concentration calibrators were not entered into the linear regression analysis to avoid biasing the low ng/mL l-MPH determinations found for the normal metabolizer urines.

LLOQ was found to be 10 ng/mL when using 2 μL injections from 500 μL reconstituted 2 mL urine extracts. The accuracy and precision were determined at three concentrations (20, 150 and 500 ng/mL) in five replicates. The results in

Table 1
Intra- and inter-day precision and accuracy of GC–MS analysis of MPH enantiomers in human urine

| | Spiked concentration (ng/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 20 | | 150 | | 500 | |
| | d-MPH | l-MPH | d-MPH | l-MPH | d-MPH | l-MPH |
| Batch 1 ($n=5$) | | | | | | |
| Observed intra-day mean (ng/mL) | 19.28 ± 0.79 | 19.66 ± 0.61 | 143.04 ± 5.59 | 145.89 ± 6.83 | 499.25 ± 7.15 | 526.61 ± 26.23 |
| Intra-day precision (%) | 4.07 | 3.12 | 3.91 | 4.68 | 1.43 | 4.98 |
| Intra-day accuracy (%) | 96.40 | 98.31 | 95.36 | 97.26 | 99.85 | 105.32 |
| Batch 2 ($n=5$) | | | | | | |
| Observed intra-day mean (ng/mL) | 18.78 ± 0.68 | 20.04 ± 2.15 | 149.31 ± 3.48 | 146.38 ± 8.25 | 520.80 ± 16.64 | 517.80 ± 34.03 |
| Intra-day precision (%) | 3.64 | 10.70 | 2.33 | 5.64 | 3.20 | 6.57 |
| Intra-day accuracy (%) | 93.89 | 100.21 | 99.54 | 97.59 | 104.16 | 103.56 |
| Batch 3 ($n=5$) | | | | | | |
| Observed intra-day mean (ng/mL) | 20.74 ± 0.98 | 20.98 ± 1.76 | 143.03 ± 1.99 | 136.79 ± 11.30 | 520.15 ± 43.52 | 500.70 ± 36.49 |
| Intra-day precision (%) | 4.74 | 8.40 | 1.39 | 8.26 | 8.37 | 7.29 |
| Intra-day accuracy (%) | 103.71 | 104.89 | 95.35 | 91.19 | 104.03 | 100.14 |
| Inter-day ($n=15$) | | | | | | |
| Observed inter-day mean (ng/mL) | 19.60 ± 1.15 | 20.23 ± 1.62 | 145.13 ± 4.78 | 143.02 ± 9.49 | 513.40 ± 27.24 | 515.04 ± 32.12 |
| Inter-day precision (%) | 5.88 | 8.03 | 3.30 | 6.64 | 5.31 | 6.24 |
| Inter-day accuracy (%) | 98.00 | 101.14 | 96.75 | 95.35 | 102.68 | 103.01 |

