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# Enantioselective gas chromatographic assay with electron-capture detection for *dl*-ritalinic acid in plasma

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## ABSTRACT

Enantioselective gas chromatographic assays for the quantitation of methylphenidate and its major metabolite ritalinic acid in plasma are described. The procedures involved the extraction of methylphenidate enantiomers from alkanised plasma. The plasma was then washed to ensure complete removal of methylphenidate before saturation with sodium carbonate to promote the extraction of ritalinic acid enantiomers with ethyl acetate–isopropanol (60:40) solvent mixture. Subsequently, ritalinic acid enantiomers were converted back into methylphenidate enantiomers by Fisher–Speier esterification. N-Heptafluorobutyryl-L-prolyl chloride, a chiral acylating reagent, was used to convert the enantiomers of methylphenidate into their corresponding diastereomeric amide derivatives, which were separated cleanly on an achiral capillary column (OV-225) and quantitated with electron-capture detection. The assays were sensitive, reliable and reproducible.

## INTRODUCTION

Methylphenidate (MPH) (Ritalin®) (Fig. 1) is widely used to treat children with attention deficit-hyperactivity disorder. MPH resembles dextro-amphetamine in its pharmacological actions, although it is less potent and has a shorter duration of action [1]. MPH is metabolised rapidly and extensively in both humans and animals, to form predominantly the de-esterified product, commonly known as ritalinic acid (RA) [2–4].

MPH is a racemic mixture of *threo*-enantiomers, the absolute configurations of which have been determined [5]. *d-threo*-MPH has been reported to be pharmacologically more potent than the corresponding *l*-antipode [6,7]. It is only recently, however, that enantioselective methods have appeared for the analysis

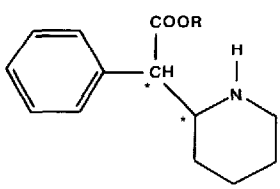


Fig. 1. Structures of ritalinic acid (R = H) and methylphenidate (R = $CH_3$) showing asymmetric carbon atoms (★).

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of MPH enantiomers in biological fluids [8,9]. The use of an enantioselective assay method revealed that the plasma levels of *d*-MPH were several times greater than those of *l*-MPH both in children with attention deficit-hyperactivity disorder [9] and adults [8] after oral doses of racemic MPH.

By contrast, the analytical procedures reported for RA are essentially non-stereoselective [10–14]. Consequently there are no data on stereoselective aspects of the dispostion of RA following the administration of MPH. In this paper we report an enantioselective, sensitive and reliable method for the analysis of both MPH and RA enantiomers in plasma. The application of the method to a single-dose pharmacokinetic study in a human adult after oral administration of racemic MPH is also described.

## EXPERIMENTAL

*dl-threo*-MPH and *dl-threo*-RA were kindly donated by Ciba-Geigy (Basel, Switzerland). Pure reference standards of *d-threo*-MPH and *l-threo*-MPH were prepared according to a previously reported procedure [8].

*d*-Ephedrine and L-tryptophan (Aldrich, Montreal, Canada) and isopropanol, ethylacetate and *n*-pentane (BDH, Toronto, Canada) were purchased from the respective commercial outlets. Heptafluorobutyryl-*l*-prolyl chloride (HPC) was synthesized in our laboratories [8]. All other chemicals and solvents used were of analytical grade and were used without further purification.

The gas chromatography (GC) was performed on a Hewlett Packard Model 5840A gas–liquid chromatograph equipped with a $^{63}$Ni electron-capture detector and a Hewlett Packard Model 5840A integrator. The separation of the various analytes was achieved on a 30 m × 0.33 mm I.D. capillary OV-225 column (Terochem Labs., Edmonton, Canada).

