JPET Fast Forward. Published on April 13, 2004 as DOI: 10.1124/jpet.104.067116

Title page

Methylphenidate is stereoselectively hydrolyzed by human carboxylesterase CES1A1.

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Running title page

Hydrolysis of methylphenidate by human carboxylesterase.

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Number of text pages: 42

Number of tables: 3

Number of figures: 5

Number of references: 41

Number of words in the abstract: 222

Number of words in the introduction: 746

Number of words in the discussion: 1353

Abbreviations: CES1A1 (gi: 179927), carboxylesterase 1A1 or hCE-1 or HU1a; CES2 (gi: 7262375), carboxylesterase 2 or hCE-2 or hiCE; CES3 (gi: 7019977), carboxylesterase 3; MP, methylphenidate; RA, ritalinic acid; LC/MS, liquid chromatography/mass spectrometry; ER, endoplasmic reticulum.

Section assignment: Absorption, Distribution, Metabolism, & Excretion

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Abstract

Methylphenidate is an important stimulant prescribed to treat attention-deficit hyperactivity disorder. It has two chiral centers, but most current commercial formulations consist of the racemic mixture of the *threo* pair of methylphenidate isomers (*d*-, *l*-*threo*-methylphenidate). The *d*-isomer is the pharmacologically active component. Numerous studies reported that oral administration of the methylphenidate racemate undergoes first-pass stereoselective clearance in humans with *l*- methylphenidate being eliminated faster than *d*-methylphenidate. Accordingly, the kinetics of hydrolysis of individual enantiomers by purified native and recombinant human liver carboxylesterases CES1A1 and CES2 and a colon isoenzyme CES3 were examined with an LC/MS assay. The expression of CES1A1, CES2 and CES3 in *Sf9* cells and the methods for purification of the three isoenzymes are reported. CES1A1 has a high catalytic efficiency for both *d*- and *l*- enantiomers of methylphenidate. No catalytic activity was detected with CES2 and CES3 for either enantiomer. The catalytic efficiency of CES1A1 for *l*-methylphenidate (k_{cat} /K_m = 7.7 $mM^{-1}min^{-1}$) is greater than *d*-methylphenidate (k_{cat} /K_m = 1.3 – 2.1 $mM^{-1}min^{-1}$). Hence, the catalytic efficiency of CES1A1 for methylphenidate enantiomers agrees with stereoselective clearance of methylphenidate reported in human subjects. Both enantiomers of methylphenidate can be fit into the 3-dimensional model of CES1A1 to form productive complexes in the active site. We conclude that CES1A1 is the major enzyme responsible for the first pass stereoselective metabolism of methylphenidate.

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Introduction

Methylphenidate is one of the most commonly prescribed psychostimulant for children in the United States with attention-deficit hyperactivity disorder (ADHD) (Findling et al., 1998; Wender, 1998). It is also used in some other neurological conditions, including depression in medically ill elderly, traumatic brain injury, and narcolepsy (Challman et al., 2000). The pharmacokinetic properties and molecular mechanism of action of methylphenidate have been extensively studied. Methylphenidate has two chiral centers (Figure 1), therefore there are four isomers, a *d,l-erythro*-methylphenidate pair and a *d,l-threo*-methylphenidate pair. Only the racemic mixture of *threo* pair of enantiomers (*d,l-threo*-methylphenidate) is used therapeutically because it has less adverse effects than the *erythro* pair (Markowitz et al., 2003). The mechanism of action of methylphenidate is thought to result from the increased level of extracellular dopamine in brain by blocking its reuptake through the pre-synaptic dopamine transporter (Gatley et al., 1996; Volkow et al., 2001). In a double-blind, 4-way randomized crossover study, *l*-methylphenidate showed no difference versus placebo in improving the sustained attention in humans. Moreover, a 5-mg dose of *d-threo*-methylphenidate has the same attention improvement effect as a 10-mg dose of *d,l-threo*-methylphenidate racemic mixture. Hence, the clinical effect of methylphenidate is attributed to the *d*-enantiomer (Srinivas et al., 1992(1)). A new formulation of methylphenidate Focalin (dexmethylphenidate) was introduced, which only contains enantiopure *d-threo*-methylphenidate (Markowitz et al., 2003).

The predominant metabolic pathway of methylphenidate is de-esterification to form pharmacologically inactive *d*- or *l*-ritalinic acid (Patrick et al., 1987). Numerous studies

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reported that *d,l*-methylphenidate was stereoselectively metabolized in humans (Lim et al., 1986; Srinivas et al., 1987), rats, and dogs (Egger et al., 1981). In both intravenous and oral administration studies, the racemic *d,l*-methylphenidate undergoes stereoselective clearance, with plasma concentrations of *d*-methylphenidate being higher than those of *l*-methylphenidate (Srinivas et al., 1993). In oral formulations including IR (immediate release), SR (sustained release), and OROS® (osmotic, controlled-released oral delivery system), the AUC_{inf} values of *l*-methylphenidate were 10 to 40 fold lower than *d*-methylphenidate (Srinivas et al., 1993; Modi et al., 2000). After oral administration, the plasma concentrations of *d*-methylphenidate are immediately higher than those of *l*-methylphenidate at 0.5 hour after oral dose. For intravenous administration, it only was 1.5 hours after dosing that the significant differences between plasma concentrations of the two enantiomers were observed. The higher concentration of *l*-ritalinic acid in both human plasma and urine is present in the first 2 hours after oral but not intravenous methylphenidate administration (Srinivas et al., 1990 and 1992(2)). Taken together, these results suggest that with oral administration, *d*- and *l*-methylphenidate undergo stereoselective clearance to ritalinic acid prior to entering systemic circulation with *l*-methylphenidate being eliminated faster than *d*-methylphenidate.

The specific hydrolases responsible for methylphenidate metabolism in humans have not been identified. The stereoselective clearance of methylphenidate indicates that methylphenidate hydrolysis is primarily enzyme catalyzed. Zhang *et. al.* reported that a porcine liver carboxylesterase hydrolyzes *l*-methylphenidate with higher efficiency than

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d-methylphenidate (Zhang et al., 2003). Therefore, we propose that human liver carboxylesterase isoenzymes stereoselectively catalyze the hydrolysis of methylphenidate enantiomers.