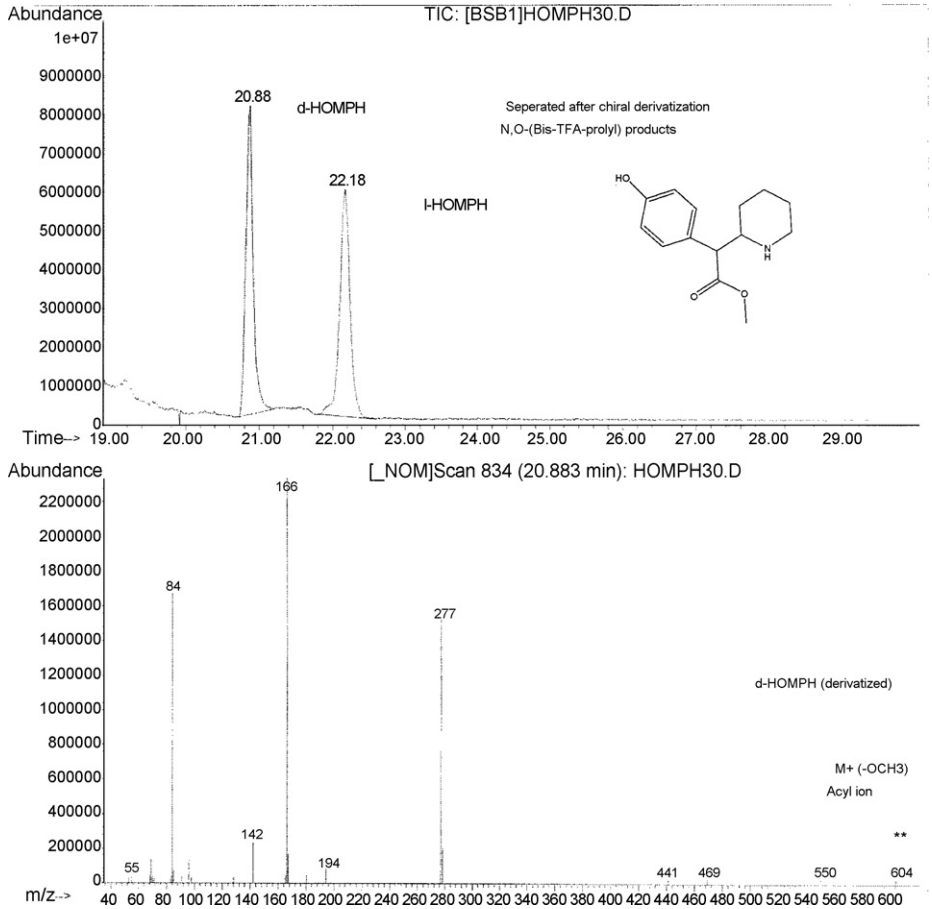


Fig. 5. GC–MS-NICI ion chromatogram of reference standard *N*,*O*-bis-TFP-dl-HO-MPH. The d-(*R*:*R*′)-HO-MPH ($t_R$ = 20.88 min) and l-(*S*:*S*′)-HO-MPH ($t_R$ = 22.18 min) configurations were tentatively assigned (see Section 3). *p*-Hydroxylation is a minor MPH metabolic pathway in humans but was investigated as an alternative metabolic pathway for the MPH esterase PM.

Table 1 showed the accuracy and precision of the developed method meet generally accepted requirements [21]. The recovery of MPH at the concentrations of 40, 300 and 1000 ng/mL and piperidine-deuterated dl-MPH (1000 ng/mL) were found to be 62.71 ± 9.30%, 63.75 ± 3.30%, 70.01 ± 9.95%, and 64.55 ± 6.50%, respectively.

### 3.2. Differential excretion of d- and l-MPH in a PM

The PM was found to have excreted nearly 70 times more l-MPH, and 5 times more d-MPH, than the mean values for 10 normal metabolizers of dl-MPH (Table 2). The isomeric MPH concentrations quantified from the PM in duplicate sample analyses were 958 and 1059 ng/mL for l-MPH with 1176 and 1194 ng/mL for d-MPH. All subjects in the present study had previously been phenotyped through plasma analyses [9].

### 3.3. Potential alternate metabolic pathways

Reference standards of dl-HO-MPH·HCl (Fig. 5) and the MPH lactam (Fig. 6) were analyzed by GC–MS to explore the possibility of alternative metabolic pathways being favored in an individual with impaired esterase activity. The GC–MS of reference standard dl-HO-MPH demonstrated baseline resolution of the two isomers following chiral derivatization. Tentative assignment of the d-(*R*,*R*) and l-(*S*,S)-HO-MPH configurations to the $(TFP)_2$-HO-MPH isomers generating chromatographic peaks at $t_R$ = 20.9 and 22.2 min, respectively, was based on the corresponding elution orders of enantiomeric TFP-MPH [22] and TFP-ethylphenidate [23].

Following glucuronide cleavage with glusulase, and chiral derivatization, GC–MS analysis of urine from the MPH PM and from a normal metabolizer revealed chromatographic peaks corresponding to the HO-MPH isomers. However, neither sample appeared to contain more than low ng/mL concentrations of HO-MPH, albeit more readily detectable for the PM. This qualitative assessment did not include an internal standard and was not explored further owing to the largely negative finding.

The MPH lactam reference standard eluted at $t_R$ = 11.5 min (Fig. 6) but was not detectable in either the PM nor normal MPH metabolizer urine samples.

