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Stereoselective determination of trihexyphenidyl in human serum by LC–ESI–MS

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Abstract

The antiparkinsonian drug trihexyphenidyl (THP) is currently manufactured and administered as a racemate. However, stereochemistry can play significant role in the drug's pharmacokinetics, biotransformation, metabolism, interaction with cellular and tissue components and overall effect on human body. It is necessary to consider such a drug as a mixture of two compounds (drug enantiomers), with their own effect on the human body. The present paper describes a simple and sensitive LC–MS method for the stereoselective determination of THP in human serum. In this study, the sample was prepared by a solid-phase extraction (SPE) procedure. The enantiomer separation was done using native β-cyclodextrin stationary phase LC column. The combination of ESI–MS detection and SPE showed excellent sensitivity and selectivity of the method. The limits of detection of <0.1 ng/ml can be easily achieved, which is 7000 times lower than the detection limits achievable by a UV detection method. The method has at least a 3-order of magnitude linear dynamic range for both enantiomers (concentrations up to 1323 ng/ml were tested). This is 24 times wider than the therapeutic range of THP (peak THP plasma concentration of 55 ng/ml was previously reported). The recoveries of THP enantiomers from the human serum were $>95\%$.

Keywords: Chiral separations; LC; ESI–MS; Trihexyphenidyl; β-cyclodextrin; Solid-phase extraction

1. Introduction

Parkinson's disease is a slowly progressive degenerative neurologic disorder characterized by a resting tremor, mask-like faces, shuffling gait, loss of postural flexes, and muscle rigidity and weakness [1]. The pathological changes are associated with the destruction of neurons in the basal ganglia and the depletion of neurotransmitter dopamine containing structures. These changes cause the imbalance between dopamine and acetylcholine that subsequently leads to increased excitement of this part of the brain [1]. Treatment of this disease is usually associated with application of some antiparkinsonian drugs such as trihexyphenidyl (THP). THP belongs to a group of

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synthetic anticholinergic drugs. Anticholinergic drugs compete with the neurotransmitter acetylcholine for its receptor sites at synaptic junctions. This results in the inhibition of the transmission of certain nerve impulses. THP, like many anticholinergic drugs, reduces Parkinson's syndrome. According to previously published results [2], THP shows some side-effects including bradycardia, disturbances of recent memory, and myasthenia gravis. In the case of psychotic patients, it demonstrated hallucinogenic and euphoric properties that led to its frequent abuse either alone or in the combination with some other opiates and narcotics. The other side-effects of common anticholinergic drugs like THP include blurred vision, constipation, dryness of the mouth, mental disturbances, slurred speech, and urinary urgency or retention [1].

THP is a chiral drug shown in Fig. 1. It is currently administered in the form of racemate in its clinical applications. From a stereochemical point of view, such a drug must be considered as a mixture of two enantiomers, with their own effect toward their target sites; as well as the overall effect on the human body. As reported by Waelbroeck et al. [3], many receptor binding sites are asymmetrical, and hence generally capable of discriminating optical isomers of chiral drugs. The stereochemistry can play a significant role in the drug's pharmacokinetics, biotransformation, metabolism, as well as its interactions with cellular and/or tissue components. It must be carefully considered and investigated in order to understand its role in the drug efficacy. It has been already revealed by in vivo kinetic studies, as well as the studies with isolated tissue, that the transport of compounds across the epithelia may be stereochemically dependent [4]. Hence, the metabolic pathways of each enentiomer may be different. As shown by previous stereoselective studies of THP enantiomers with muscarinic receptor subtypes, (R)-(−)-THP demonstrated considerably higher receptor affinities than the (S)-(+)-THP enantiomer [5–8]. However, as noted by Waelbroeck et al. [6], the conclusions about the potency of the 'high affinity' enantiomer with respect to the affinity ratios of the two enantiomers should be drawn only with the greatest caution, since no correlations between these two were observed. Hence, the enantiomers of a certain drug can show different pharmacological activities and in some cases even qualitative differences [9].

