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Short communication

Enantioseparation of (±)-*threo*-methylphenidate in human plasma by cyclodextrin-modified sample stacking capillary electrophoresis

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ABSTRACT

The (±)-*threo*-methylphenidate ((±)-*threo*-MP) is widely used for treatment of attention-deficit hyperactivity disorder (ADHD). According to clinical evidence, (+)-*threo*-MP possesses higher potency than (−)-*threo*-MP. Due to ppb level in plasma, till now, none of the capillary electrophoresis (CE) methods have been able to provide adequate sensitivity for therapeutic (±)-*threo*-MP monitoring. In this study, a cyclodextrin-modified field-amplified sample stacking CE method (CD-FASS-CE) for enantioseparation of (±)-*threo*-MP in human plasma was established for clinical applications. Phosphate buffer (50 mM, pH 3.0) was filled into uncoated fused silica capillary (40 cm, 50 μm I.D.), followed by a water plug (0.5 psi, 10 s). Electrokinetic injection (6 kV, 200 s) was used to load samples and to enhance sensitivity. Stacking and separation were performed at 20 kV and 200 nm using phosphate buffer (50 mM, pH 3.0) containing 20 mM HP-β-CD and 30 mM triethanolamine. Analytes were separated simultaneously by using CD-FASS-CE and had a lower detection limit of equal to the sub-ppb level. Linear calibration curves were obtained from 1 to 80 ng/mL (r = 0.998). The limit of detection for both isomers was 600 pg/mL. RSD and RE of precision and accuracy in intra- and inter-day assays were below 7.89%. This method was further applied to analyze (±)-*threo*-MP in four healthy Asian volunteers and that provided some relevant information for clinical treatments.

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is one of the most common disorders among school-going children with a prevalence rate of about 5–10% in the general population [1]. It has enormous impact on an individual's psychological development, education, relationships and family. ADHD in childhood often continues into adolescence and adulthood [2–4]. Popular medication for ADHD is racemate of *d*-threo-(R,R)-methylphenidate ((+)-*threo*-MP) and *l*-threo-(S,S)-methylphenidate ((−)-*threo*-MP), known as (±)-*threo*-MP [1,5–7]. Though the mode of action in humans is not completely understood as yet, it is thought to block re-uptake of norepinephrine and dopamine into the presynaptic neuron and increase the release of these monoamines into the extraneuronal space. According to clinical evidence, (+)-*threo*-MP possesses higher potency than (−)-*threo*-MP [5–10]. Due to the different performances of (+)- and (−)-*threo*-MP, metabolism and disposition in vivo have become an interesting issue. The enantioselective metabolism of (±)-*threo*-MP results in markedly higher plasma concentrations of (+)-*threo*-MP [6]. Therefore, it is necessary to establish a sensitive and reliable method for further clinical assessments.

A number of analytical methods were used for determination of (±)-*threo*-MP in biological samples [11–26]. The most used detector is mass spectrometer (MS). However, it is not so popular for routine analysis. An assay employing gas chromatographic (GC) methods with flame ionization detection (FID) requires large volume blood samples (10 mL) and lacks sensitivity [25]. One assay is based on GC–chemical ionization MS where the limit of quantitation (LOQ) was 1 ng/mL [22]. A GC method with a dual nitrogen–phosphorus FID was able to reach the detection limit of 1 ng/mL in plasma [16]. Unfortunately, the sample preparation was time-consuming and required an extensive kit prior to GC analysis. HPLC-fluorescence is perhaps the first non-MS detection method that can meet the requirements of selectivity and sensitivity [18].

Some stacking CE methods were developed to enhance sensitivity [27–30], such as field amplified sample stacking (FASS) and dynamic pH junction or large volume sample stacking (LVSS). Electrokinetic injection is supposed to load more ionic samples than the hydrodynamic mode. Our aim is to develop the first stacking

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CE method using UV detector for enantiomeric separation of (±)-*threo*-MP for clinical monitoring. On-line stacking CE coupled with chiral separation was investigated and optimized. The application in four volunteers also provided some dosing information.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade, available commercially. (±)-*threo*-MP was purchased from Cerilliant Co. (Round Rock, TX, USA). Amphetamine (A), methamphetamine (MA), codeine (C), ritalinic acid (RA), risperidone (as internal standard, IS) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were from Sigma–Aldrich Co. (St. Louis, MO, USA). α-CD, β-CD, γ-CD and other derivative CDs were from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Sodium dihydrogen phosphate (NaH_2PO_4), n-hexane, HCl, triethanolamine (TEA) and NaOH were purchased from Merck Co. (Merck, Darmstadt, Germany). Milli-Q water (Millipore, Bedford, MA, USA) was used for preparation of buffer and related aqueous solutions.