## 4. Discussion

A MPH PM was recently identified by the unprecedented high plasma concentrations of l-MPH and by the absence of plasma d- or l-ethylphenidate when dl-MPH was dosed with ethanol. d-MPH concentrations were also considerably higher than normal values [9]. Based on the present study, the especially high concentration of l-MPH detected in the plasma of the PM (Fig. 2) generalizes to the urine l-MPH concentration when compared to that from 10 normal subjects. Accordingly, the enantiospecific GC–MS method developed here can serve as an expedient to phenotyping MPH metabolism status. Broader population based studies will be necessary to establish whether this MPH PM represents a rare genetic defect or is the first evi-

Table 2
MPH excreted in 10 h urine following a 0.3 mg/kg oral dose of dl-MPH·HCl[a,b]

| | *d*,l-MPH dose (0.3 mg/kg) | Total urine volume (mL) | Urinary d-MPH conc. (ng/mL) | Urinary l-MPH conc. (ng/mL) | Average d-MPH conc. (ng/mL) | Average l-MPH conc. (ng/mL) | [a]% d-MPH dose excreted unchanged | [a]% l-MPH dose excreted unchanged | % total dose excreted unchanged |
|---|---|---|---|---|---|---|---|---|---|
| Poor metabolizer | | | | | | | | | |
| 1 | 22.5 | 2550 | 1176 | 1059 | 1184 ± 11 | 1009 ± 71 | 10.5 | 9.0 | 9.7 |
| Duplicate | | | 1192 | 958 | | | | | |
| Normal males | | | | | | | | | |
| 1 | 27.5 | 2185 | 418 | ND | 325 ± 133 | 25 ± 12 | 3.0 | ND | 1.5 |
| 2 | 27.5 | 1720 | 278 | 18.2 | | | 2.0 | 0.1 | 1.1 |
| 3 | 20.0 | 2840 | 392 | 43.1 | | | 3.9 | 0.4 | 2.2 |
| 4 | 25.0 | 2155 | 425 | 24.2 | | | 3.4 | 0.2 | 1.8 |
| 5 | 32.5 | 2230 | 110 | 15.8 | | | 0.7 | 0.1 | 0.4 |
| Normal females | | | | | | | | | |
| 1 | 17.5 | 1790 | 146 | 18.3 | 176 ± 42 | 20 ± 2 | 1.7 | 0.2 | 0.9 |
| 2 | 22.5 | 1560 | 154 | ND | | | 1.4 | ND | 0.7 |
| 3 | 22.5 | 2770 | 225 | ND | | | 2.0 | ND | 1.0 |
| 4 | 22.5 | 2550 | 218 | 21.4 | | | 1.9 | 0.2 | 1.1 |
| 5 | 20.0 | 2450 | 138 | ND | | | 1.4 | ND | 0.7 |

[a] Based on a half of the dose (50:50 mixture of *d*,l-MPH).
[b] Average of duplicate determinations; ND: not detected; MPH concentrations calculated as free base.

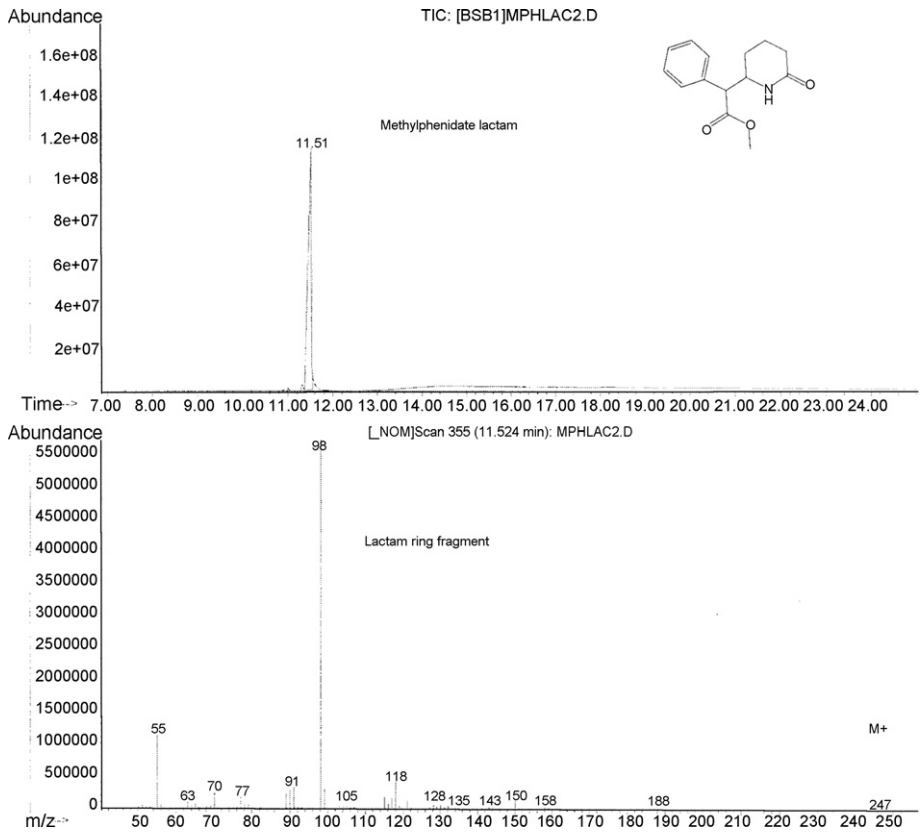


Fig. 6. GC–MS-EI total ion monitoring of the MPH lactam metabolite reference standard. This metabolite was not detectable in urine. This 6′-position oxidative metabolite is a minor metabolic pathway in humans but was investigated as an alternative metabolic pathway for the PM.

