Enantioselective Analysis of Ritalinic Acids in Biological Samples by Using a Protein-Based Chiral Stationary Phase

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Purpose. This study was to develop and validate a new chiral HPLC-UV method for the quantitative analysis of enantiomeric ritalinic acid (RA) in human plasma.

Methods. An α_1-acid glycoprotein column was used with the mobile phase containing 0.4% acetic acid and 0.1% dimethyloctylamine, pH 3.4. The detection of enantiomeric RAs was at 220 nm.

Results. A baseline separation for d- and l-RA was achieved by a separation factor of 2.08. Methylphenidate, the precursor of RA, was eluted with the front solvent, and thus does not interfere in the analysis of RA in our method. The assay was successfully applied for the *in vitro* analysis of enantiomeric ritalinic acids produced by human and dog plasma and dog liver.

Conclusions. Data demonstrated that the esterase(s) in human plasma metabolize d-methylphenidate faster than its l-isomer. The yielded intrinsic clearances (Cl_{int}) are 1.02 and 2.17 μl/min/mg protein, respectively, for d and l-methylphenidate.

KEY WORDS: ritalinic acid; methylphenidate; chiral separation; HPLC.

INTRODUCTION

Methylphenidate (d- and l-threo-methyl-α-(2-piperidyl) acetate) is a piperidine-derived central nervous system stimulant. It is the most commonly prescribed psychoactive medication given to children in North America for the treatment of attention deficit hyperactivity disorder (ADHD). Methylphenidate (MPH) has two asymmetric centers, and exists as four enantiomers (1). Methylphenidate is extensively metabolized through hydrolysis by esterase, producing ritalinic acid (RA) as its major metabolites. The structures of d- and l-threo-MPH and d- and l-threo-RA are shown in Fig. 1. It was found that the d- and l-threo pair of enantiomers is pharmacologically more active than the d- and l-erythro isomers, and d-threo-MPH is more active than its l-threo antipode (2). MPH has been reported to undergo enantioselective metabolism in human adults and children with ADHD as well as rats and dogs (3–7). Hydrolytic cleavage, a Phase I metabolic pathway, brought about by various tissue esterases, was shown to be partly responsible for this stereoselectivity (2). Previous *in vitro* work using dog and human plasma demonstrated the stereoselectivity existing in its enzymatic hydrolysis; d-MPH is preferred as a substrate of esterase.

Earlier chiral separation of d- and l-MPH used gas chromatography with a variety of detectors, such as electron capture detector and mass spectrometry (8,9). All of these techniques are limited in their direct application since they require the derivatization of the analyte to improve volatility, enhance detection or allow the separation of diastereomers using achiral stationary phases. In this latter process it is essential that the derivatization steps are quantitative and the derivatizing agent is optically pure. Since the mid of 1980s, chiral separations using newer techniques of HPLC and capillary electrophoresis (CE) have been successfully applied to directly analyze non-volatile, mixtures of chiral compounds. Such techniques avoid the extra steps of derivatization and remove the need for optically pure reagents. In some cases these techniques can demonstrate excellent resolution characteristics (10,11).

In this article, we report a direct chiral HPLC-UV method, which was fully validated for the quantification of d and l-threo-RA enantiomers in human plasma and enzyme incubation mixtures. To this end three chiral methods for separation of d and l-RA have been investigated, and the method using α_1-acid glycoprotein (AGP) bonded column was demonstrated to be the best one for the quantitative analysis of RA in biologic samples. The developed method was applied to the determination of the enantiomeric composition of d- and l-RA in biologic samples. Hydrolysis of MPH mediated by plasma and a commercially available carboxyl esterase (E-3128, from porcine liver) was characterized.

MATERIALS AND METHODS

Materials

Racemic MPH·HCl was purchased from the USP Convention Inc. (Nucrotechnics, Montreal, Canada). Pure d- and l-threo RA were gifts from Copley Pharmaceuticals, Inc. (Boston, MA, USA,). CIBA-Geigy Company (Basel Switzerland) kindly donated the d, l-threo racemic mixture of RA. The chiral-AGP column was obtained from ChromTech AB Company (Sweden). 5-Hydroxyindole acetic acid (5-HIAA) used as internal standard, and dimethyloctylamine (DMOA) were of analytical grade and purchased from SIGMA Chemical Co. (St. Louis MI). All other chemicals were of analytical grade while organic solvents were of HPLC grade and used without further purification. Esterase E-3128 purified from porcine liver was purchased from SIGMA Chemical Co. (St. Louis MI, USA).