Human liver carboxylesterases (E.C.3.1..1.1) play an important role in metabolism, detoxification of ester drugs, such as cocaine, heroin and CPT-11 (Satoh et al., 1998). Carboxylesterases belong to a multigene family, based on the phylogenetic analysis of the sequences of the isoenzymes. The human isoenzymes are classified into 3 main families: CES1, CES2, and CES3 (Satoh et al., 1998). Two major isoenzymes were identified in human liver, CES1A1 (Shibata et al., 1993;Brzezinski et al., 1994) and CES2 (Pindel et al., 1997). A third carboxylesterase, CES3, was initially reported in the NEDO human cDNA sequencing project (gi:7019977) and it is expressed in human liver and colon (Sanghani et al., 2004). There is very limited information on the substrate specificity of CES3. While CES1A1 and CES2 share broad and overlapping substrate specificity, they do exhibit very different catalytic efficiencies for selected substrates. For example in cocaine hydrolysis, CES1A1 hydrolyzes only the methyl ester and CES2 hydrolyzes only the benzoyl ester (Brzezinski et al., 1994; Pindel et al., 1997). CES1A1 may also hydrolyze methylphenidate methyl ester, because both methylphenidate and cocaine have a methyl ester linkage.

In this study, an improved procedure is described for expression and purification of CES1A1, CES2 and CES3 from *Sf9* insect cells. CES1A1 and CES2 are also purified from human liver (Humerickhouse et al., 2000; Sanghani et al., 2003). The kinetics of *d*-

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and *l*-methylphenidate hydrolysis by the purified native and recombinant CES1A1, CES2 and CES3 isoenzymes are compared.

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Materials and Methods

Human liver cDNA was purchased from BD Biosciences Clontech (Palo Alto, CA). The PCR kit was purchased from Roche (Indianapolis, IN) and primers were ordered from Invitrogen (Carlsbad, CA). The baculovirus DNA and transfer vector was from BD Biosciences Pharmingen (San Diego, CA). *Sf9* insect cells and cell culture media were from Invitrogen (Carlsbad, CA). *d*- and *l*-methylphenidate and ritalinic acid were purchased from Sigma Chemical Co. (St. Louis, MO). The internal standard ritalinic acid-d_5 was purchased from Isotech Inc. (Miamisburg, OH). Unless otherwise indicated, all general chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA).

Cloning of CES1A1. The CES1A1 cDNA was amplified from human liver Marathon-Ready cDNA by PCR. The primer sequence, 5'ACGATGTGGCTCCGTGCCTTT3' (sense) and 5'TCACAGCTCTATGTGTTCTGTCTG' (antisense), were based on the published cDNA sequence of human liver carboxylesterase 1 (Munger et al., 1991). The PCR reaction proceeded through 35 cycles, each cycle consisting of 1 min at 95°C for denaturation, 30 seconds at 62°C for annealing and 3 min at 72°C for extension. The PCR product was incorporated into pCR®-Blunt II –TOPO vector and transformed into TOP 10 *E. coli* cells (Invitrogen, Carlsbad, CA). After plasmid isolation by using Quantum Prep® Plasmid Miniprep kit (Bio-Rad), the plasmid DNA was sequenced with vector sequencing primers (M13 forward primer: 5'GTTTTCCCAGTCACGAC; M13 reverse primer: 5'CAGGAAACAGCTATGAC) and internal primers (internal 1: 5'GTGGTGACCATTCAATATCGCC; internal 2: 5'GCTGACTCTCCAAAGATGG;

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internal 3: 5'TGCAGTTGATGAGCTATCCA; internal 4: 5'GACAGTGTCGTCTGTTCCTCC). The cDNAs for CES2 (Kedishvili et al., unpublished) and CES3 (Sanghani et al., 2004) were similarly cloned.

Expression and purification of recombinant CES1A1, CES2 and CES3. Methods for cloning and expression of CES1A1 are similar to those described previously (Kroetz et al., 1993; Morton et al., 2000). The CES1A1 cDNA was excised from pCR®-Blunt II – TOPO vector by *BamH* I and *Not* I digestion and incorporated into baculovirus expression vector pAcMP3 (Pharmingen, San Diego, CA) which was digested by the same enzymes. The CES1A1 construct (1.3 μg) and 0.5 μg of linearized baculovirus DNA were co-transfected into 2×10^6 *Sf9* insect cells by calcium phosphate precipitation at 27°C for 4 hours. The medium was collected and cell debris was removed by centrifugation at 500 × g for 5 min at 4°C. The supernatant contained CES1A1 recombinant virus, which was isolated and titered by plaque assay as described previously (Gonzalez et al., 1991). The recombinant baculovirus was similarly prepared for CES2 (Kedishvili et al., unpublished) and CES3 (Sanghani et al., 2004).

Two liters of *Sf9* cells ($1.8\text{-}2\times10^6$ cells/ml) were transfected with CES1A1, CES2 or CES3 recombinant baculovirus with a MOI (multiplicity of infection) of about one. After incubation for 63-66 hours at 27°C, the cells were harvested by low speed centrifugation (500 × g) and rinsed with PBS. The cell suspension was sonicated and cell debris was removed by centrifugation at 100,000 × g for 1 hour. The supernatant was applied to a Concanavalin A affinity chromatography column (Sigma Chemical Co., St. Louis, MO)

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equilibrated with 10 column volumes of 20 mM Tris, 0.2 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4. Bound protein was eluted with a methyl-α-D-mannopyranoside gradient from 0 to 0.2 M in 200 ml buffer and fractions were assayed for esterase activity with 4-methylumbelliferyl acetate. The active fractions were pooled and concentrated and loaded onto a preparative polyacrylamide gel electrophoresis unit (Bio-Rad, Model 491). The stacking gel contained 4% polyacrylamide and the resolving gel contained 6% polyacrylamide. The proteins were electrophoresed at a constant current of 30 mA. Fractions with carboxylesterase activity were pooled, concentrated and ethylene glycol was added to a final concentration of 10%. The purified enzyme was stored at 4°C. The recombinant CES2 (Kedishvili et al., unpublished) and CES3 (Sanghani et al., 2004) were similarly expressed and purified.

