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DEVELOPMENT OF ENANTIOSELECTIVE GAS CHROMATOGRAPHIC QUANTITATION ASSAY FOR *dl-threo*-METHYLPHENIDATE IN BIOLOGICAL FLUIDS

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SUMMARY

An enantioselective gas chromatographic quantitation assay was developed for the enantiomers of *dl-threo*-methylphenidate in plasma and urine. *dl-threo*-Methylphenidate and the internal standard were acylated with N-heptafluorobutyryl-1-prolylchloride under Schotten—Baumann conditions prior to gas chromatographic separation on achiral mixed stationary phases. The derivatives were detected by means of a nitrogen—phosphorus detector. Linear and reproducible calibration curves were obtained over the concentration ranges 0.43—43.25 and 2.16—216.25 ng/ml enantiomer in plasma or urine, respectively. This enantioselective gas chromatographic quantitation assay was applied in a single oral dose disposition study of *dl-threo*-methylphenidate in a healthy adult volunteer. Stereoselective differences were observed in the plasma concentration—time profiles and cumulative urinary excretion profiles following oral doses of 20 and 40 mg of *dl-threo*-methylphenidate hydrochloride. Only *d-threo*-methylphenidate was detectable in plasma after 4 h.

INTRODUCTION

dl-threo-Methylphenidate [*dl-threo*-methyl 2-phenyl-2-(2-piperidyl)acetate] is a cyclic amphetamine analogue which has pharmacological properties similar to those of dextroamphetamine [1]. Methylphenidate is the drug of choice for the management of children diagnosed as having attention deficit disorder, with or without hyperactivity. This neurobehavioural problem was reported to affect about 5—20% of all children in North America [2—4].

dl-threo-Methylphenidate is a drug with a high intrinsic clearance [5]. After oral therapeutic doses, the drug is rapidly hydrolysed to ritalinic acid [6] resulting in low plasma concentrations (1—20 ng/ml) of the parent drug. It is

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only recently that analytical procedures have been developed for racemic methylphenidate with sufficient sensitivity and selectivity to study the disposition of methylphenidate in children or adults. These analytical procedures involved gas chromatography (GC) utilizing the nitrogen—phosphorus detection (NPD) [7,8] or mass spectrometric (MS) detection [9—12]. Recently, Gualtieri et al. [13] reported that there was no significant correlation between blood levels of racemic methylphenidate and clinical response in children with attention deficit disorder. It was speculated that one reason for the lack of correlation may be attributed to non-enantioselectivity of the quantitation assay employed.

It is now well recognized that biochemical processes at the molecular level exhibit stereoselectivity. This is not at all surprising since animal studies have indicated that L-amino acids in the diet are preferentially incorporated into the tissues [14—16]. Hence, macromolecules in biological systems should be viewed as asymmetric entities. Stereoselectivity in molecular interactions in biological systems have been traced to stereospecific interactions with the corresponding receptor and stereospecific differences in transport mechanisms or metabolic pathways [17].

The common practice of viewing a racemic drug as though it were a single pure compound has led to the neglect of stereoselectivity in clinical pharmacology and pharmacokinetics [18]. The measurement of total racemic drug instead of the concentrations of its enantiomers in therapeutic drug monitoring is not only misleading, but can also lead to erroneous interpretation of data and, hence, to less efficacious pharmacotherapy. The recognition of the need for chiral assay is clearly demonstrated by a recent court ruling requiring the identification of a specific drug enantiomer, *l*-cocaine [19], and this ruling may set precedence for the need to quantitate enantiomers of a racemic drug in the future.

Nothing is known about the stereoselective disposition of *dl-threo*-methylphenidate in children or adult human, although investigation of stereoselective metabolism in dog revealed that only the primary metabolite, ritalinic acid, was excreted in equi-enantiomeric proportions [20]. This paper now reports the development of an enantioselective GC assay for *dl-threo*-methylphenidate in biological fluids and its application to the study of plasma concentration–time profiles of *d*- and *l-threo*-methylphenidate after the administration of racemic drug to a human adult.

EXPERIMENTAL

Chemicals

Racemic *dl-threo*-methylphenidate, U.S.P. (Ritalin) was kindly donated by Ciba-Geigy (Basle, Switzerland) and the enantiomers were separated by the method described previously [21]. The following were obtained from Aldrich (Montreal, Canada); triethylamine (99+%, Gold Label), L-(−)-proline (99+%, Gold Label), *dl*-α-methylbenzylamine (99%), *d*-α-methylbenzylamine (98%), *l*-α-methylbenzylamine (98%), heptafluorobutyric anhydride (HFBA), thionyl chloride (99+%), Gold Label), 1-adamantanemethylamine (98%), sodium tetraborate decahydrate (ACS reagent), sodium bicarbonate (99+%) and sodium

carbonate (anhydrous powder, 99+%). Acetone, methanol and cyclohexane, distilled-in-glass grade, were obtained from BDH (Toronto, Canada).