### *Assay procedure*

A 2-ml plasma sample (standard, quality control or a sample from a dosed volunteer) in borosilicate glass tubes (125 mm × 16 mm) was spiked with *d*-ephedrine (200 ng/ml) to serve as an internal standard in the analyses of *d*- and *l*-MPH. The sample was mixed for 5 s (Vortex Genie, Fischer Scientific, Edmonton, Canada) after which 0.6 ml of a saturated solution of sodim tetraborate was added (pH 8.0–9.0); the sample was mixed again followed by the addition of 5 ml *n*-pentane. The tube was capped and the contents were mixed for 10 min on a rotating-type mixer set at 1600 rpm (IKa Vibrax VXR, Fischer Scientific) and then centrifuged (Model TJ-6 centrifuge, Beckmann Instruments, Palo Alto, CA U.S.A.) at 1720 *g* for 5 min at 4°C. The lower aqueous layer was set aside for analysis of RA. The upper organic phase which contained MPH and the internal standard *d*-ephedrine was then pipeted into a clean tube containing 1 ml of 0.1 *M* hydrochloric acids. Both *dl*-MPH and *d*-ephedrine were derivatized with HPC as described previously [9].

A portion of the aqueous phase (1 ml) from the above was subjected to two washings with 5 ml of an *n*-pentane–methylene dichloride mixture (80:20) to remove any trace of MPH. A solution (100 μl) of L-tryptophan (2.5 ng/μl) was added to the aqueous phase as an internal standard for RA, followed by addition of 4 ml of a mixture of isopropanol–ethyl acetate (40:60) and 1 g of anhydrous $Na_2CO_3$. The tube was capped and the contents were mixed for 10 s (Vortex mixer) and then shaken for 15 min on a rotating-type mixer set at 1600 rpm after which the layers were separated by centrifugation at 1720 *g* for 10 min at 4°C. The organic layer was transferred into a clean tube and evaporated to dryness under a gentle stream of nitrogen at 65°C. The residue contained the RA enantiomers which were converted back to the parent drug enantiomers by carrying out the Fischer–Speier esterification reaction by heating at 80°C for 20 min with 0.2 ml of a mixture (2:1, v/v) of methanol and sulphuric acid [14]. The mixture was then cooled on ice after which the pH was adjusted by careful addition of 3 ml of a solution containing $NaHCO_3$–$Na_2CO_3$ (7:3, w/w, pH 9.5). The esterified RA was then derivatised with HPC and extracted as described previously [10].

*Standard curves for MPH and RA enantiomers*

A series of plasma samples were spiked with *dl*-MPH in the range 0.28–45.0 ng (free base) of each enantiomer per ml and *dl*-RA in the range 1.4–75 ng (free base) of each enantiomer per ml. Seperate standard curves for *d*- and *l*-enantiomers of both MPH and RA were constructed by chromatographing the spiked standards and plotting peak-height ratios of each of the enantiomers of the drug to the appropriate internal standard *versus* the concentration of each enantiomer.

*Analytical conditions*

GC conditions used in the separation of the various analytes were according to the previously published procedure [9]. Briefly, the column oven temperature (175°C) was held for 1 min and thereafter increased at a rate of 7°C/min to a final temperature of 260°C, which was held for 5 min. The injection port temperature was 280°C, the detector temperature 300°C, the argon–methane (carrier gas) flow-rate 1 ml/min, the argon–methane (make up gas) flow-rate 60 ml/min, the split vent flow-rate 28 ml/min, the septum vent flow-rate 2 ml/min and the head pressure on the column 1 bar.

*Intra- and inter-assay variation*

Intra-assay variability in the analysis of MPH and RA were determined by analysing on the same day (in triplicate) a series of plasma samples spiked with various concentrations (ng/ml for each enantiomer) of MPH (0.28–45) and RA (1.4–75). Inter-assay variability was determined similarly except that samples were analysed in triplicate on three separate days.

*Human study*

A healthy male volunteer weighing 70 kg was given 40 mg MPH·HCl (four 10-mg tablets, Ritalin) orally after an overnight fast. A predose blood sample (15 ml) was obtained immediately before ingestion of the drug. Further blood samples (15 ml each) were drawn into heparinised evacuated tubes (Vacutainers®) at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 h. Caution was exercised to avoid the contact of the blood specimens with the rubber stopper of the vacutainers. The blood samples were immediately centrifuged and the plasma was separated and stored at −20°C until analysis.

*Pharmacokinetic calculations*

Areas under the plasma concentration *versus* time curves ($AUC_0^t$) were calculated by linear trapezoidal rule. Areas to infinity ($AUC_0^\infty$) were obtained by adding to $AUC_0^t$ the quotient of the last plasma concentration measured and the terminal slope value [15]. Plasma elimination half-life values ($t_{1/2}$) for the enantiomers were estimated from the terminal slope of the plasma concentration–time curves.