Purification of native human liver carboxylesterase CES1A1 and CES2. All buffers used in purification were purged with helium and contained 1 mM benzamidine and 1 mM dithiothreitol. Approximately 80 g frozen human liver obtained at autopsy was homogenized in 180 ml of 50 mM HEPES, pH 6.8. The DEAE ion exchange chromatography and Concanavalin A affinity chromatography procedures were described previously (Humerickhouse et al., 2000). The recovered activity pool was applied to a preparative polyacrylamide gel electrophoresis unit and eluted as described above for recombinant enzymes. Two peaks of activity were recovered: the first peak contained CES2 and the second peak contained CES1A1. The purified enzymes were concentrated and 10% ethylene glycol was added. The enzymes were stored at 4°C.

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Carboxylesterase activity and protein assays. Carboxylesterase activity was determined by incubating 5 μl enzyme (purified or homogenate supernatant) with 0.5 mM 4-methylumbelliferyl acetate in 90 mM KH_2PO_4, 40 mM KCl, pH 7.4 at 37°C in the total volume of 1.0 ml. Formation of the hydrolysis product 4-methylumbelliferone was monitored with a spectrophotometer at 350 nm. Rates of hydrolysis (μmol/min) were calculated by linear regression of absorbance versus time using an extinction coefficient of 12.2 cm^{-1} mM^{-1} for 4-methylumbelliferone. The specific activity was expressed as μmol/min per mg protein. Protein concentrations were determined by Bio-Rad protein assay with bovine serum album as standard.

SDS-polyacrylamide gel electrophoresis and western blot analysis. The stacking gel contained 4% acrylamide with 0.1% SDS and the resolving gel contained 10% acrylamide with 0.1% SDS. Protein samples were boiled with β-mercaptoethanol and electrophoresed at 10 mA constant current at room temperature. Gels were stained with Coomassie Blue. For western blot analysis, the gel was electroblotted overnight onto a nitrocellulose membrane using a Bio-Rad mini transfer-blot cell. The membrane was then blocked for 1 hour with 1% BSA in TBS to reduce the background, rinsed briefly with TTBS, and incubated for 1 hour with 1:1000 dilution of rabbit anti-CES1A1 antibody in TTBS. After washing three times with TTBS, the membrane was incubated for 1 hour with ^{125}I-labeled protein A (9.7 mCi/mg) diluted 1:2500 in TTBS. The membrane was washed three times with TTBS. Immunoreactive protein was visualized by autoradiography.

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Deglycosylation of CES1A1, CES2, and CES3 by Endoglycosidase H. Ten μg of each CES1A1, CES2, and CES3 was mixed with 15 mU endoglycosidase H in 50 μl of 50 mM citrate buffer with 1 mM EDTA, 1 mM benzamidine, and 1 mM DTT, pH 6.5. The reaction mixture was incubated at 37°C for 70 hours. Untreated isoenzymes were similarly incubated without endoglycosidase H. The activities of both treated and untreated isoenzymes were measured with 4-methylumbelliferyl acetate as the substrate. Both treated and untreated isoenzymes were analyzed by SDS-PAGE to determine their molecular weight change.

Assay of carboxylesterase-mediated methylphenidate hydrolysis to ritalinic acid by LC/MS. The CES1A1-catalyzed hydrolysis reaction of *l*-methylphenidate to ritalinic acid was performed by incubating 20 μg/ml (100 μg/ml for *d*-methylphenidate) purified native or recombinant enzyme with varying amount of the methylphenidate enantiomers (4, 8, 16, 32, 64, 128, 256 μM) in 20 mM Hepes, 10% ethylene glycol (v/v) at pH 7.0 for 20 min at 25°C. The total reaction volume was 0.5 ml. The incubation time was 20 min for CES1A1 and 24 hours for CES2 and CES3 at 25°C. A control reaction was prepared without added carboxylesterase. The reaction was stopped by adding 10% trichloroacetic acid and ritalinic acid-d_5 (4 μM) was added as the internal standard. Precipitated protein was removed by centrifugation, and 0.55 ml supernatant was recovered for ritalinic acid assay. For the hydrolysis of methylphenidate by CES2 and CES3, 100 μg/ml purified native and recombinant CES2 or CES3 was added to 128 μM *d*- or *l*-methylphenidate.

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A Strata X33µm Polymeric Sorbent Solid Phase Extraction cartridge (Phenomenex, Torrance, CA) was equilibrated sequentially with 1 ml of methanol and 1 ml of 2% aqueous phosphoric acid. After loading 0.5 ml of the reaction supernatant onto the SPE cartridge, it was washed with 1 ml of 5% methanol and 2% acetic acid (v/v) in water. The sample was eluted with 0.4 ml 50% methanol, 2% acetic acid. The recovery rate was about 90% for all analytes. Ritalinic acid and methylphenidate were separated by liquid chromatography with a Luna C18 column (150 x 4.6 mm, 3 um; Phenomenex, Torrance, CA) (Doerge et al., 2000). Thirty µl of the extract was loaded onto the column. The gradient elution was performed by increasing methanol concentration from 20% to 80% in 0.1% aqueous acetic acid over 6.5 minutes. The flow rate was 0.8 ml/min and the entire effluent was introduced into the MS APCI (atmospheric pressure chemical ionization) probe for ionization. Positive ions were acquired in selected ion monitoring mode. The MS conditions (QP8000A single quadrapole, Shimadzu Scientific) were as follows: probe temperature 400°C, CDL temperature 250°C, and nebulizer gas flow 2.5 l/min. The following $[M+H]^+$ ions were measured: *d*-methylphenidate (m/z 234), *l*-methylphenidate (m/z 234), ritalinic acid (m/z 220), ritalinic acid-d_5 (m/z 225). The standard curve for ritalinic acid was constructed by adding varying concentration of ritalinic acid from 0.0625 to 64 µM and a fixed amount of ritalinic acid-d_5 internal standard (4 µM) to the reaction buffer without enzyme. The area ratios of different amounts of the ritalinic acid to the corresponding internal standard ritalinic acid-d_5 were plotted versus the ritalinic acid concentration. The standard curve was linear from 0.0625 to 64 µM with an R^2 value greater than 0.99. The limit of detection was 0.01 µM and the

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limit of quantification was 0.025 μM. The standard curve was used to quantify the methylphenidate hydrolysis product ritalinic acid formed in enzyme assays.