HPLC-UV Assay

The HPLC system consisted of an isocratic HPLC pump (Model 590, Waters), an autoinjector (SIL-10A, Shimadzu) fitted with an injection loop of 200 μl and directly connected to the chiral AGP column (150 × 4.0 mm id.). Detection of analyte was achieved using a variable wavelength UV detector (Model 481, Waters) set at 220 nm. A reversed-phase mode was used, and the mobile phase consisted of 0.4% acetic acid containing 0.1% DMOA, pH 3.4, which DMOA was used as an organic modifier. The mobile phase was degassed before use. The flow-rate was 1 ml/min and an ambient column temperature was used. AGP column used in the experiments are stable within at least 3 months.

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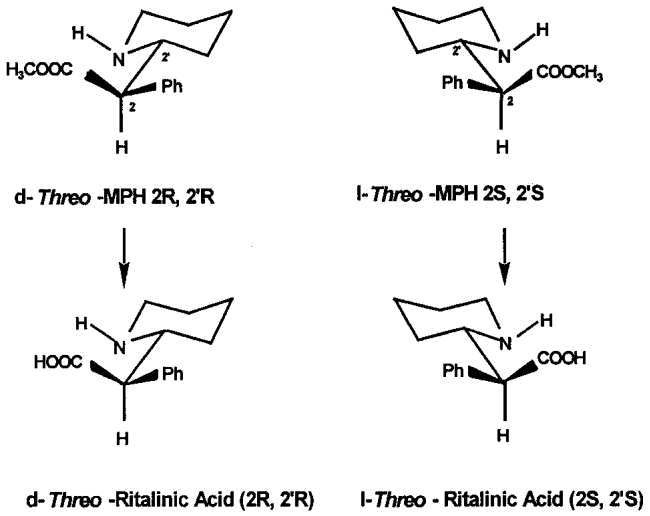

Fig. 1. Structures of d, l-threo-methylphenidate and d, l-threo-ritalinic acid

Method Validation in Incubation Mixtures With Human Plasma and Purified Esterase

Stock solutions of d- and l-RA (5 mg/ml for each enantiomer) were prepared in 0.35 M HCl. These solutions were stable under storage at 4°C for at least 2 weeks. The stock solution of d, l-MPH (1mg/ml) was freshly prepared for each analytical run. All solutions were prepared in borosilicate glass vials (Kimble Glass INC., Vineland, NJ, USA). For the preparation of the calibration samples in plasma, d- and l-RA was diluted to give standard solutions of 25, 50, 100, 175 and 250 mg/L in human plasma or in distilled deionized water. The concentration of the internal standard (5-hydroxyindole acetic acid) was 100 μg/ml. The sample numbers (n) for each concentration were 6,2,6,2 and 6 corresponding to from 25 to 250 mg/L, which pattern satisfies FDA recommended guidelines for method validation. Each calibrator was placed in 1.5-ml Eppendof tubes, and 100 μl of 1 M perchloric acid was added to precipitate plasma protein. The mixture was vortex-mixed, and then centrifuged at 13,300*g* for 10 min. The supernatant of the deproteinized solution was transferred to injection vials for HPLC analysis.

Standard curves were also obtained with the concentration of 5, 7, 10, 20, 50, 100 μg/ml in the presence of 100 μg/ml internal standard and esterase E-3128 (ca. 40 μg protein) in final volume of 500 μl of 10 mM potassium phosphate buffer with pH 7.4. Other sample treating procedures are the same as those in human plasma.

Stability of d- and l-RA and Methylphenidate in Stock Solutions. Stock solutions of d- and l-RA (5 mg/ml for each enantiomer) were prepared in 0.35 M HCl. This stock solution was examined to be stable under storage at 4°C for at least 2 weeks. It was found that d- and l-MPH can be automatically hydrolyzed in neutral or acidic solutions, and thus their stock solutions (1mg/ml) have to be freshly prepared. For the preparation of calibrators, d- and l-RA were freshly diluted to give standard solutions of 25, 50, 100, 175 and 250 mg/L in 1 ml of human plasma or in distilled deionized water.