2.2. Sample preparation

Stock solutions of (±)-*threo*-MP and IS were prepared in water at concentration of 1 mg/mL, and were diluted with plasma as the working solution. All stock solutions were stored at 4 °C. Blank plasma was collected from healthy volunteers in our laboratory. All plasma samples were stored at −70 °C until analysis. Liquid-liquid extraction was performed by adding 1 ml of n-hexane into 400 μL aliquot of plasma containing (±)-*threo*-MP and IS. After vortexing and a 10-min centrifugation at 12,000 × *g*, 900 μL of the supernatant was dried in a vacuum centrifuge. The residue was reconstituted with water (100 μL) and then analyzed in CE.

2.3. CE system

CE separations were performed on a Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with a UV detector. The data were analyzed by Karat 3.2 software. All separations were performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm id and 30 cm effective length (total length 40 cm). The running buffer was phosphate/TEA/HP-β-CD solution adjusted to pH 3.0, and the detector wavelength was set at 214 nm. The required pH value was adjusted with 1.0 M HCl solution. Before separation, the new capillary was preconditioned with 1.0 M HCl solution for 15 min, water for 10 min, 1.0 M NaOH solution for 15 min and water for 10 min. Temperature of the capillary was controlled at 25 °C by liquid coolant. The capillary was rinsed with 0.1 M NaOH for 5 min, water for 5 min, and running buffer for 10 min before each run.

2.4. CD-FASS-CE

The capillary was first filled with phosphate buffer (50 mM, pH 3.0), followed by a water plug (0.5 psi, 10 s). Electrokinetic injection (6 kV, 200 s) was used to load samples and to enhance sensitivity. Stacking and separation were performed at 20 kV and 200 nm, using phosphate buffer (50 mM, pH 3.0) containing 20 mM HP-β-CD and 30 mM TEA.

2.5. Method validation

Calibration curves were established by spiking (±)-*threo*-MP in 400 μL blank human plasma to obtain concentrations of 1, 5, 10, 40 and 80 ng/mL. The limits of detection (LOD) were determined by spiking the reference standards with decreasing concentrations of each analyte until the ratio of signal-to-noise equaled to 3 (S/N = 3). Accuracy and precision were determined by five-replicate analysis of blank human plasma spiked at different concentrations (2, 20, and 60 ng/mL) of (±)-*threo*-MP. Selectivity assay was evaluated by simultaneous separation of amphetamine (A), methamphetamine (MA), codeine (C), (±)-*threo*-MP and its acidic metabolite, ritalinic acid (RA).

2.6. Applications

Informed consents were obtained from the volunteers who received a single oral dose (0.3 mg/kg) of immediate-release (±)-*threo*-MP (Ritalin®, Novartis, Pharmaceuticals, Summit, NJ.). Blood samples were collected prior to oral administration of (±)-*threo*-MP (0 hr), and 0.5 h, 1 h, 2 h and 3 h after oral dosing. Blood collection tubes were green-top blood sample tubes (6 mL) which contained sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Samples were immediately centrifuged, and the plasma was transferred to vials and stored at −70 °C.

3. Results and discussion

3.1. Optimization of CE

Some stacking methods have been tried. FASS provided better sensitivity than others. Longer injection time probably increased the sensitivity. At 6 kV, injection time longer than 200 s could not increase the sensitivity significantly. Therefore, 200 s was used. Various CDs (α-CD, β-CD, γ-CD and HP-β-CD) were tried at a concentration of 20 mM, which were dissolved in a 30 mM TEA-50 mM phosphate buffer (pH 3.0) under 20 kV and 25 °C. In general, the cavity size of CD is crucial to chiral separation. If the cavity size of CD is much larger or smaller than the size of the chiral drugs, the inclusion between CD and drugs may be ineffective. Therefore, the choice of a proper CD for chiral separation plays a very important role. HP-β-CD is widely used because of its good inclusion and solubility [31]. The results showed that both α-CD and γ-CD were not able to interact with (±)-*threo*-MP racemates (data not shown). Since β-CD was better but had poor solubility, HP-β-CD was selected. Here, HP-β-CD in the range of 0 mM to 30 mM was tested. Higher concentrations (greater than 20 mg/mL) of HP-β-CD resulted in better resolution, but longer migration time. Finally, HP-β-CD was set at 20 mg/mL.