Kinetic analysis of methylphenidate hydrolysis by CES1A1, CES2 and CES3. For steady-state kinetic analysis with purified carboxylesterase isoenzymes, the ritalinic acid (m/z 220) area and the corresponding internal standard ritalinic acid-d_5 (m/z 225) area from each methylphenidate hydrolysis assay were obtained from the LC/MS chromatogram. The ratio of ritalinic acid to ritalinic acid-d_5 was used to calculate the amount of ritalinic acid produced in each assay based on the standard curve. The amount of enzymatic ritalinic acid production was obtained by subtracting the control non-enzymatic production from the total ritalinic acid production. This difference was used to calculate the velocity of the hydrolysis reaction based on the enzyme added (20 μg/ml of CES1A1 for *l*-methylphenidate hydrolysis, and 100 μg/ml of CES1A1 for *d*-methylphenidate hydrolysis) and incubation time (20 min). Each substrate concentration was examined in duplicate assays. All data were fit to the Michaelis-Menten equation and kinetic parameters were calculated by non-linear regression analysis with GraFit software (Erithacus Software Limited).

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Results

Human liver carboxylesterase CES1A1 cDNA (Shibata et al., 1993) was cloned from human liver cDNA by PCR, inserted into a baculovirus transfer vector and co-transfected with baculovirus DNA into insect cells. *Sf9* cells infected with CES1A1 recombinant virus produced high carboxylesterase activity, 78 μmol/min total activity per liter cell culture (Table 1), with 4-methylumbelliferyl acetate as substrate, compared to the uninfected *Sf9* cells, 26 μmol/min total activity per liter cell culture. Western blot analysis confirmed the expression of recombinant CES1A1 in *Sf9* cells (data not shown). The enzyme was purified to >95% homogeneity by Concanavalin A affinity chromatography and preparative non-denaturing polyacrylamide gel electrophoresis with a yield of 2.5 mg protein per liter of *Sf9* cells at a concentration of 2×10^6 cells/ml (Table 1). The recombinant CES1A1 had the same subunit mass as native CES1A1 (60 kDa) on SDS-PAGE (Figure 2A). The specific activity of purified recombinant CES1A1 (7 μmol/min/mg) is consistent with the native CES1A1 purified from human liver (Brzezinski et al., 1994).

CES2 was similarly cloned and expressed in *Sf9* cells. Infected cells generated 680 μmol/min total activity per liter cells above the uninfected cells activity (Table 1). The enzyme was purified by Concanavalin A affinity chromatography and preparative non-denaturing polyacrylamide gel electrophoresis. The yield was 1.9 mg protein per liter of *Sf9* cells at a concentration of 2×10^6 cells/ml (Table 1). Recombinant CES2 had the same subunit mass as native CES2 (60 kDa) in SDS-PAGE (Figure 2B). The recombinant CES2 had about the same specific activity with 4-methylumbelliferyl acetate (120

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μmol/min/mg) as native CES2 purified from human liver (Pindel et al., 1997). CES3 was expressed and purified to homogeneity by method similar to CES1A1 and CES2 (Sanghani et al., 2004). The purified recombinant CES3 had a specific activity of 1.5 μmol/min/mg with 4-methylumbelliferyl acetate as substrate. CES3 also had a subunit molecular weight of about 60 kDa (Figure 2A). The amino acid sequence identities of all expressed carboxylesterases were confirmed by MALDI-MS analysis (data not shown).

CES1A1, CES2, and CES3 were deglycosylated by treatment with endoglycosidase H. The deglycosylated enzymes had greater mobility than the native enzymes in SDS-PAGE (Figure 2). Deglycosylation resulted in a removal of approximately 3 kDa of carbohydrate residues. The deglycosylation of CES1A1 and CES3 had no significant effect on catalytic activity (Figure 2 legend). The CES2 catalytic activity was decreased by only 19% after deglycosylation (Figure 2 legend).

The hydrolysis of *d*- and *l*-methylphenidate by human carboxylesterase isoenzymes was examined by an LC/MS assay. The substrate methylphenidate (m/z=234, MP) was well separated from the product ritalinic acid (m/z=220, RA) in the LC/MS chromatogram (Figure 3). The area of ritalinic acid peak was used to generate a standard curve to quantify product formation in the enzyme assay. Methylphenidate was spontaneously hydrolyzed in the assay buffer. The spontaneous RA formation was linear with reaction time for 24 hours and methylphenidate concentration (4-256 μM). The first order rate constant for non-enzymatic hydrolysis was the same for *d*- and *l*-methylphenidate, 5×10^{-4} min^{-1}. In the enzymatic hydrolysis assay, the spontaneous RA production was subtracted

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from the total RA production (Table 2). The CES1A1 enzymatic RA production was linear with reaction time for 1 hour (most reactions were for 20 min) and enzyme concentration (10-100 μg/ml). The K_m and V_{max} values of CES1A1 for methylphenidate were determined by measuring the rate of enzymatic formation of ritalinic acid at different methylphenidate concentrations and fitting the data to the Michaelis-Menten equation (Fig. 4 and Table 3). In all cases, the reaction conditions were adjusted so that less than 10% of the substrate was consumed during the time of measurement. The k_{cat} values were obtained by multiplying V_{max} by the enzyme subunit molecular weight of 60 kDa.

As shown in Table 2, only CES1A1 had hydrolytic activity with methylphenidate. Recombinant CES1A1 had about 6-fold higher catalytic efficiency with *l*-methylphenidate than *d*-methylphenidate (Table 3). The activity of native versus recombinant CES1A1 was similar. Native and recombinant CES2, and recombinant CES3, demonstrated very little or no detectable catalytic activity for *d*- and *l*-methylphenidate (Table 2).

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Discussion

The expression of recombinant CES1A1, CES2, and CES3 in *Sf9* insect cells creates a reproducible source of enzyme to study their structure/function relationships among this family of related carboxylesterase isoenzymes. Although enzymes can be purified from human liver (Humerickhouse et al., 2000), recent studies indicate the presence of genetic variants (Munger et al., 1991; Shibata et al., 1993; Wu et al., 2003) that could influence enzyme expression or catalytic activity. The first successful expression of human carboxylesterase CES1A1 in the *Sf9* insect cell system was reported by Kroetz (Kroetz et al., 1993). They found that inhibition of N-glycosylation of the CES1A1 in insect cells prevents formation of active enzyme. While enzyme can be expressed in *E. coli*, it was not glycosylated and not fully active (Zhang and Bosron, unpublished). In this study, we expressed recombinant CES1A1, CES2 and CES3 in the *Sf9* insect cell system. The expressed CES1A1 and CES2 isoenzymes had the same specific activity as the native enzymes purified from human liver (Table 1). Deglycosylation of the three enzymes by endoglycosydase H (Figure 2) had minimal effect on catalytic activity. In the reported crystal structure of CES1A1 (Bencharit et al., 2003), the sugar moieties are located at the trimer interface of CES1A1 and presumably stabilize subunit association. Hence, glycosylation could assist the correct folding and subunit association to yield the native, active CES1A1. However, after the proper protein folding is achieved, the removal of the carbohydrate moiety may not change the overall protein structure dramatically. Whether glycosylation has any major effect on CES2 or CES3 folding (both monomers) is not known.