dence of a distinct polymorph (i.e., being found in a population frequency of at least 1%).

Chiral derivatization GC methods for enantiospecific urinary MPH analysis have been reported using nitrogen phosphorous detection [11] or electron capture detection [12] and in-house synthesized *N*-heptafluorobutyrylprolyl chloride. The present method benefits from the molecular specificity of MS detection and utilizes commercially available *N*-trifluoroprolyl chloride to form GC separable MPH derivatives. Achiral GC methods for urinary MPH analyses have also been reported [24–26] and the total MPH isomer concentrations reported in such earlier studies (Table 3) are generally consistent with those reported here for normal metabolizers.

Carboxylesterase-1 (CES1A1) rapidly hydrolyzes dl-MPH and results in a large distortion of plasma MPH isomers concentration, with d-MPH ≫ l-MPH [9]. In most individuals, this metabolic pathway accounts for the short MPH elimination half-life of 2–3 h [5]. Consequently, multiple daily dosing is generally required when using immediate-release MPH to ameliorate the symptoms ADHD throughout the day [5]. Alternatively, a single dose of modified –release MPH is required [27]. Most MPH formulations used at the present time are racemic mixtures of d- and l-MPH (Fig. 1; $R = CH_3$). However, enantiopure d-MPH formulations have recently become available [3]. d-MPH is generally regarded as the active isomer in the treatment of ADHD, while l-MPH has no demonstrable therapeutic effects [28]. Nevertheless, several lines of experimental evidence support at least some pharmacological activity being attributable to l-MPH [5]. This activity now includes the enantioselective interaction of l-MPH with ethanol to yield the transesterification product ethylphenidate (Fig. 1, $R = CH_2CH_3$). This drug interaction is accompanied by a significant elevation in plasma d-MPH [9].

In the MPH PM, plasma l-MPH actually exceeded that of d-MPH, and the maximum plasma concentration ($C_{max}$) of d-MPH in the PM was approximately twice that of the normal subject mean values. Further, only in the PM was no ethylphenidate detectable when administered concomitant ethanol–MPH [9]. Taken together, these metabolic anomalies of the PM are consistent with a *CES1A1* [29] null or reduced activity allele. This hypothesis has recently been supported by preliminary genotyping studies [10]. In that this esterase also prominently clears cocaine from the systemic circulation, it may be speculated that such PM(s) are pharmacogenetically predisposed to idiosyncratic cocaine responses, including overdose.

Low esterase activity has had clinical implications for response to other ester containing drugs such as succinylcholine. In this latter precedent, a bimodal distribution of cholinesterase activities was found in humans [30].

The majority of an oral dose of dl-MPH can be accounted for in the urine as the deesterified form of MPH, ritalinic acid (Fig. 1). Relatively low concentrations of MPH have been reported in urinary elimination studies (Table 3), however, these concentrations still greatly exceed those of plasma

Table 3
Literature urinary MPH values in humans

| Reference | Method | Dose | MPH reported | Comments |
|---|---|---|---|---|
| [11] | GC-NPD | 20 mg | d-MPH: ~400 μg l-MPH: ~5 μg | Chiral derivatization; 11 h cumulative; adult |
| [12] | GC-ECD | 40 mg | d-MPH: 53 μg l-MPH: 5 μg | Chiral derivatization; 16 h cumulative; adult |
| [24] | GC–MS | 0.4 mg/kg | 1.17 μg/mL | Achiral; 3 h; adult |
| [18] | GC–MS | 0.3 mg/kg | 0.051–0.274 μg/mL | Achiral; 24 h; adult |
| [25] | GC-FID | Unknown | 0.8–40 μg/mL | Achiral; forensic |
| [26] | GC-FID | 10 mg | 1.58 μg/mL | Achiral; 6 h; children |

GC: gas chromatography; NPD: nitrogen phosphorous detection; ECD: electron capture detection; MS: mass spectrometry; FID: flame ionization detection.