Chromatography

A Hewlett-Packard (Avondale, PA, U.S.A.) Model 5840A gas chromatograph equipped with dual nitrogen—phosphorus and flame-ionization detectors, a packed column compatible Model 18835B capillary inlet system and a Model 5840A integrator terminal was used. The column used was a 1.83 m × 2 mm I.D. glass column packed with 1.5% OV-7/1.5% OV-210 on 60—80 mesh Chromosorb AW-DMCS (Supelco Canada, Oakville, Canada).

Initial oven temperature was 220°C, held for 1 min, programmed at 2°C/min to 250°C and held for 5 min. The injector and nitrogen—phosphorus detector temperatures were set at 280° and 300°C, respectively. The carrier gas was helium and the flow-rate was set at 30 ml/min.

A VG Micromass 7070E mass spectrometer was interfaced via a single-stage glass jet separator to a Hewlett-Packard 5790A gas chromatograph and was equipped with a VG 2025 data system.

Synthesis of N-heptafluorobutyryl-l-prolyl chloride (HPC)

The chemicals and reagents were further purified by distillation prior to use in the synthesis of HPC. HFBA, if needed, can be purified by reflux over phosphorus pentoxide and then distilled in glass [22].

HPC was synthesised by a modification of the reported procedure of Wiecek et al. [23]. Fresh, doubly distilled HFBA (0.5 ml), dissolved in anhydrous diethyl ether (3 ml), was added to 0.12 g of L-(−)-proline in a round-bottom flask maintained at −78°C in a dry ice—acetone bath. After 10 min, the flask was allowed to stand at room temperature for 2 h. The diethyl ether and unreacted HFBA were removed under vacuum at room temperature. A solution of doubly distilled thionyl chloride (1.6 ml) in dry benzene (3 ml) was added to the residue at 0°C and the reaction mixture was left to stand at room temperature for 2.5 h. The benzene and unreacted thionyl chloride were then removed under vacuum at room temperature. The residue was dissolved in dichloromethane (DCM) (1 ml) and the solvent was removed under vacuum. The latter process was repeated twice. The residue was then dissolved in an appropriate volume of DCM to give a concentration of 0.02 *M*.

The optical purity of HPC was determined by GC separation of the diastereomers formed from the reaction of HPC with optically pure enantiomers and racemic α-methylbenzylamine, respectively. Aliquots of HPC were sealed under nitrogen in 10-ml vials and stored at −20°C.

Working solutions

Aqueous stock standard solutions of *dl-threo*-methylphenidate·HCl and an internal standard (adamantanemethylamine·HCl) at concentrations of 4.33 μg/ml enantiomer (free base) and 8.19 μg/ml (free base), respectively, were prepared fresh every week. Human plasma and urine samples containing *dl-threo*-methylphenidate at various concentrations were obtained by serial dilution of the aqueous stock solution.

Assay procedure

The PTFE-lined, screw-capped test tubes were washed with chromic acid and then rinsed thoroughly with tap water. The tubes were neutralized with detergent and rinsed with tap water, deionized water, acetone and finally with methanol. The tubes were then oven-dried.

The extraction procedure used was a modification of the reported procedure of Huffman et al. [24]. To a 15-ml PTFE-lined, screw-capped test tube were added a 1-ml plasma or urine sample and 80 μl of aqueous adamantanemethylamine·HCl solution (819 ng/ml free base). The sample was gently mixed (Vortex-Genie) and 0.5 ml of saturated sodium tetraborate solution were added (approximate pH 9). The mixing was repeated and then 6 ml of cyclohexane were added. The tube was tightly capped and rotated at 1600 rpm for 10 min on a rotating-type tube mixer (IKA-VIBRAX VXR). After centrifugation at 1720 *g* for 10 min at 4°C (Model T-J6 centrifuge, Beckman Instruments), the organic layer was transferred to a 10-ml PTFE-lined, screw-capped test tube containing 300 μl of 1.0 *M* hydrogen chloride in methanol. The solvent was evaporated under a gentle stream of nitrogen at 75°C.

The residue was mixed with 1 ml of 5% (w/v) $NaHCO_3$—Na_2CO_3 (7:3) buffer and then allowed to stand in an ice-bath for 10 min. HPC (20 μl) was added and the tube was tightly capped. The contents of the tube were mixed by rotation at 1600 rpm for 10 min on a rotating-type tube mixer and then allowed to stand in the ice bath for another 20 min. Cyclohexane (4 ml) was then added, and the tube was tightly capped prior to mixing on a rotating-type tube mixer at 1600 rpm for 10 min. After centrifugation at 400 *g* (Fisher Safety centrifuge) for 5 min, the organic layer was transferred to another test tube. The solvent was evaporated at 75°C under a gentle stream of nitrogen. The residue was reconstituted in 50—100 μl of ethyl acetate and aliquots of 3—6 μl were analysed by GC—NPD.