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The average yield of the three expressed enzymes in this study is low, only about 2 mg from 1 liter insect cells. All three carboxylesterases contain the C-terminal four amino acid ER retention signal (Satoh et al., 1998). Morton et al. (2000) studied expression of a rabbit liver carboxylesterase in insect cells. Expression of the C-terminal truncated rabbit liver carboxylesterase resulted in secretion of the enzyme into the serum-free cell culture media (Morton et al., 2000). The major advantage of the expression of truncated carboxylesterase as a secreted protein was that it increased yield and simplified purification from cell media (Morton et al., 2000).

CES1A1 and CES2 have been shown to exhibit different substrate specificity (Satoh et al., 2002). For example, CES1A1 catalyzes the hydrolysis of the methyl ester of cocaine, and CES2 catalyzes the hydrolysis of the benzoyl ester of cocaine (Brzezinski et al., 1994; Pindel et al., 1997). CES1A1 hydrolyzes meperidine, whereas CES2 does not (Zhang et al., 1999). CES2 hydrolyzes aspirin and procaine, whereas CES1A1 does not (Takai et al., 1997). Even in the overlapping substrates such as 4-methylumbelliferyl acetate, heroin and CPT-11, the catalytic efficiencies are significantly different (Pindel et al., 1997; Humerickhouse et al., 2000). Based on the analysis of the substrates structures, we proposed that CES1A1 preferentially cleaves the ester linkage with small alcohol group and large acyl group (e.g., cocaine methyl ester and meperidine), and CES2 prefers the substrate with small acyl group and large alcohol group, e.g., cocaine benzoyl ester, CPT-11, heroin (Bosron et al., 2002). We predicted that CES1A1 would hydrolyze methylphenidate, because methylphenidate has a similar methyl ester linkage as cocaine. The kinetics results in this study demonstrate that CES1A1 has high catalytic efficiency,

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k_{cat}/K_M = 1.3-7.7 $mM^{-1}min^{-1}$ with *d*- or *l*- methylphenidate, whereas CES2 and CES3 are not active with methylphenidate as substrate (Table 3).

A recently resolved crystal structure of CES1A1 with the cocaine analog homatropine revealed that the cocaine methyl ester linkage (small alcohol group) is oriented toward the active site serine of CES1A1 and fits well into the selective small, rigid substrate binding pocket (Bencharit et al., 2003). In an attempt to identify potential structural correlations for the higher catalytic efficiency *of d- versus l*-methylphenidate, (Table 3), we docked both *d*- and *l*- methylphenidate into CES1A1 active site by using the experimentally observed position of homatropine as a guide (Figure 5). Both enantiomers can be modeled into the active site and interact well with the surrounding residues. The modeled distance between the hydroxyl group of Ser221 and the carbonyl carbon of methylphenidate is within the range for nucleophilic attack from Ser221 (2.6Å). We were not able to identify any obvious structural differences that might account for the six-fold difference in the stereoselectivity of hydrolysis from this simple docking experiment, although it is possible that the enzyme might exhibit a preference for a particular orientation of the benzyl versus the piperidine substituents of methylphenidate within the substrate pocket. In our docking experiment we have treated these as interchangeable interactions, but it is likely their different chemical characteristics will influence the actual position of the substrate when bound to the enzyme. A crystal structure of CES1A1 in complex with each methylphenidate enantiomer is needed for a more thorough explanation for the observed stereoselectivity for hydrolysis.

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Orally administrated *d,l*-methylphenidate undergoes significant stereoselective, and first-pass clearance (Hubbard et al., 1989; Srinivas et al., 1993). One of the possible explanations is that methylphenidate is stereoselectively metabolized in the gastrointestinal tract or liver prior to entering the systemic circulation as suggested by Zhang et al. (Zhang et. al., 2003). Accordingly, we studied the stereoselectivity of the three major human carboxylesterase isoenzymes, CES1A1, CES2 and CES3, which are known to be expressed in human liver and gastrointestinal tissues. We found that CES1A1 has a high catalytic efficiency for both *d*- and *l*- methylphenidate, whereas CES2 and CES3 have no catalytic activity with either enantiomers (Tables 2 and 3). The catalytic efficiency of CES1A1 for *l*-methylphenidate is 3.6-6 fold greater than *d*-methylphenidate (Table 3). The K_m value of the purified CES1A1 for methylphenidate, 40-126 μM, is several orders of magnitude higher than the pharmacological methylphenidate concentration in blood, 25-64 nM (Challman et al., 2000). Therefore, CES1A1 is expected to exhibit first-order kinetics in the hydrolysis of methylphenidate *in vivo*. We also found that *d*- and *l*-methylphenidate have the same first-order rate constant (5×10^{-4} min^{-1}) in their spontaneous hydrolysis, thus their stereo-specific clearance is entirely due to their different enzymatic hydrolysis rates.

The absorption of orally dosed methylphenidate is complete and rapid through gastrointestinal tract (Faraj et al., 1974**)**. However, the bioavailability of the intact drug is low and variable, due to the extensive first-pass, stereoselective metabolism (Markowitz et al., 2003). We were unable to find evidence for stereoselective absorption of the drug in gastrointestinal tissues. The three carboxylesterase isoenzymes examined in this study

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are expressed both in liver and gastrointestinal tissues. For CES1A1, the relative abundance is liver >> stomach > colon (Satoh et al., 2002). CES2 is relatively more abundant in small intestine and colon than CES1A1 (Satoh et al., 2002), and CES3 is mainly expressed in liver and colon (Sanghani et al., 2004). However, CES2 and CES3 do not have hydrolytic activity with methylphenidate. Hence, the expression of CES1A1 in liver and stomach is consistent with presystemic stereoselective metabolism of methylphenidate.