Hydrolysis of MPH Mediated By Human And Dog Plasma, Dog Liver And Purified Enzyme E-3128 d- and l-MPH (5 mg/L to 100 mg/L for each isomer) was incubated with esterase E-3128 (ca. 40 μg protein) in 500 μl of potassium phosphate buffer (10 mM, pH 7.4), for 2 h. d- and l-MPH (50 mg/L for each isomer) was also incubated with dog liver and plasma of human and dog, respectively, in 500 μl of potassium phosphate buffer (10 mM, pH 7.4), for 2 h. The enzymatic reaction was terminated by the addition of 100 μl of 0.1 M perchloric acid. MPH was also found to be hydrolyzed non-enzymatically, and thus a parallel blank incubate was prepared using a denatured enzyme sample (incubated in boiling water for 2 min). After centrifugation at 13,300*g* for 10 min, the supernatants were determined for levels of d and l-RA.

RESULTS AND DISCUSSION

Establishment and optimization of the HPLC enantioseparation method for the determination of d, l-threo-RA enantiomers. In our study, an AGP column that is derived from α_1-acid glycoprotein was used. The separation of the enantiomers was optimized by altering the concentration and pH value of the buffer, as well as the concentration and nature of the organic solvent. It has been reported that a small amount of DMOA in the mobile phase can significantly improve chiral separations, and lead to an increased α value in the separation of chiral compounds possessing a carboxyl moiety, such as NSAIDS and naproxen (12,13). The optimal mobile phase selected was a mixture of 0.4% acetic acid and 0.1% DMOA. The highest separation factor (α) that was reached for the corresponding separation of d- and l-RA was 2.08 (Fig. 2) obtained from aqueous pure sample solution. The separation was accomplished within a short time of 6 min.

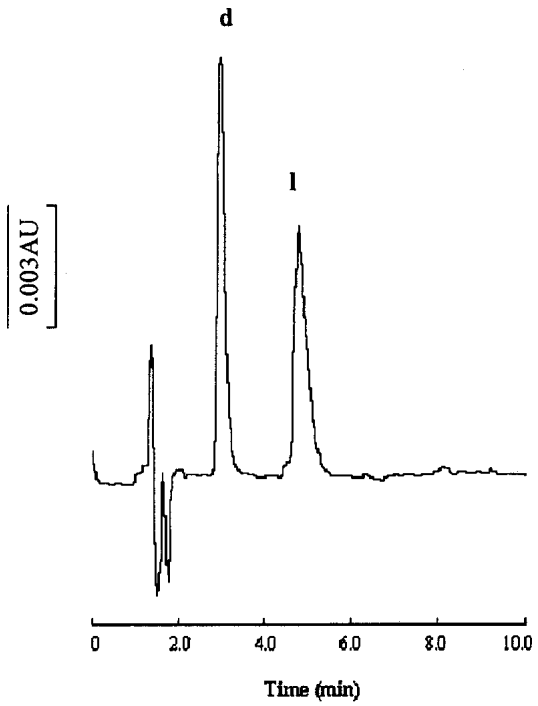

Fig. 2. Separation pattern of d, l-ritalinic acid on an AGP column. Sample was obtained from standard solution. Column: Chiral-AGP column (150 × 4.0 mm, ChromTech AB); Mobile phase: 0.4% acetic acid, pH 3.4, containing 0.1% DMOA. Peaks: 1, d-ritalinic acid, 2, l-ritalinic acid.

MPH, the precursor of RA, was demonstrated to be eluted very quickly (almost co-eluted with front solution), and thus it does not interfere in the analysis of RA in our method.

Method Validation In Human Plasma

The HPLC-UV method was validated in order to quantitatively analyze the metabolites in the plasma. The linearity of HPLC analyses of the standards was examined and demonstrated a good linear relationship between concentration and the ratio of peak height of analyte over that of the internal standard. The range of concentration obtained was 1.25 μg to 12.5 μg per injection. The correlation coefficients for d- and l-RA enantiomers were 0.994 and 0.992, respectively. The detection limits for d- and l- RA were 100 and 150 ng per injection, respectively, at a signal-to-noise ratio of three.

Intra- and inter-day precision of the assays for d- and l-RA were determined. Blank pooled human plasma was spiked with different amounts of d- and l-RA and internal standard. After the removal of proteins, the amount of RA enantiomers in the supernatants was detected. The intra-day and inter-day precision of this assay is shown in Table I. The C.V. of the intra-day analysis for d- and l-RA was found to be 2.73–5.45% and 5.89–6.98%, respectively, and the inter-day analysis (within 3 days) gave the C.V. of 1.36–4.19%, and 2.19–5.79%, respectively. Table II summarizes the accuracy of the method for d- and l-RA. The accuracy for d- and l-enantiomers, expressed as the difference between the amount added and that observed was 90.9–99.2% and 86.5–98.6% in the measured ranges of 25 to 250 mg/L. The reproducibility and accuracy satisfy recommended guidelines for assay precision and accuracy (14). The limit of quantitation (LOQ) was 25 mg/L. The recovery for both d- and l-RA in human plasma was higher than 85%.