The capillary's temperature affects viscosity of the buffer solution as well as the inclusion complex between analytes and CDs. In this study, migration time and resolution were examined from 15 °C to 25 °C, of which 25 °C was found to be the most suitable temperature. The buffer pH affected the degree of ionization of analytes and electroosmotic flow (EOF) in the capillary. At lower pHs, (±)-*threo*-MP was fully protonated and the EOF was suppressed. The enantiomeric resolution increased as the EOF decreased. Here, pH 1.5 to pH 3.5 in phosphate/TEA/HP-β-CD buffer were tested. Lower pH buffer (pH 2.0) showed higher current (exceeding 100 μA). However, when pH value was altered from 3.0 to 3.5, the order of IS peak shifted behind the (±)-*threo*-MP because IS possessed two pK_a values (pK_{a_1} 8.2 and pK_{a_2} 3.1) that resulted in different degrees of ionization after the pH value was changed. Finally, pH 3.0 was selected.

The peak tailing was a troublesome issue. After many trials, TEA reduced the difficulty as it is a strong cationic molecule that can be used to reduce adsorption of analytes onto the capillary wall [32]. Using a phosphate/HP-β-CD buffer without TEA resulted in significant peak tailing (data not shown). Analytes possessing positive charges under the given pH value might adsorb on the capillary

Table 1
Regression analysis for determination of (±)-*threo*-MP in plasma.

Compound	Regression equation	Coefficient of correlation (r)
Intra-day[a]		
(+)-*Threo*-MP	$Y=(0.025\pm0.001)X-(0.009\pm0.002)$	0.999
(−)-*Threo*-MP	$Y=(0.025\pm0.001)X-(0.008\pm0.002)$	0.998
Inter-day[b]		
(+)-*Threo*-MP	$Y=(0.022\pm0.001)X-(0.025\pm0.001)$	0.999
(−)-*Threo*-MP	$Y=(0.021\pm0.001)X-(0.064\pm0.002)$	0.998

[a] Regression equations of intra-batch analysis were calculated from assay values of prepared standard on a single day ($n=3$).
[b] Regression equations of inter-batch analysis were calculated from assay values of prepared standard on five different days.

Table 2
Precision and accuracy for determination of (±)-*threo*-MP in human plasma in an intra-day ($n=3$) and inter-day ($n=5$) analysis.

	Conc. known (ng/mL)	Conc. found (ng/mL)	RSD (%)	RE[a] (%)
Intra-day				
(+)-*Threo*-MP	2	1.9 ± 0.05	2.6	−5.0
	20	20.3 ± 0.2	1.0	1.5
	60	60.0 ± 0.5	0.8	0.0
(−)-*Threo*-MP	2	1.9 ± 0.04	2.1	−5.0
	20	20.4 ± 0.3	1.4	2.0
	60	59.8 ± 0.4	0.7	−0.3
Inter-day				
(+)-*Threo*-MP	2	2.0 ± 0.1	5.0	0.0
	20	20.1 ± 0.2	0.1	0.5
	60	60.4 ± 0.5	0.8	0.7
(−)-*Threo*-MP	2	2.0 ± 0.1	5.0	0.0
	20	20.1 ± 0.2	1.0	0.5
	60	59.9 ± 0.4	0.7	−0.1

[a] RE (%) = (concentration found − concentration known)/(concentration known) × 100.

wall. Zero to 40 mM of TEA were tried. Better resolved peaks were observed at concentrations greater than 30 mM. Thirty mM TEA was set for separation.

3.2. Method validation

In this research, LOD for both isomers was 600 pg/mL, lower than those detected by other CE methods [19,22]. The calibration curves were established by using blank plasma spiked with (±)-*threo*-MP at concentrations from 1 ng/mL to 80 ng/mL (Table 1). The curves were linear with correlation coefficients greater than 0.998. Three concentrations (2, 20 and 60 ng/mL) in five replicates

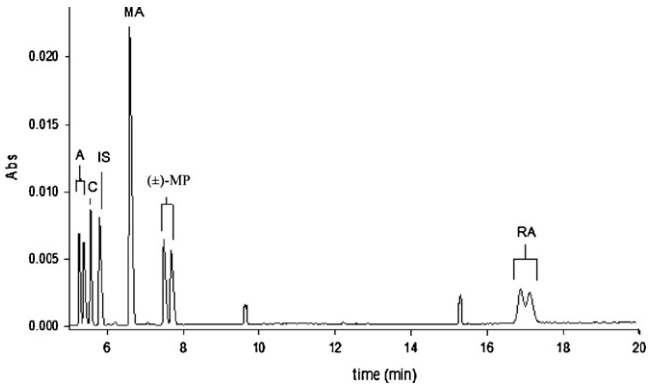

Fig. 1. Electropherogram of standards spiked in plasma for the selectivity test. CE conditions: 50 mM phosphate containing 20 mM HP-β-CD and 30 mM TEA in uncoated fused silica capillary (40 cm × 50 μm I.D.). Peaks: A, amphetamine; C, codeine; MA, methamphetamine; (±)-*threo*-MP), (±)-*threo*-methylphenidate; RA, ritalinic acid; IS, internal standard.