Preparation of reagents

Samples of N-heptafluorobutyryl-*l*-propyl-adamantanemethylamine (HFBP-ADM) and N-heptafluorobutyryl-*l*-propyl-*dl-threo*-methylphenidate (HFBP-methylphenidate) were prepared under the Schotten- Bauman conditions described under *Assay procedure*, except that pure adamantanemethylamine·HCl or *dl-threo*-methylphenidate·HCl was added to the $NaHCO_3$—Na_2CO_3 buffer instead of the residue from an extract. After checking the purity of the samples (thin-layer chromatography, GC—NPD, GC—MS), standard solutions of HFBP-ADM and HFBP-methylphenidate in cyclohexane were prepared.

Effect of amount of HPC on reactivity

dl-threo-Methylphenidate·HCl (20 ng) was reacted with various volumes of 0.02 *M* HPC separately (5, 10, 15, 30 and 50 μl) as described above. Standard solution containing 102 ng HFBP-ADM was added to the cyclohexane as an external standard. The residue obtained upon evaporation of the solvent was dissolved in 100 μl ethyl acetate and analyzed by GC—NPD immediately. Each set of reaction conditions was studied in duplicate.

Effect of reaction time on diastereomer formation

dl-threo-Methylphenidate·HCl (20 ng) was reacted with 20 μl of 0.02 *M* HPC as described above. HFBP-ADM (102 ng) was added to the cyclohexane as external standard. The reaction was stopped at various times (5, 10, 15, 30, 60 and 120 min) by extraction of the diastereomers with cyclohexane. Samples, in duplicate, were stopped at each reaction time. The residue, thus obtained, was reconstituted in 100 μl ethyl acetate and analyzed by GC–NPD immediately.

Stability of diastereomeric derivatives of dl-threo-methylphenidate

dl-threo-Methylphenidate·HCl (50.35 ng) and adamantanemethylamine·HCl (80 mg) were reacted with 20 μl of 0.02 *M* HPC as described above. The residues obtained upon evaporation of the solvent were stored at various lengths of time (24, 48 and 72 h). Following storage, the residues of each set of storage conditions, in duplicate, were analysed by GC—NPD.

Dynamic linear range of nitrogen—phosphorus detector responses

dl-threo-Methylphenidate·HCl and adamantanemethylamine·HCl, 2 μg each, were reacted separately with 100 μl of 0.02 *M* HPC as described above. The derivatives of *dl-threo*-methylphenidate and adamantanemethylamine were then serially diluted with ethyl acetate prior to analysis by GC—NPD.

Calibration curves

Calibration curves for *d-* and *l-threo*-methylphenidate were constructed by analysing a series of plasma and urine samples spiked with *dl-threo*-methylphenidate in the concentration range of 0.43–43.25 and 2.16—216.25 ng/ml enantiomer (free base), respectively. Extraction and derivatization of the samples were carried out as described above.

Recovery studies

A series of plasma and urine samples, spiked with various amounts of *dl-threo*-methylphenidate·HCl, were processed as described under *Assay procedure*. An accurately measured volume of the cyclohexane layer was removed in the extraction step and 80 ng of HFBP-ADM were added to the cyclohexane layer as external standard. The residue obtained was dissolved in 50–100 μl of ethyl acetate and analysed by GC—NPD. The peak-height ratios obtained were compared with the peak-height ratios obtained when equal amounts of *dl-threo*-methylphenidate were derivatized with 20 μl of 0.02 *M* HPC without prior extraction.

The recovery of adamantanemethylamine from plasma and urine was determined in the same way as for *dl-threo*-methylphenidate except that 200 ng of HFBP-methylphenidate were used as the external standard.

Intra-assay and inter-assay variability studies

Intra-assay variability of *dl-threo*-methylphenidate, in plasma and urine, was determined by analysing samples of various concentrations in quadruplicate by GC—NPD. Inter-assay variability of *dl-threo*-methylphenidate, in plasma and

urine, was determined by analysing samples of various concentrations in quadruplicate on four separate days.

Interference studies

A series of plasma samples were spiked with the following drugs and analysed in the same way as for *dl-threo*-methylphenidate: phenobarbital, phenytoin, carbamazepine, theophylline, amphetamine, dextroamphetamine, pemoline, thioridazine, chlorphentermine and chlorpromazine.

Experimental protocol

A healthy adult male volunteer (64.5 kg), after an overnight fast, was administered orally on two separate occasions, an extemporaneously prepared capsule containing 20 or 40 mg *dl-threo*-methylphenidate·HCl with 250 ml of water. There was a period of two weeks between the oral administration of the two doses. Blood samples were drawn at 0, 0.5, 1.3, 2.2, 3.1, 4, 6.1, 8.1 and 10 h into heparinized evacuated test tubes (vacutainers) for the 20-mg dose. For the 40-mg dose, the blood samples were taken at 0, 0.5, 1.3, 1.7, 2.1, 3, 4.3, 6, 8, 10 and 13 h. Extreme care was taken to avoid contact of the blood with the rubber stoppers during collection of venous blood. The blood samples were immediately centrifuged at 1720 *g* for 10 min at 4°C and the separated plasma was stored in 20-ml glass scintillation vials at −20°C until analysis.