Although the stereoselective determination of THP has not been reported in the literature yet, the drug itself was already subject of many analytical studies. The nonstereoselective methods for THP determination in biological fluids utilize mainly gas chromatography (GC) [2,10,11]. The method developed by Knitz et al. [2] used an OV-17 packed column with a nitrogen-phosphorus sensitive detector. The reported detection limit was 2 ng/ml. The improved detection limit of 1 ng/ml was reported by Desage et al. [10], using mass spectrometric electron impact single ion monitoring detection (MS–EI–SIM), a DB-1 capillary column, and splitless injection. Owen et al. [11] reported further decrease of the detection limit (50 pg/ml) using a capillary column with the chemically bonded OV-17 stationary phase, splitless injection, and flame thermionic detection. There was also an attempt for the enantiomeric separation of THP by capillary zone electrophoresis with γ-cyclodextrin as the chiral solvating agent, but the resolution was not satisfactory [12]. Schjelderup et al. [13] reported that the optical resolution of racemic THP can be achieved by fractional crystalization via formation of

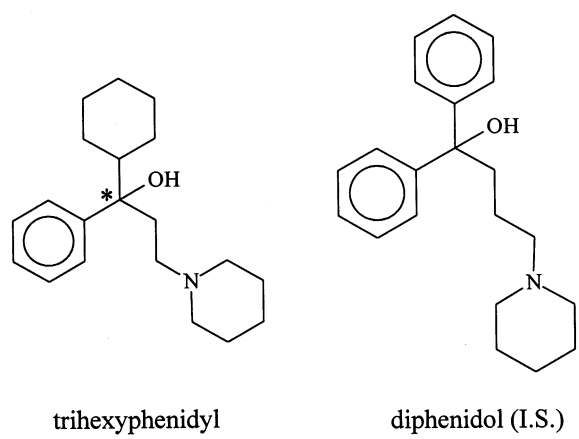

Fig. 1. Chemical structures of trihexyphenidyl and diphenidol (internal standard).

diastereoisomeric salts with (R,R)-tartaric acid, or (S)-mandelic acid. However, this method is rather tedious and macroscale. It suitable for preparative applications rather than trace clinical analysis. Further, simultaneous determination of both THP enantiomers is also not possible since the non-stereoselective secondary analytical technique (e.g. GC method) must be used for THP determination.

In the previous studies, liquid-liquid extraction was used for the isolation of THP from the biological samples after the pH adjustment (pH 9–11). The extraction solvents used were cyclohexane [2], heptane [10], and ethyl acetate-hexane (1:3) [11]. These procedures are usually time-consuming and often suffer from poor reproducibilities and recoveries.

This paper describes a stereoselective method for the determination of THP enantiomers in human serum with ESI–MS detection. The method uses solid-phase extraction for sample preparation that is suitable for the analysis of small molecules (e.g. pharmaceutical drugs, like THP) in the presence of macromolecular contaminants (e.g. proteins).

2. Experimental

2.1. Materials

DL-trihexyphenidyl hydrochloride, diphenidol hydrochloride (internal standard, I.S.), human serum (catalog no. S-7023), HPLC grade acetonitrile, and methanol were obtained from Sigma (St. Louis, MO). HPLC grade glacial acetic acid was from J.T. Baker (Phillipsburg, NJ). Triethylamine (99 + %) was purchased from Aldrich (Milwaukee, WI). (R)-(−)-trihexyphenidyl hydrochloride and (S)-(+)-trihexyphenidyl hydrochloride were synthesized and donated by Professor Arne Jørgen Aasen (Department of Pharmacy, University of Oslo, Oslo, Norway). The mobile phase was filtered through the fluoropore PTFE membrane filter, type FH, 0.5 μm pore (Millipore, Bedford, MA) prior to use. De-ionized water was from the NANOpure water system (Branstead/Thermolyne, Dubuque, IA). ACS grade cyclohexane for serum extraction was from Fischer (Itasca, IL). Immediately prior to injection to the HPLC system, each sample was filtered through a sterile Whatman nylon syringe filter (4 mm, 0.2 μm pore) from Fisher.