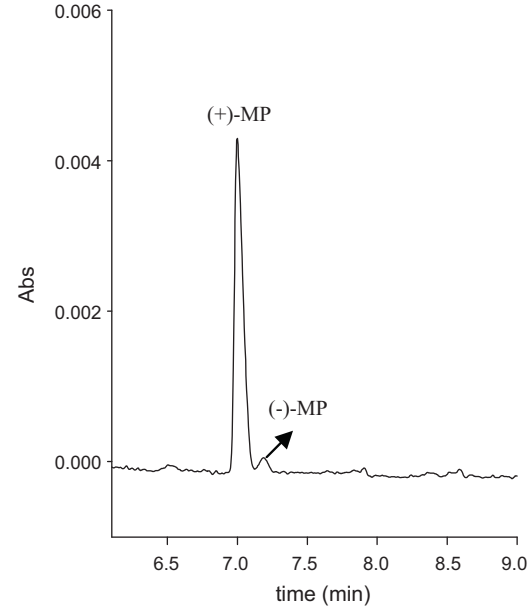

Fig. 2. Electropherogram of analysis of (±)-*threo*-MP in one volunteer's plasma. CE conditions are the same as Fig. 1.

were used to validate the accuracy and precision of the developed method (Table 2). Relative standard deviations (RSD) and relative errors (RE) of inter- and intra-day assays were less than 5.0 and 2.0, respectively, indicating good precision and accuracy.

Selectivity was examined by separation of (±)-*threo*-MP and other related substances. (±)-*threo*-MP in terms of both structure and pharmacological effects was similar to commonly abused drugs such as A, MA and C. These substances and the inactive metabolite, ritalinic acid (RA), were checked and the absence of interference of (±)-*threo*-MP was confirmed. As shown in Fig. 1, (±)-*threo*-MP was well separated from A, C, MA and RA. This method shows good selectivity for (±)-*threo*-MP and related interferences.

3.3. Applications

This method was applied to four healthy volunteers. Electropherogram of one real sample is shown in Fig. 2. The front peak, determined as (+)-*threo*-MP, was higher. The developed CE method was successfully applied to human plasma of four healthy

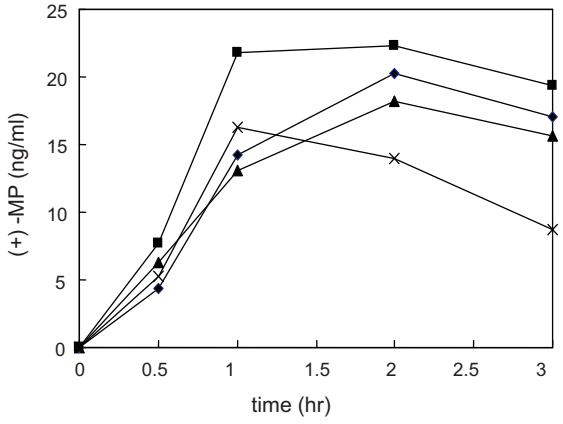

Fig. 3. Plasma concentration–time curves of (+)-*threo*-MP of four healthy volunteers after oral administration of Ritalin® (0.3 mg/kg). Volunteer I (25 year, female, ♦), volunteer II (26 year, male, ■), volunteer III (40 year, female, ▲), volunteer IV (30 year, male, ×).

volunteers each of whom was given 0.3 mg/kg of (±)-*threo*-MP. When collecting samples at 0.5 h, 1 h, 2 h and 3 h after dosing, (+)-*threo*-MP concentrations obtained were between 4.3 ng/mL and 22.3 ng/mL (Fig. 3). The (−)-*threo*-MP concentrations, however, were not completely examined through this method because their presence in human plasma after metabolism is minor, as mentioned in previous studies. The time to maximum plasma concentration (t_{max}) was 1–2 h. Peak plasma concentrations (C_{max}) of (+)-*threo*-MP were 14.2–22.3 ng/mL. The data were found to be in agreement with the previous study [33].

4. Conclusion

In this paper, development of a stacking CE method for chiral separation of (±)-*threo*-MP using CD as a chiral selector is proposed. To the best of our knowledge, this is the first on-line stacking CE–UV method without MS or LIF, and can provide sufficient sensitivity for (±)-*threo*-MP in clinical monitoring. Several parameters that affect chiral separation have been examined. The results were obtained in less than 8 min, with a satisfactory resolution. The method was applied to four healthy volunteers and the acquired pharmacokinetics data were found coincident with previous studies. This method showed excellent selectivity, linearity, precision and accuracy. With this method, more detailed pharmacokinetic databases of ADHD patients in Taiwan can be established.

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