Urine was also collected. The time, volume and pH were recorded immediately after collection. It was then stored in glass scintillation vials at −20°C until analysis.

RESULTS AND DISCUSSION

GC has emerged as a promising analytical tool for separation and quantitation of enantiomers in biological fluids. These enantioselective GC analytical methods can generally be classified as direct and indirect methods. Direct methods involve separation of the enantiomers on chiral stationary phases. In this case, derivatisation with achiral reagents is employed purely for the purpose of improving the chromatography or detector sensitivity [25,26]. One of the drawbacks associated with presently available chiral stationary phases is their low-temperature stability which makes them unsuitable for higher-boiling-point compounds, such as methylphenidate. The indirect methods, such as that reported here, require derivatisation of the enantiomers with optically pure chiral reagents prior to GC separation on achiral stationary phases [27–29]. The drawbacks of indirect methods include the possible formation of unequal amounts of the two diastereomers as a result of different reaction rate constants and the possible occurrence of false positive results due to trace isomeric contamination of the chiral reagent [30].

An enantioselective analytical method, based on derivatisation with a chiral reagent, can be developed, provided two criteria are satisfied. They are: first, the chiral reagent must be optically pure (or its optical purity must be known and constant) and, second, there must be no inversion of configuration during transformation of the enantiomers into the diastereomers or during chromatography [23]. Based on these premises, an enantioselective GC quantitation

assay for *dl-threo*-methylphenidate in biological fluids was developed utilizing L-(−)-proline as the resolving agent. The rationale for using L-proline instead of other amino acids, is that L-proline does not racemize during acylation or peptide synthesis since oxazolone formation is not possible [31]. Furthermore, the rigid conformation of the prolyl–peptide bond is thought to enhance differences in the physical properties of its diastereomeric derivatives and consequent enhancement of chromatographic resolution.

Souter [32] reported that N-heptafluorobutyryl-substituted L-prolyl derivatives tend to elute earlier than their trifluoroacetyl counterparts, without any significant diminution in separation. In the case of *dl-threo*-methylphenidate, we observed that the heptafluorobutyramide eluted earlier than either the trifluoroacetamide, pentafluoropropionamide or pentafluorobenzamide. Based on this property and also on its ease of synthesis, HPC was selected as the resolving agent. HPC was found to be optically pure and stable for at least four months, when stored at −20°C in sealed vials under an atmosphere of dry nitrogen.

It was observed that purification of HFBA, as described under Experimental, resulted in cleaner chromatograms. Racemizations associated with either synthesis of the chiral reagents and/or during the formation of diastereomers

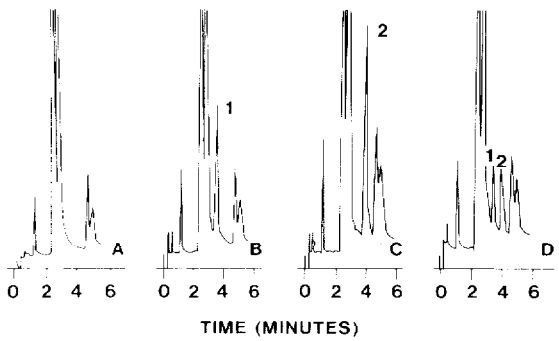


Fig. 1. Determination of optical purity of N-heptafluorobutyryl-*l*-propyl chloride (HPC) reagent by GC—NPD. (A) Blank; (B) after reaction with optically pure *d*-α-methylbenzylamine; (C) after reaction with optically pure *l*-α-methylbenzylamine; (D) after reaction with racemic α-methylbenzylamine. The numbered peaks are HPC derivatives of (1) *d*-α-methylbenzylamine and (2) *l*-α-methylbenzylamine.

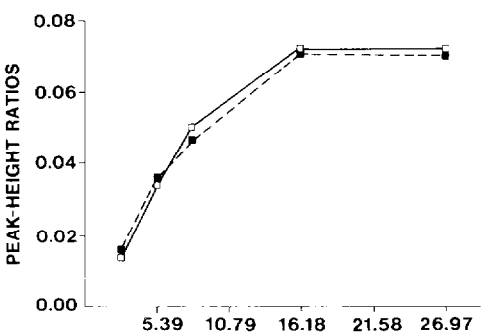


Fig. 2. Effect of varying HPC-to-methylphenidate ratios on the production of HPC-methylphenidate derivatives of *d-threo*-methylphenidate (□) and *l-threo*-methylphenidate (■).

have been well documented [33—35]. The criteria used for assessment of the optical purity of HPC were similar to those published previously [33—35]. HPC, with optical purity of at least 99% (Fig. 1), was required for use in quantitation of *dl-threo*-methylphenidate. In our hands, racemization was observed during acylation of *dl-threo*-methylphenidate by HPC in organic media with triethylamine as a catalyst, even at −78°C. One possible explanation is that racemization was promoted through abstraction of mildly acidic proton at the 2-position of HPC by triethylamine [36].