## RESULTS AND DISCUSSION

The development of the present enantioselective analytical method was influenced by the reports of Nakajima *et al.* [14] who described a non-stereoselective analytical procedure for MPH and RA. The method is based on (i) the extraction and measurement of MPH followed by (ii) the extraction of RA and its subsequent conversion into MPH. While the introduction of stereoselectivity is the most important departure from the method of Nakajima *et al.* [14], the present method also offers a number of other significant alternatives. For example, the present GC–electron-capture detection (ECD) method does not depend upon the availability of a combined gas chromatograph–mass spectrometer.

*Extraction of RA enantiomers from plasma*

RA is an amphoteric metabolite which has proved to be very difficult to extract from biological fluids. Nakajima *et al.* [14] solved the problem by using 100% isopropanol to extract the metabolite from plasma saturated with $K_2CO_3$. This procedure gave a very high recovery of RA (90%) but suffers from two drawbacks. Firstly, it is necessary to use reduced pressure in order to evaporate isopropanol efficiently. Secondly, this polar solvent extracts a multitude of endogenous compounds which may interfere with the chromatography of the analytes. In the present method the mixed solvent system [40% (v/v) isopropanol in ethyl acetate] gave a reasonable recovery of RA (70%) and could be evaporated in 30–60 min at 65°C under a gentle stream of nitrogen.

In the earlier method, an external standard was used for the quantitation of RA [14]. In the present study, several compounds were subjected to the processes

of extraction and derivatisation under similar conditions to that of RA to help in the selection of a suitable internal standard. Several small molecules such as ephedrine, chlorphentermine, adamantanamine and methyladamantanamine were found to be unsuitable because of volatility which led to appreciable loss during the work-up procedure. Consequently, the possibility of using an amino acid similar to RA as an internal standard was investigated. One important advantage was that an amino acid internal standard could undergo both esterification and acylation reactions employed in the derivatization of RA. Thus the use of amino acid provided an ideal comparison of both extraction and derivatization processes of the RA enantiomers. The three criteria considered in the selection of an amino acid were: (i) reproducibility of its recovery, (ii) chromatography of the derivatized compound, and (iii) length of its retention on the column. L-Tryptophan was found most suitable of the various amino acids examined with an estimated recovery similar to that of RA. Separation of the peaks of the derivatised RA enantiomers and L-tryptophan are shown in Fig. 2. Chromatograms were clean and showed no interference from the endogenous plasma constituents at the retention times corresponding to the peaks of the derivatised analytes.

Standard curves constructed for each RA enantiomer with L-tryptophan as internal standard (Table I) were linear in the range 75–1.4 ng/ml for each enantiomer of RA in plasma. The regression line for *d*-RA could be described by the equation $y = 0.0525x + 0.0094$ ($r^2 = 0.999$) whereas the corresponding