Methylphenidate is usually prepared as a chiral drug composed of *d,l-threo*-methylphenidate racemic mixture. *d*-Methylphenidate has been shown to be more potent than its *l*-isomer (Maxwell RA et al., 1970; Patrick et al., 1987). Some *in vivo* studies supported that the higher clinical activity of *d*-methylphenidate is due to its higher potency for the dopamine transporter (Ding et al., 1997: Challman et al., 2000). However, the decreased catalytic efficiency of CES1A1 for *d*- versus *l*-methylphenidate may also contribute to the increased potency of *d*-methylphenidate *in vivo*. The decreased catalytic efficiency of CES1A1 for *d*- versus *l*-methylphenidate is consistent with the higher plasma concentration of *d*-methylphenidate after oral or intravenous administration of the racemic drug (Hubbard et al., 1989; Srinivas et al., 1993). Several pharmacokinetic and pharmacodynamic studies of methylphenidate demonstrated the significant interindividual variation in dosage and clinical effectiveness in children with attention-deficit hyperactivity disorder (Hungund et al., 1979; Shader et al., 1999). It is possible that these variations are related to the interindividual variability in the hydrolysis of methylphenidate in patients (Srinivas et al., 1992(1)). Therefore, the pharmacogenetic

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studies of CES1A1 could be useful in the effort to predict the effectiveness, dosage requirement and toxicity of methylphenidate and other drugs metabolized by CES1A1.

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Acknowledgment

We thank Drs. Hiremagalur N. Jayaram and Paresh C. Sanghani for helpful discussion of the manuscript. We also thank the INGEN Protein Expression Core in the Department of Biochemistry & Molecular Biology, Indiana University, for assistance in enzyme expression and purification.

JPET Fast Forward. Published on April 13, 2004 as DOI: 10.1124/jpet.104.067116
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Footnotes

Financial support: This research was funded, in part, by NIDA grant R01DA006836.

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Legends for figures

Figure 1. Fischer projections of *d*- and *l-threo*-methylphenidate. The asymmetric carbon atoms are shown with '*.'

Figure 2. Deglycosylation of recombinant CES1A1, CES2 and CES3. Ten μg of each enzyme was treated with 15 mU endoglycosidase H at 37°C for 70 hours. Two μg of each treated and untreated enzyme was loaded on 10% SDS-PAGE. A. CES1A1 and CES3, lane 1 = molecular weight standard; lane 2 = untreated CES1A1 (6.9 μmol/min/mg); lane 3 = treated CES1A1 (6.7 μmol/min/mg); lane 4= untreated CES3 (6.3 μmol/min/mg); lane 5= treated CES3 (6.3 μmol/min/mg). B. CES2, lane 1 = untreated CES2 (117 μmol/min/mg); lane 2 = treated CES2 (95 μmol/min/mg); lane 3 = endoglycosidase H.

Figure 3. LC/MS chromatogram for methylphenidate, ritalinic acid, and d_5-ritalinic acid. In the selected ion monitoring mode (SIM), the intensities of methylphenidate (MP) peak (m/z=234), ritalinic acid (RA) peak (m/z=220) and internal standard ritalinic acid-d_5 (RA-d_5) peak (m/z=225) are plotted versus their retention time from Luna C18 liquid chromatography column. The Y axis is peak intensity and the X axis is retention time in minutes.

Figure 4. Michaelis-Menten plot of kinetics data of CES1A1 with *d*-methylphenidate. The K_m and V_{max} value of purified native CES1A1 with *d*-methylphenidate was obtained from duplicate LC/MS assays containing 100 μg/ml CES1A1 incubated with various

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concentrations of substrate at 25°C for 20 min. Activity data were fit to the Michaelis Menten equation (solid line). The V_{max} was 3.2±0.1 nmol/min/mg, K_m was 89.9±6.6 μM. The data sets were transformed and represented in Lineweaver-Burke plots (Inset).

Figure 5. *d*- and *l*- Methylphenidate were modeled into CES1A1 active site using the experimentally observed position of homatropine as a guide (Bencharit et al., 2003). All residues within 4Å of methylphenidate (MP) are shown. The distances from Ser221 hydroxy group to carbonyl carbon of methylphenidate are 2.6 Å for both *d*-MP (Panel A) and *l*-MP (Panel B). The distances from His468 imidazole group to the methyl ester linkage of methylphenidate are 4.6 Å for both *d*-MP and *l*-MP.

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Tables

Table 1 Purification of recombinant CES1A1 and CES2

Enzyme		Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)
CES1A1	Whole cell lysate	155	400	0.4
	Concanavalin A	59	17	3.5
	Preparative PAGE	35	5	7
CES2	Whole cell lysate	1360	440	3.1
	Concanavalin A	960	17	56
	Preparative PAGE	470	3.8	124

Carboxylesterase activity was measured with 4-methylumbelliferyl acetate as the substrate. Purification data were from a 2 liter preparation of *Sf9* cells at the density of 2×10^6 cells/ml.

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Table 2 Examples of the hydrolysis of *d*- and *l*-methylphenidate (*d*-MP and *l*-MP) by CES1A1, CES2, and CES3.

Enzyme		Total RA (μM)	Control RA (μM)	Enzymatic RA (μM)
CES1A1 (native)[a]	*l*-MP	24.6	1.3	23.3
	d-MP	6.8	1.2	5.6
CES1A1 (recombinant)[a]	*l*-MP	21.9	1.3	20.6
	d-MP	6.3	1.2	5.1
CES2 (native)[b]	*l*-MP	43.9	43.2	0.7
	d-MP	44.2	45.7	-0.15
CES2 (recombinant)[b]	*l*-MP	43.5	43.2	0.3
	d-MP	44.3	45.7	-1.4
CES3 (recombinant)[b]	*l*-MP	54.4	54.1	0.3
	d-MP	48.6	47.9	0.7

d- and *l*-Methylphenidate (128 μM) were incubated with 100 μg/ml CES1A1, CES2, or CES3, respectively, in the total volume of 0.5ml 20 mM Hepes buffer, pH 7.0, at 25°C for [a] 20 min or [b] 24 hours. This is a single experiment to demonstrate the enzymatic vs. nonenzymatic hydrolysis of methylphenidate. Replicate kinetic analysis is shown in Table 3.