Method Validation in Enzyme Buffer Solution

The chiral HPLC-UV method was also validated for the determination of d- and l-RA that are produced from MPH by the esterase E-3128.

To determine the linearity of the method, various concentrations of diluted solution of racemic RA were quantitatively analyzed. Standard curves were obtained with the concentrations of 5, 7, 10, 20, 50, 100 μg/ml (final concentration)

Table I. The Intra-Day and Inter-Day Precision of this Assay for d- and l-Ritalinic Acids

Spiked concentration (μg/ml)	d-RA		l-RA	
	Amount (μg/ml)	CV (%)	Amount (μg/ml)	CV (%)
intra-day				
250 (n = 6)	265.04 ± 7.23	2.73	263.08 ± 18.37	6.98
100 (n = 6)	101.57 ± 5.53	5.45	102.28 ± 6.02	5.89
25 (n = 6)	22.71 ± 0.67	2.94	21.63 ± 1.46	6.74
Inter-day				
250 (n = 18)	253.23 ± 10.61	4.19	251.25 ± 9.32	3.71
175 (n = 6)	163.45 ± 2.23	1.36	165.15 ± 4.06	2.46
100 (n = 18)	105.54 ± 2.68	2.53	109.20 ± 2.39	2.19
50 (n = 6)	50.39 ± 1.09	2.16	50.02 ± 1.44	2.88
25 (n = 18)	23.23 ± 0.89	3.83	21.15 ± 1.23	5.79

* Inter-day CVs were determined for three independent experiments.

Table II. Accuracy of the Assay for d- and l-Ritalinic Acids

Spiked concentration (μg/ml)	d-RA		l-RA	
	Amount (μg/ml)	Accuracy (%)	Amount (μg/ml)	Accuracy (%)
250 (n = 6)	265.04 ± 7.23	93.98	263.08 ± 18.37	93.69
175 (n = 2)	171.94	98.25	166.49	95.14
100 (n = 6)	101.57 ± 5.53	98.43	102.28 ± 6.02	97.72
50 (n = 2)	49.58	99.17	49.31	98.62
25 (n = 6)	22.71 ± 0.67	90.85	21.63 ± 1.46	86.53

in the presence of 100 μg/ml internal standard and esterase E-3128 (ca. 40 μg protein) in a final volume of 500 μl of 10 mM potassium phosphate buffer, pH 7.4. A good linear relationship was obtained over a range of concentration (5–100 μg/ml). The correlation coefficients for d- and l-RA were 0.999 and 0.995, respectively. Quality control samples were prepared in duplicate using 10, 50, and 100 μg/ml directly diluted from the stock solutions. The accuracy from the QC samples was shown higher than 85% of the normal values.

Hydrolysis Catalyzed By Human and Dog Plasma

The hydrolysis of d- and l-MPH was investigated using enzymes obtained from human and dog plasma. After *in vitro* incubation for up to two hours in dog and human plasma, the concentrations of d- and l-RA was determined using the HPLC-UV method described above. The specific activities are summarized in Fig. 3. As can be seen, esterases both in human and dog plasma catalyze the hydrolysis of MPH. It was confirmed that there is stereoselectivity in the enzyme activity to hydrolyze d- and l-MPH. In contrast with liver, esterases in

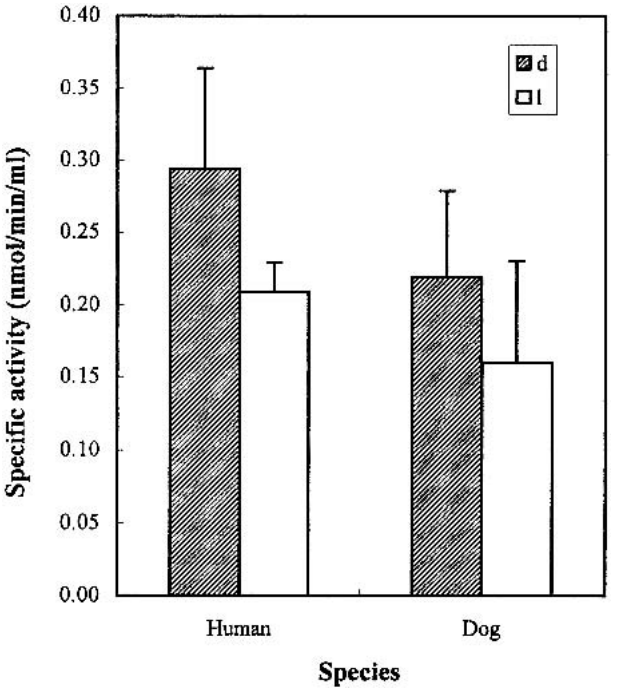

Fig. 3. Enzyme hydrolysis of methylphenidate by plasma of dog and human. *, $p < 0.05$, significant difference between d- and l-methyphenidates.