The reaction of pentafluorobenzoyl chloride with phenolic or amino compounds in aqueous media has been reported previously [37,38]. This is the first report of the application of Schotten—Baumann conditions to the acylation of an amine with a chiral derivatising agent. The advantages of carrying out the derivatisation in an aqueous medium were that racemization was minimal and there was much less interference from unreacted HPC than there was when the reaction was carried out in organic media. Furthermore, the production of diastereomers was reproducible under the aqueous derivatisation conditions described under Experimental. The acylation reaction reached a plateau when the ratio of HPC to each enantiomer of *threo*-methylphenidate was 16 180:1 or greater (Fig. 2). Thus, 20 μl of 0.02 *M* HPC was found to be adequate for the acylation of *dl-threo*-methylphenidate in the ng/ml range without undue interference from reagent peaks. The use of more than 20 μl of reagent, however, did lead to interference peaks in the chromatograms, with no further enhancement of the peak heights of methylphenidate diastereomer derivatives. Fig. 3 shows that the acylation reactions were maximal in 10 min. The tendency for the peak-height ratios to decline after 10 min may have been due to slow hydrolysis of the ester function in the alkaline conditions. The HFPB-methylphenidate derivatives were found to be stable for up to 72 h when stored under the conditions shown in Table I.

The mixed stationary phase of 1.5% OV-7 and 1.5% OV-210 was employed to ensure separation of a reagent peak from the peaks owing to the derivatised enantiomers of methylphenidate. Fig. 4 shows gas chromatograms of derivatised methylphenidate and adamantanemethylamine standards extracted from spiked control samples of plasma or urine. Also shown are chromato-

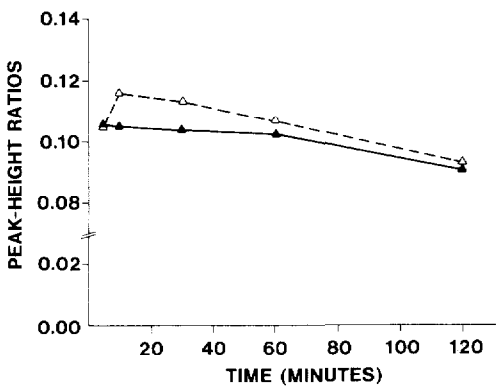


Fig. 3. Effect of reaction time on the formation of HPC derivatives of (△) *d-threo*-methylphenidate, and (▲) *l-threo*-methylphenidate.

TABLE I

STABILITY OF N-HEPTAFLUOROBUTYRYL-*l*-PROLYL-(±)-*threo*-METHYLPHENIDATE AS A FUNCTION OF STORAGE TIME AND TEMPERATURE

Time (h)	Enantiomer response (peak-height ratios)*					
	24°C		4°C		−20°C	
	d	*l*	*d*	*l*	*d*	*l*
0	0.221**	0.204**	0.221**	0.204**	0.221**	0.204**
24	0.225	0.200	0.194	0.180	0.213	0.186
48	0.242	0.221	0.209	0.194	0.230	0.199
72	0.213	0.191	0.225	0.208	0.222	0.211

*Peak-height ratios determined for drug/internal standard. *threo*-Methylphenidate enantiomer concentration was 21.63 ng/ml; mean of duplicate samples.
**Baseline samples were analysed immediately after preparation.

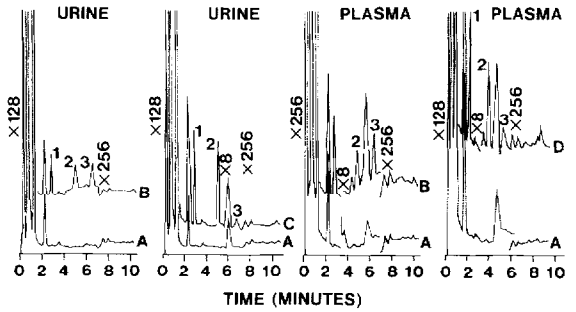
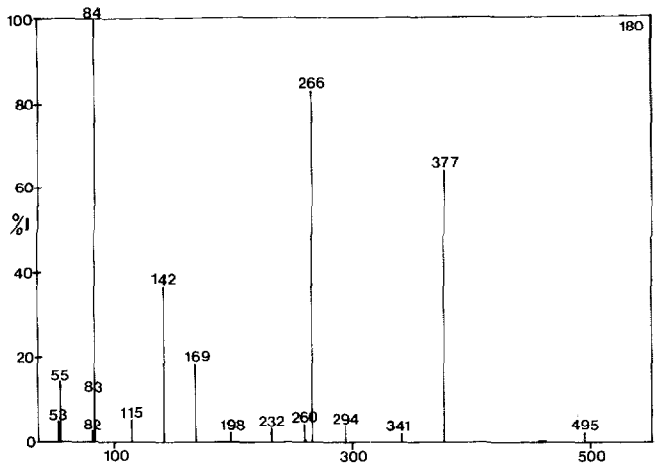