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Table 3 Kinetic constants for enzymatic hydrolysis of *d*- and *l*-methylphenidate (*d*-MP and *l*-MP)

Enzyme		k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} min^{-1})
CES1A1 (native)	*l*-MP	0.34±0.02	43.8±5.8	7.7±0.7
		0.28±0.01	40.1±5.4	7.0±0.7
	d-MP	0.19±0.01	89.9±6.6	2.1±0.1
		0.18±0.01	80.0±5.8	2.3±0.1
CES1A1 (recombinant)	*l*-MP	0.31±0.01	40.1±5.4	7.8±0.8
		0.36±0.02	47.1±5.6	7.6±0.6
	d-MP	0.16±0.01	126.2±12.0	1.3±0.1
		0.17±0.01	110.4±11.3	1.6±0.1
CES2 (native and recombinant)	*l*-, *d*-MP	0	N/A	0
CES3 (recombinant)	*l*-, *d*-MP	0	N/A	0

The values of kinetic constants were reported as mean±standard error. Two individual sets of data were reported for each enzymatic reaction. The standard error of each constant was less than 14% of the mean value.

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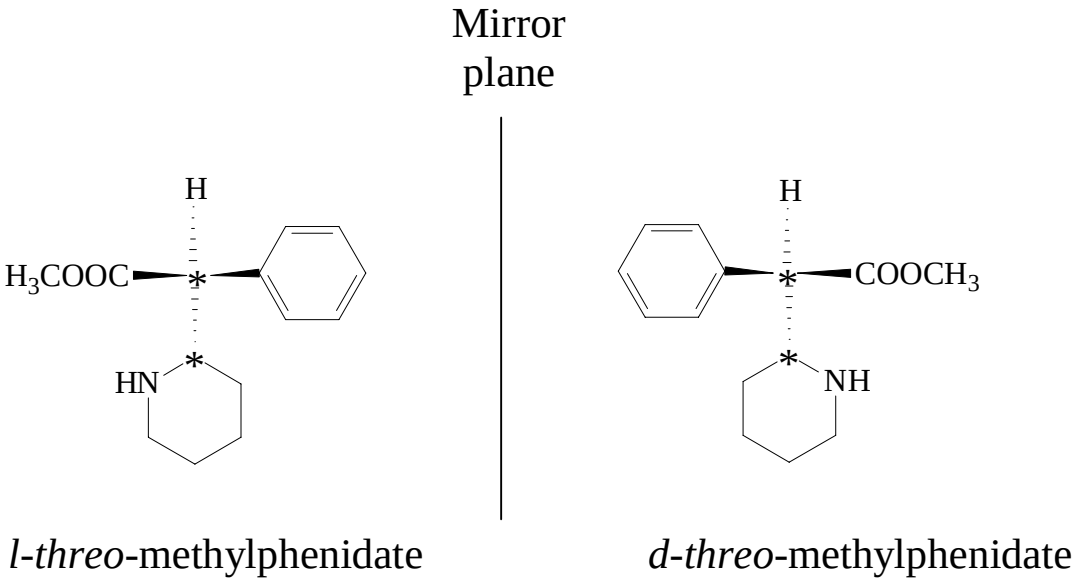
Figures



l-threo-methylphenidate *d-threo*-methylphenidate

Figure 1.

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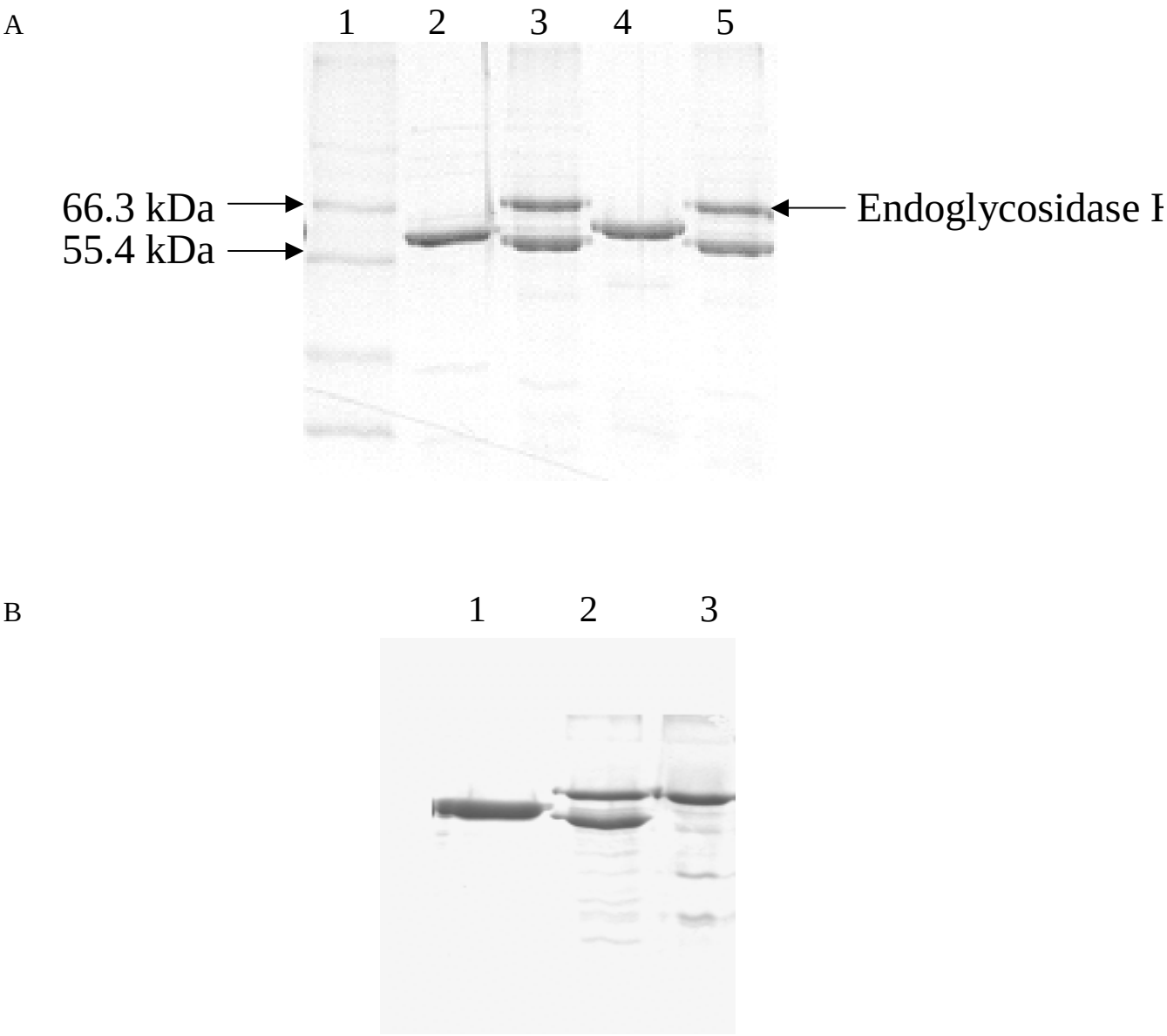

Figure 2.