[5]. Thus, urine MPH analysis avoids most analytical sensitivity issues. The minor MPH metabolites HO-MPH and MPH lactam have been reported to be primarily, if not exclusively, eliminated in the urine as their corresponding carboxylic acids [14,15]. The alkalinized urine liquid–liquid extraction protocol used in the present study prevented recovery of such zwitterionic and/or acidic biotransformation products. Accordingly, the absence of appreciable HO-MPH and MPH lactam in the test urine samples does not, in and of itself, preclude the possibility of such oxidative biotransformations from occurring. These pathways might compensate for the impaired esterase function of the MPH PM. While intact esters of HO-MPH and MPH lactam do not appear to be pertinent to MPH urinary mass balance, even in the PM, the present report offers characterization of the GC–MS properties of the metabolites HO-MPH (Fig. 5) and MPH lactam (Fig. 6) to serve as reference analytical chemistry in the ongoing exploration of MPH metabolism.

Piperidine-deuterated MPH was chosen as the internal standard rather than methyl-deuterated MPH [27,31]. This standard allowed acquisition of the EI generated high abundance TFP-piperidyl fragment which favors higher sensitivity (higher relative abundance fragment ion) when compared to any methyl-deuterated MPH EI ions. Furthermore, use of this internal standard avoided any potential deuterium isotope effect differentially influencing hydrolytic loss during sample work-up. Additionally, methyl-deuterated dl-MPH was found to generate artifactual unlabeled MPH in an LC system containing methanol (unpublished observation).

Use of stable isotope analogues for analytical control in drug analysis from biological samples can optimize not only accuracy and precision, but also may enhance sensitivity through the carrier effect [32]. Deuterated MPH avoids the common, but problematic use of ethylphenidate (Fig. 1) as an internal standard in MPH analysis. Ethylphenidate has previously been identified as a metabolite in humans receiving a single dose of dl-MPH and ethanol [9,33], and this metabolite is readily detectable in urine of such subjects [34]. In the context of MPH–ethanol co-abuse, detection of urinary ethylphenidate could serve as a forensic biomarker in a fashion analogous to urinary anhydroecgonine ethyl ester detection indicating co-abuse of ethanol and smoked cocaine [35].

The present GC–MS-EI method provides a routine, rapid and non-invasive approach to phenotyping MPH metabolizer status with the potential for improving rational ADHD drug selection for individuals with compromised esterase-mediated MPH clearance. The special significance of these results are not so much the establishment of absolute urinary concentrations of d- and l-MPH, but rather the ability to identify a MPH PM by the *relative* amounts of d-MPH versus l-MPH in urine. A very pronounced qualitative difference in the urinary MPH chromatogram for the PM is readily apparent upon comparison to that of a representative normal metabolizer (Fig. 3). The PM excreted almost equal amounts of d- and l-MPH, while all 10 normal metabolizers excreted only a small fraction of l-MPH relative to d-MPH. Accordingly, a preliminary guideline for phenotyping via the present method is proposed: When the urinary ratio of d-MPH to l-MPH approaches unity, the subject becomes a candidate for PM status; when the ratio of d-MPH to l-MPH is $\geq$10, the subject likely exhibits wild-type esterase function.

Acknowledging the limitations of evaluating urine from only 11 total subjects in the present study, it is noted that there was no indication of any intermediate or ultrarapid metabolic phenotypes in this small population. There were clear differences between subjects known to be normal metabolizers ($n = 10$) and the subject ($n = 1$) that had previously been identified as a PM.

The apparent bimodal distribution of esterase activity in this preliminary investigation contrasts with that of some other Phase I drug metabolizing enzymes systems such as the unimodal distribution of CYP3A4 or tetramodal distribution of CYP2D6. Nevertheless, such guidelines for delineation of normal versus PMs of MPH would be expected to be further refined as urinary values from a much larger population are determined.

In the pilot therapeutic drug monitoring study by Jonkman et al. [7], children with ADHD who displayed elevated l- and d-MPH plasma concentrations exhibited significantly less efficacy. However, little if any such plasma l-MPH-efficacy inverse correlations have been reported for transdermal dl-MPH. l-MPH reaches at least 10 times greater plasma concentrations by the transdermal route than by the oral route, but still only attains 40–60% of the plasma concentration of d-MPH [8]. If a correlation between therapeutic effects and MPH PM status is found to exist, alternative pharmacotherapies such as atomoxetine or amphetamine may be appropriate in this subset of esterase compromised patients.

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