Fig. 4. GC—NPD profiles of derivatised extracts of: (A) blank urine or plasma; (B) spiked urine (43.16 ng/ml enantiomer) or plasma (2.15 ng/ml enantiomer); (C) 10.75-h urine from a healthy adult volunteer dosed with 20 mg methylphenidate; (D) 0.5-h plasma from healthy adult volunteer dosed with 40 mg methylphenidate. Peaks are HPC derivatives of (1) adamantanemethylamine, (2) *d-threo*-methylphenidate and (3) *l-threo*-methylphenidate.

grams obtained after extraction of plasma or urine from a healthy adult volunteer dosed with racemic *threo*-methylphenidate. Mass spectra (Fig. 5) of the compounds giving rise to peaks 2 and 3 (Fig. 4) were similar and contained ions diagnostic of derivatised methylphenidate. Therefore, it may be concluded that peaks 2 and 3 represent the derivatised enantiomers of *threo*-methylphenidate.

A linear relationship was obtained between the peak height and the amount of HFBP-methylphenidate and HFBP-ADM injected into the gas chromatograph. The equations of the regression lines of the nitrogen—phosphorus detector response for *d*- and *l-threo*-methylphenidate in the range 95.2—474.8 pg were definable by the expressions of $y = 0.0025x - 0.0285$ ($n=4$) and $y = 0.0024x - 0.0626$ ($n=4$), respectively. The equation of the regression line of the nitrogen—phosphorus detector response for adamantanemethylamine in the range 56.1—935.68 pg ($n=4$) was determined to be $y = 0.0813x + 0.0017$. The correlation coefficients for the regression lines were determined to be at least 0.995.



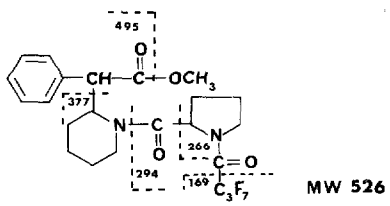


Fig. 5. GC—MS (electron impact, 70 eV) of the HFBP derivative of *l-threo*-methylphenidate. The mass spectrum of the corresponding *d-threo*-methylphenidate derivative was identical.

TABLE II

CALIBRATION CURVE DATA FOR PLASMA

Quantity of each *threo*-methylphenidate enantiomer* (ng)	*n*	Plasma peak-height ratios** (mean ± S.D.)	
		d	*l*
0.43	4	0.0105 ± 0.0001	0.0089 ± 0.0007
2.16	6	0.0287 ± 0.0018	0.0265 ± 0.0011
4.33	6	0.0515 ± 0.0027	0.0490 ± 0.0022
10.81	5	0.1166 ± 0.0053	0.1134 ± 0.0074
21.63	6	0.2346 ± 0.0064	0.2289 ± 0.0086
43.25	6	0.4951 ± 0.0245	0.4830 ± 0.0233
Correlation coefficient		0.9993	0.9994
Slope		0.0113	0.0110
y-Intercept		0.0008	0.0004
Mean coefficient of variation (%)		4.18	5.31

**threo*-Methylphenidate (free base) used as the racemate mixture.

****Peak-height ratios determined for drug/internal standard. Standard deviations calculated for *n* aliquots for each weight of *threo*-methylphenidate (free base).

Calibration curve data for *d*- and *l-threo*-methylphenidate in plasma and urine are given in Tables II and III. The calibration curves, based on peak-height ratios, were linear from 0.43 to 43.25 and 2.16 to 216.25 ng/ml enantiomer in

plasma or urine, respectively. A satisfactory correlation coefficient of at least 0.999 for each enantiomer was obtained. The mean coefficient of variation, in each case, was determined to be less than 10%. The lowest quantitation limits for each enantiomer in plasma and urine were determined to be 0.43 and 2.16 ng/ml, respectively.

dl-threo-Methylphenidate and adamantanemethylamine were quantitatively (>80%) and reproducibly (coefficient of variation, <10%) recovered from plasma and urine at the concentrations examined (Table IV). No change in extraction efficiency was observed over the concentration range studied.