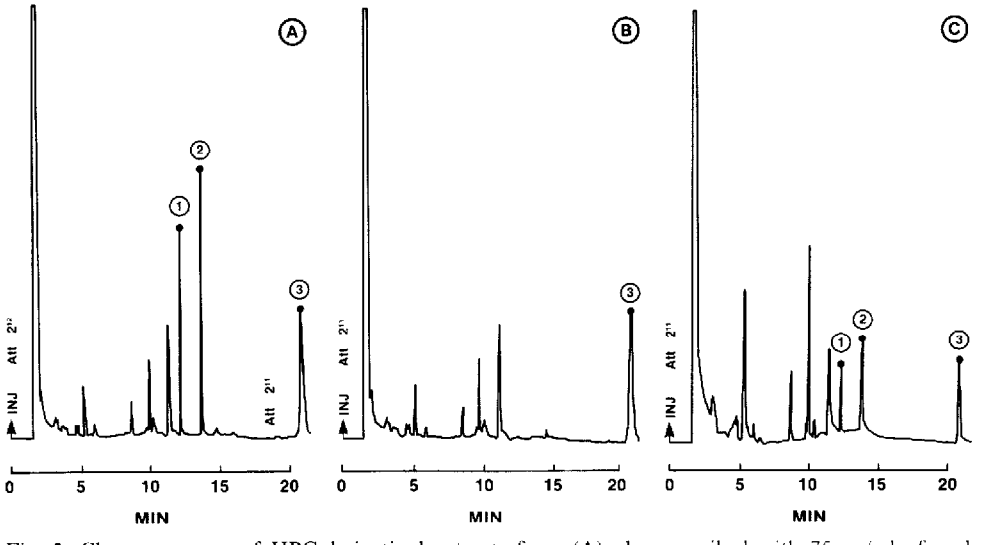


Fig. 2. Chromatograms of HPC-derivatised extracts from (A) plasma spiked with 75 ng/ml of each enantiomer of ritalinic acid, (B) pre-dosed blank plasma and (C) plasma sample obtained at 8.0 h from a healthy volunteer after ingestion of 40 mg of *dl*-methylphenidate hydrochloride. The peaks correspond to derivatives of (1) *d*-RA, (2) *l*-RA and (3) L-tryptophan.

TABLE I

STANDARD CALIBRATION CURVE DATA FOR RITALINIC ACID ENANTIOMERS IN PLASMA

| Concentration of each enantiomer (ng/ml) | $n$ | Peak-height ratios of derivatised RA enantiomer to internal standard (mean ± S.D.) | |
|---|---|---|---|
| | | *d*-RA | *l*-RA |
| 75 | 3 | 3.9253 ± 0.1497 | 4.6427 ± 0.3712 |
| 37.5 | 3 | 2.0119 ± 0.3345 | 2.5239 ± 0.2128 |
| 11.25 | 3 | 0.6448 ± 0.0385 | 0.7639 ± 0.0661 |
| 5.625 | 3 | 0.2598 ± 0.0189 | 0.3113 ± 0.0239 |
| 1.400 | 3 | 0.0686 ± 0.0068 | 0.0833 ± 0.0071 |
| $r^2$ | | 0.9994 | 0.9988 |
| $y$-Intercept | | 0.0094 | 0.0074 |
| Slope | | 0.0525 | 0.0619 |

equation for *l*-RA was $y = 0.0619x + 0.0074$ ($r^2 = 0.999$). Standard curves contructed in a similar way for each MPH enantiomer with *d*-ephedrine as internal standard are shown in Table II. The regression lines for *d*-MPH and *l*-MPH could be described by the equations $y = 0.0790x - 0.0061$ and $y = 0.0582x - 0.0038$, respectively. Studies carried out on intra-assay and inter-assay variabilities (Table III) revealed that the assay for RA enantiomers was precise

TABLE II

STANDARD CALIBRATION CURVE DATA FOR METHYLPHENIDATE ENANTIOMERS IN PLASMA

| Concentration of each enantiomer (ng/ml) | $n$ | Peak-height ratios of derivatised MPH enantiomer to internal standard (mean ± S.D.) | |
|---|---|---|---|
| | | *d*-MPH | *l*-MPH |
| 45 | 3 | 3.5698 ± 0.0927 | 2.6285 ± 0.1201 |
| 22.5 | 3 | 1.6082 ± 0.0368 | 1.2102 ± 0.0439 |
| 11.25 | 3 | 0.7580 ± 0.0441 | 0.5510 ± 0.0236 |
| 4.5 | 3 | 0.2991 ± 0.0195 | 0.2305 ± 0.0108 |
| 1.125 | 3 | 0.0515 ± 0.0017 | 0.0448 ± 0.0017 |
| 0.56 | 3 | 0.0269 ± 0.0017 | 0.0228 ± 0.0008 |
| 0.28 | 3 | 0.0138 ± 0.0006 | 0.0113 ± 0.0008 |
| $r^2$ | | 0.9973 | 0.9977 |
| $y$-Intercept | | −0.0061 | −0.0038 |
| Slope | | 0.0790 | 0.0582 |

and reproducible. A coefficient of variation of less than 10% was obtained for each RA enantiomer. Similar studies carried out for MPH enantiomers indicated that the assay for MPH was both precise and reproducible (Table III). A coefficient of variation of less than 10% was obtained for each enantiomer. Quality control studies (Table IV) carried out for both MPH and RA enantiomers revealed that the experimentally determined values were within 10% of the actual spiked concentration in each sample examined.