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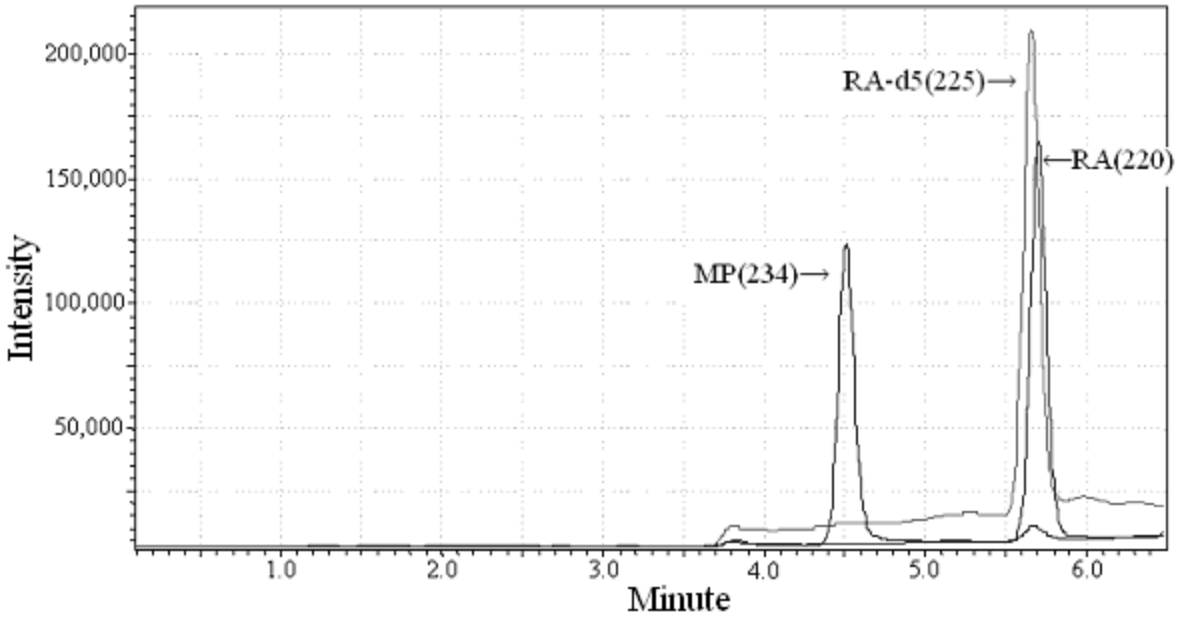

Figure 3.

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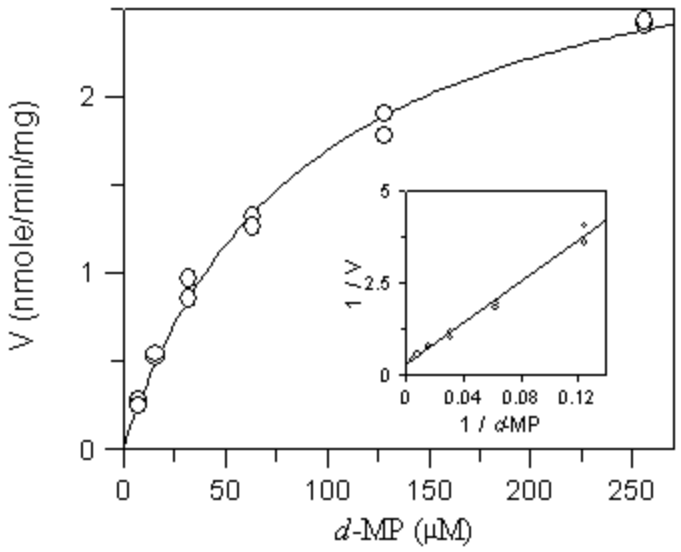

Figure 4.

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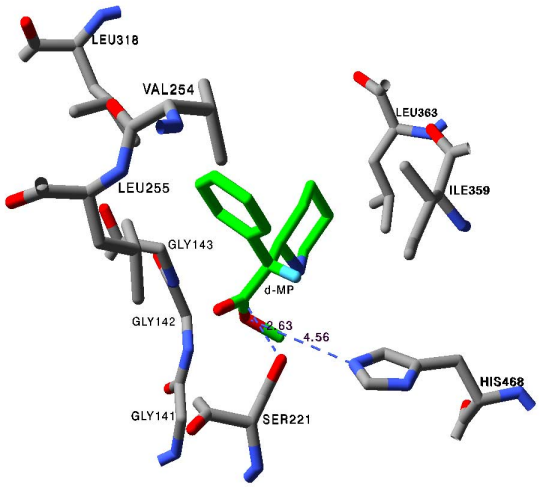

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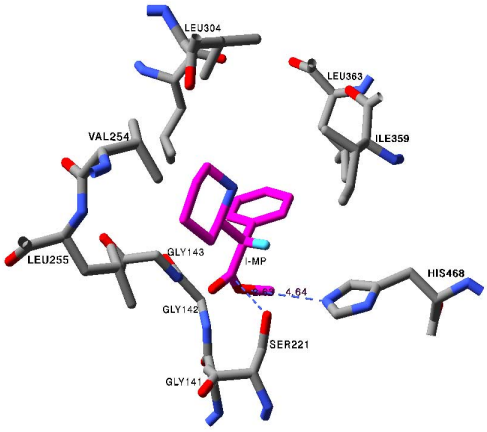

B

Figure 5.