Intra- and inter-assay coefficients of variation for plasma (Table V) and urine (Table VI) were less than 10% over the wide concentration range examined. It

TABLE III

CALIBRATION CURVE DATA FOR URINE ($n=6$)

Quantity of each *threo*-methylphenidate enantiomer* (ng)	Urine peak-height ratios** (mean ± S.D.)	
	d	*l*
2.16	0.0233 ± 0.0020	0.0219 ± 0.0021
4.33	0.0404 ± 0.0032	0.0388 ± 0.0013
21.63	0.2115 ± 0.0132	0.2124 ± 0.0134
86.50	0.8319 ± 0.0427	0.8440 ± 0.0410
216.25	2.2066 ± 0.1779	2.2006 ± 0.1431
Correlation coefficient	0.9997	0.9999
Slope	0.0102	0.0102
y-Intercept	−0.0116	−0.0097
Mean coefficient of variation (%)	7.16	6.14

**threo*-Methylphenidate (free base) used as the racemate mixture.

******Peak-height ratios determined for drug/internal standard. Standard deviations calculated for *n* aliquots for each weight of *threo*-methylphenidate (free base).

TABLE IV

RECOVERY OF (±)-*threo*-METHYLPHENIDATE AND ADAMANTANEMETHYLAMINE FROM PLASMA AND URINE

C.V. = Coefficient of variation; $n=4$.

Quantity of each enantiomer added to biological fluid (ng)	Mean recovery from plasma (%)		Mean C.V. (%)		Mean recovery from urine (%)		Mean C.V. (%)	
	d	*l*	*d*	*l*	*d*	*l*	*d*	*l*
Methylphenidate								
2.16	88.09	86.06	4.33	6.29	–	–	–	–
4.29	–	–	–	–	95.00	94.07	9.00	10.25
43.25	98.37	97.08	8.45	9.69	–	–	–	–
214.52	–	–	–	–	84.32	83.44	3.01	2.78
Adamantanemethylamine								
65.52	95.81		5.19		94.08		3.45	

TABLE V

STATISTICAL EVALUATION OF THE PRECISION AND REPRODUCIBILITY OF THE ASSAY FOR (±)-*threo*-METHYLPHENIDATE IN PLASMA

C.V. = Coefficient of variation; $n = 4$.

Amount of each enantiomer added (ng/ml)	Amount of each enantiomer found (mean ± S.D.) (ng/ml)		C.V. (%)	
	d	*l*	*d*	*l*
Intra-assay variability				
43.08	39.18 ± 1.32	39.21 ± 2.83	3.37	7.22
21.54	19.25 ± 0.07	19.55 ± 1.43	0.36	7.32
8.62	8.64 ± 0.60	8.44 ± 0.42	6.94	4.98
4.31	3.58 ± 0.14	3.72 ± 0.04	3.91	1.08
Inter-assay variability				
43.08	39.87 ± 2.26	41.66 ± 3.56	5.67	8.55
21.54	20.22 ± 0.68	20.54 ± 0.92	3.36	4.48
8.62	9.37 ± 0.65	8.81 ± 0.78	6.94	8.85
4.31	4.84 ± 0.48	4.62 ± 0.24	9.92	5.19

TABLE VI

STATISTICAL EVALUATION OF THE PRECISION AND REPRODUCIBILITY OF THE ASSAY FOR (±)-*threo*-METHYLPHENIDATE IN URINE

C.V. = Coefficient of variation; $n = 4$.

Amount of each enantiomer added (ng/ml)	Amount of each enantiomer found (mean ± S.D.) (ng/ml)		C.V. (%)	
	d	*l*	*d*	*l*
Intra-assay variability				
216.25	227.08 ± 21.08	232.67 ± 18.10	9.28	7.78
17.30	17.37 ± 1.15	16.66 ± 0.62	6.64	3.69
Inter-assay variability				
216.25	209.43 ± 11.78	204.10 ± 10.22	5.62	5.00
17.30	17.57 ± 0.80	16.75 ± 1.04	4.56	6.21

was concluded that the precision and reproducibility of the enantioselective GC method for *dl-threo*-methylphenidate in plasma and urine were satisfactory.

None of the following compounds, when added to plasma or urine, interfered with the assay: dextroamphetamine, pemoline, amphetamine, phenobarbital, phenytoin, carbamazepine, theophylline, thioridazine, chlorphentermine and chlorpromazine.

A pilot disposition study in a healthy adult volunteer, after oral administration of 20 or 40 mg *dl-threo*-methylphenidate·HCl, was carried out to demonstrate the applicability of the method to measure plasma and urine concentrations of the enantiomers. The plasma concentrations of the *d*-enantiomer were consistently found to be greater than those of the *l*-antipode at the two doses

examined (Fig. 6). After about 4 h, only *d*-enantiomer was quantifiable in the plasma. It showed a monoexponential decay with elimination half-lives of 3.6 and 3.2 h for 20- and 40-mg doses, respectively.

The simple pharmacokinetic parameters after oral doses of *dl-threo*-methylphenidate·HCl in a healthy adult volunteer were determined from the plasma concentration—time profiles and are summarized in Table VII. The $T_{\frac{1}{2}}$, K and AUC were not calculated for the *l*-enantiomer since its plasma concentrations were below the quantitation limit of the enantioselective assay after 4 h. The time for the plasma concentrations of both enantiomers to reach maximum was approximately 2 h. The peak concentrations of the *d*-enantiomer were approximately fourteen-fold greater than those of the *l*-antipode at the two doses examined.