TABLE III

STATISTICAL EVALUATION OF THE ACCURACY AND REPRODUCIBILITY OF THE ASSAY FOR METHYLPHENIDATE AND RITALINIC ACID ENANTIOMERS IN PLASMA

| No. | Concentration of each enantiomer (ng/ml) | Concentration of each enantiomer after analysis (mean + S.D.) (ng/ml) | |
|---|---|---|---|
| | | *d* | *l* |
| *Methylphenidate* | | | |
| *Intra-assay variability*[a] | | | |
| 1 | 45 | 45.26 ± 1.18 | 45.33 ± 1.62 |
| 2 | 22.5 | 21.07 ± 0.49 | 21.02 ± 0.64 |
| 3 | 11.25 | 10.53 ± 0.54 | 11.09 ± 0.31 |
| 4 | 4.5 | 3.99 ± 0.23 | 4.39 ± 0.22 |
| 5 | 0.56 | 0.49 ± 0.02 | 0.54 ± 0.02 |
| 6 | 0.28 | 0.29 ± 0.01 | 0.30 ± 0.02 |
| *Inter-assay variability*[a] | | | |
| 1 | 45 | 45.13 ± 0.13 | 45.18 ± 0.17 |
| 2 | 22.5 | 22.17 ± 0.96 | 22.03 ± 0.87 |
| 3 | 11.25 | 10.80 ± 0.22 | 10.94 ± 0.34 |
| 4 | 4.5 | 4.47 ± 0.47 | 4.68 ± 0.41 |
| 5 | 0.56 | 0.57 ± 0.06 | 0.58 ± 0.04 |
| 6 | 0.28 | 0.27 ± 0.02 | 0.27 ± 0.03 |
| *Ritalinic acid* | | | |
| *Intra-assay variability*[a] | | | |
| 1 | 75 | 76.01 ± 4.92 | 75.97 ± 6.79 |
| 2 | 37.5 | 35.66 ± 1.11 | 36.31 ± 1.50 |
| 3 | 11.25 | 10.88 ± 0.71 | 10.84 ± 1.06 |
| 4 | 5.63 | 5.78 ± 0.45 | 5.37 ± 0.05 |
| 5 | 1.40 | 1.61 ± 0.07 | 1.70 ± 0.09 |
| *Inter-assay variability*[a] | | | |
| 1 | 75 | 74.91 ± 0.98 | 74.99 ± 0.93 |
| 2 | 37.5 | 37.72 ± 1.88 | 38.76 ± 2.21 |
| 3 | 11.25 | 11.42 ± 0.62 | 11.47 ± 0.70 |
| 4 | 5.63 | 5.41 ± 0.33 | 5.31 ± 0.08 |
| 5 | 1.40 | 1.45 ± 0.16 | 1.51 ± 0.14 |

[a] $n = 3$.

TABLE IV

ANALYSIS OF QUALITY CONTROL SAMPLES OF PLASMA SPIKED WITH METHYLPHENIDATE AND RITALINIC ACID

| Analyte | Spiked concentration of each enantiomer (ng/ml) | Experimentally determined concentration (mean ± S.D.) (ng/ml) | |
|---|---|---|---|
| | | *d* | *l* |
| Methylphenidate | 4.5 | 4.36[a] | 4.54[a] |
| | 1.25 | 1.29[a] | 1.32[a] |
| Ritalinic acid | 5.75 | 6.01[b] | 6.25[b] |
| | 2.00 | 1.99[b] | 2.16[b] |

[a] $n = 2$.
[b] $n = 3$.