plasma have a higher activity to d-MPH than that to its l-form because esterases are different existing in plasma and liver. Results are consistent with a previous report where the remaining concentration of MPH was measured when it was incubated with human plasma (2).

Carboxyl Esterase (E-3128)-Mediated Hydrolysis

It is known that MPH is a typical substrate of carboxyl esterases. However, the enzymes involved in its hydrolysis were not well characterized, especially in their stereoselectivity. Furthermore it still remains unknown whether or not other esterases, such as cholinesterases, are also involved in the hydrolysis of MPH. In our study, three esterases were chosen as enzyme sources, which are an acetyl cholinesterase isolated from electronic eel, butyryl cholinesterase from horse serum and a carboxyl esterase from porcine liver. It was found that acetyl and butyryl cholinesterase did not catalyze the hydrolysis of MPH. Therefore we focus on the characteristics of carboxyl esterase (E-3128) on the hydrolysis of MPH.

The enzymatic hydrolysis of MPH was calculated by subtraction of the amount of RA non-enzymatically produced with the denatured enzyme samples. The production of ritalinic acids was linearly dependent on the incubation time for up to 2 h at 37°C and on the protein amount of the enzyme for up to ca, 40 μg protein in each incubate. The values of the apparent Michaelis constant, K_m, and the maximal velocity, V_{max}, were calculated with Lineweaver and Burk plot (Fig. 4). The apparent K_m and V_{max} values were 0.98 mM and 1.00 nmol/min/mg protein, respectively, for d-MPH and 2.3 mM and 5.00 nmol/min/mg protein, respectively, for l-MPH. The intrinsic clearance (Cl_{int}) for l-RA (2.17 μl/min/mg protein) was higher than that of d-isomer (1.02 μl/min/mg protein). The results show that there are enantioselective differences in the E-3128-mediated hydrolysis between d- and l-MPH enantiomers. In vivo studies have shown that the levels of l-MPH are approximately one-fifth its d-enantiomer (2). This stereoselective disposition of MPH could be caused by the stereoselective hydrolysis of MPH in the liver rather than in plasma. A direct characterization of the hydrolysis of MPH in human liver is required to validate this hypothesis.

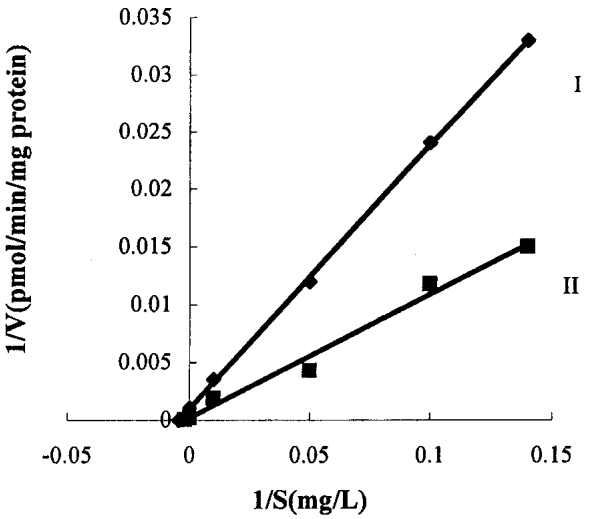

Fig. 4. Lineweaver-Burk's plot of the enzymatic hydrolysis of d- and l-methylphenidates. The reciprocal of the reaction velocity (pmol/min/mg protein) was plotted against that of the substrate concentration (mg/L). I: for d-methylphenidate; II: for l-methylphenidate.

In summary, a new chiral HPLC-UV method has been developed and validated for the quantitative analysis of enantiomeric RA in human plasma and enzyme buffer solution, and furthermore it was successfully applied for the *in vitro* enzymatic studies on the stereoselective hydrolysis of MPH to RA. The stereoselective method of d, l-RA can be used for the *in vivo* pharmacokinetic study of methylphenidate.

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