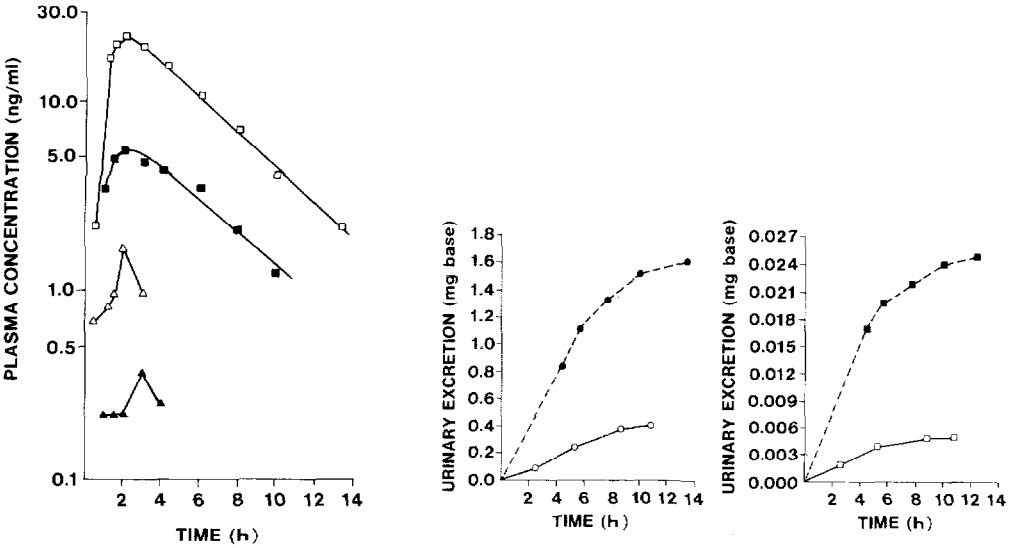


Fig. 6. Plasma concentration—time profiles of *d*- (■, □) and *l*- (▲, △) enantiomers of *threo*-methylphenidate in a healthy volunteer after single oral doses of 20 mg (■, ▲) or 40 mg (□, △) of racemic *threo*-methylphenidate·HCl on two separate occasions.

Fig. 7. Cumulative urinary excretion of *d*- (●, ○) and *l*- (■, □) enantiomers of *threo*-methylphenidate in a healthy volunteer after single oral doses of 20 mg (○, □) or 40 mg (●, ■) of racemic *threo*-methylphenidate·HCl on two separate occasions.

Cumulative urinary excretion profiles of the enantiomers of *dl-threo*-methylphenidate, at the two doses examined, were similar to the plasma profiles in that greater amounts of the *d*-enantiomer than its *l*-antipode were excreted in urine (Fig. 7). The cumulative urinary excretion of unchanged *dl-threo*-methylphenidate is within the range of 0.8 to 11% as reported by Wells et al. [39].

The present investigation clearly indicates the presence of pronounced stereoselective processes in the disposition of *dl-threo*-methylphenidate in humans after oral doses. The stereoselectivity would appear unlikely to be due to stereoselective urinary excretion since the cumulative urinary excretion profiles of the enantiomers were similar to the plasma profiles. Furthermore,

TABLE VII

(±)-*threo*-METHYLPHENIDATE PHARMACOKINETIC PARAMETERS IN A NORMAL ADULT AFTER ORAL DOSE

Abbreviations: C_{max} = peak concentration; T_{max} = time of peak concentration; $T_{½}$ = elimination half-life; K = elimination rate constant; AUC_0^∞ = area under the curve from time zero to infinity.

Parameter	Dose: 20 mg		Dose: 40 mg	
	d	*l*	*d*	*l*
C_{max} (ng/ml)	5.52	0.37	22.44	1.66
T_{max} (h)	2.17	3.05	2.08	2.08
$T_{½}$ (h)	3.61	–	3.22	–
K (h^{-1})	0.192	–	0.215	–
AUC_0^∞ ($\mu g \cdot h/l$)	39.55	–	138.42	–

stereoselective absorption can be excluded since Faraj et al. [6] demonstrated that there was essentially complete absorption of *dl-threo*-methylphenidate in humans. Therefore, the observed stereoselectivity in disposition of methylphenidate could, perhaps, be explained by stereoselectivity in metabolism. Further investigations are in progress to confirm and explain the cause(s) of the distortion of the ratio of enantiomers in the plasma and urine after the administration of racemic *threo*-methylphenidate.

CONCLUSIONS

An enantioselective GC method has been developed, with sufficient sensitivity and selectivity to measure concentrations of the enantiomers of *threo*-methylphenidate in plasma or urine, after the administration of single oral doses. Stereoselectivity was observed in the disposition of methylphenidate administrated orally, at two dosage levels, to a healthy human adult volunteer.

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