The chiral assays were applied for the quantitation of MPH and RA enantiomers in the plasma of an adult male who received an oral dose of 40 mg *dl*-MPH. Fig. 3 shows the log plasma concentration–time curve of the enantiomers of MPH and RA. The levels of both MPH and RA could be mon-

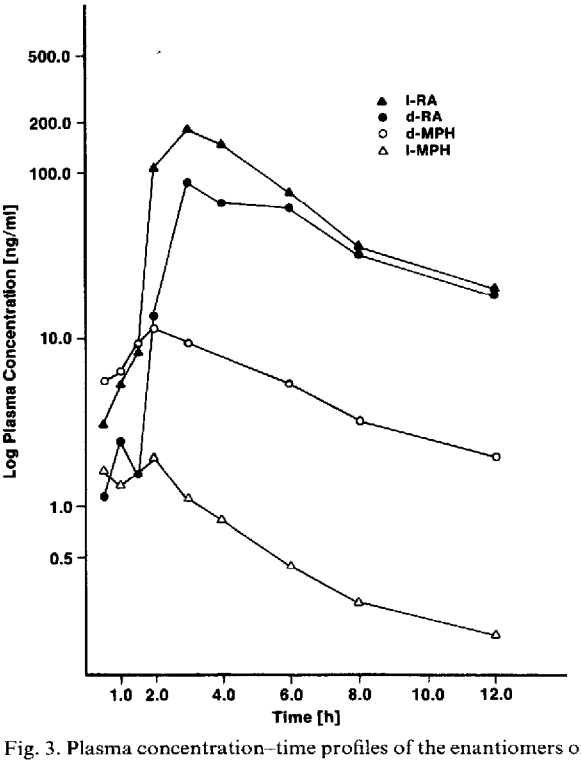


Fig. 3. Plasma concentration–time profiles of the enantiomers of methylphenidate (MPH) and ritalinic acid (RA) in a healthy adult volunteer after a single oral dose of 40 mg racemic methylphenidate hydrochloride.

TABLE V

SIMPLE PHARMACOKINETIC PARAMETERS OF ENANTIOMERS OF METHYLPHENIDATE AND RITALINIC ACID IN AN ADULT (70 kg) DOSED WITH 40 mg RACEMIC METHYLPHENIDATE HYDROCHLORIDE

| Compound | Enantiomer | $T_{max}$ (h) | $C_{max}$ (ng/ml) | $AUC_0^t$ (ng/ml per h) | $AUC_0^\infty$ (ng/ml per h) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|---|
| Methylphenidate | *d* | 2.0 | 11.71 | 65.24 | 76.91 | 4.06 |
| | *l* | 2.0 | 1.97 | 7.53 | 8.45 | 3.76 |
| Ritalinic acid | *d* | 3.0 | 80.71 | 440.57 | 538.82 | 3.67 |
| | *l* | 3.0 | 178.63 | 755.95 | 852.66 | 3.35 |

itored up to 12 h after the dosing. Table V shows some simple pharmacokinetic parameters calculated from the plasma concentration–time profiles of both MPH and RA. The times to peak concentration ($T_{max}$) for the enantiomers of MPH were 2.0 h, whereas the corresponding values for RA enantiomers were 3.0 h. The peak plasma concentration values ($C_{max}$) were higher for RA enantiomers than the corresponding MPH enantiomers. $AUC_0^t$ values calculated for the MPH enantiomers indicated that *d*-MPH (65.24 ng/ml per h) were eight-fold larger than *l*-MPH (7.53 ng/ml per h) whereas comparison of $AUC_0^t$ varies for RA enantiomers indicated *l*-RA (755.95 ng/ml per h) was 1.75-fold larger than *d*-RA (440.57 ng/ml per h).

The apparent $t_{1/2}$ values calculated for *d*-RA (3.67 h) and *l*-RA (3.35 h) were shorter than the corresponding values for *d*-MPH (4.06 h) and *l*-MPH (3.76 h). No statistical data could be obtained on the comparisons of the pharmacokinetic parameters of each pair of enantiomers due to participation of a single subject in the pilot study.

In conclusion, an enantioselective procedure has been developed for the quantitation of RA enantiomers in plasma. Previously, the configurational stability, reliability and versatility of HPC in its application as a chiral derivatising agent has been well established [16]. Furthermore the present method has been demonstrated to be reliable, sensitive and reproducible. Although marked enantioselectivity has been demonstrated in the disposition of MPH in humans [8,9,17], there appeared to be a less pronounced distortion in the enantiomeric ratio of RA in plasma. However, further studies are needed to confirm the present preliminary data.

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