2.2. Instrumentation

Initially, high concentrations of THP enantiomers were used for the method development and optimization. The HPLC system consisted of a GP40 gradient pump (Dionex, Sunnyvale, CA) equipped with a Rheodyne 9125 biocompatible injection valve with a 25 μl sample loop, a Dionex AD20 variable wavelength absorbance detector, a Cyclobond I 2000 native β-cyclodextrin (β-CD) column (250 × 4.6 mm, Advanced Separation Technologies, Whippany, NJ), and a Varian 4290 integrator (Varian, Sunnyvale, CA). For the low THP concentration study, a 10 μl sample loop, a Cyclobond I 2000 (250 × 2.0 mm) narrowbore column, and the post-column split of 1:8 were used. The split ratio was adjusted by the resistance in the two outgoing split lines. The larger flow of the split was connected to an HP1100 variable wavelength UV detector (Hewlett Packard, San Fernando, CA) and the smaller one to a Quattro II triple quadrupole ESI–MS (Micromass, Manchester, UK) with the MassLynx data acquisition system. The repeatability was evaluated using an HP1100 autosampler synchronized with the data acquisition system.

2.3. Extraction of serum samples

The liquid–liquid extraction (LLE) procedure used was developed by Knitz et al. [2] with minor modifications as follows: 0.5 ml of serum sample was alkalized with 50 μl of 1 M NaOH and extracted 4 × with 0.5 ml of cyclohexane. The mixture was centrifuged at 4000 × *g* after each extraction step. The combined cyclohexane extract was evaporated to dryness and reconstituted in 150 μl of mobile phase.

Solid-phase extraction (SPE) was done using Diazem C_8 Dual-Zone SPE cartridges (200 mg, 3 ml, MetaChem Technologies, Torrance, CA) and a VISIPREP 12-port SPE vacuum manifold (Su-

pelco, Bellefonte, PA). Before loading the serum samples, the cartridge was conditioned with 4 ml of CH_3OH followed by equilibrating with 2 ml of 15% CH_3CN in H_2O. Then, 1 ml of serum sample containing THP and I.S. was diluted with 1 ml of H_2O and loaded onto the cartridge. Care was taken to draw the sample through the cartridge at a slow rate (dropwise, < 5 ml/min) to ensure that the interaction time for the analytes with the packing material was sufficient. The cartridge was then washed with two 1-ml and one 3-ml portions of 15% CH_3CN in H_2O and dried under vacuum for $\approx 2-3$ min. Special attention was paid to insure that the packing material would not dry prior to the final washing step with 15% CH_3CN in H_2O. Elution was done with 2 ml of CH_3OH. The eluate was evaporated to dryness and reconstituted in 300 μl of mobile phase (100 μl for the detection limit study).

2.4. Chiral separation by liquid chromatography/mass spectrometry

The β-CD chiral column was operated in 'polar organic mode', which uses non-aqueous polar organic mobile phase containing CH_3CN with small amounts of CH_3OH, HAc (acetic acid), and TEA (triethylamine). Their ratios and amounts were optimized for maximum resolution of THP enantiomers using the similar approach as that of Armstrong et al. [14]. The optimization studies were done with THP.HCl concentrations of 0.3 mg/ml in the mobile phase and the UV detection at 258 nm.

First, the methanol content in the mobile phase (MP) was varied and optimized while keeping the composition of the rest of the constituents constant (MP: CH_3CN/CH_3OH/HAc/TEA — 95/CH_3OH/0.4/0.3, v/v/v/v, where CH_3OH content was 1 or 3 or 5). Similarly, the total amount of acid and base MP modifiers was studied by varying the sum of HAc + TEA (v + v) from 0.1 to 1.3, with the rest of the conditions (including the ratio of HAc/TEA, v/v) unchanged (MP: CH_3CN/CH_3OH/HAc/TEA — 95/1/HAc/TEA, v/v/v/v, HAc/TEA = 1.333, v/v). The HAc/TEA ratio that controls the enantioselectivity was investigated as well in a similar manner (MP: CH_3CN/CH_3OH/HAc/TEA — 95/1/HAc/TEA, v/v/v/v, HAc/TEA, v/v ranged from 1 to 7, HAc + TEA = 0.8, v + v). Finally, the optimal HAc + TEA total amount was further re-confirmed at an optimal HAc/TEA ratio in an identical manner (MP: CH_3CN/CH_3OH/HAc/TEA — 95/1/HAc/TEA, v/v/v/v, HAc + TEA, v + v was 0.7 or 0.8 or 0.9, HAc/TEA = 1.67, v/v). The optimized mobile phase composition (CH_3CN/CH_3OH/HAc/TEA — 95/1/0.5/0.3, v/v/v/v) was used to conduct LC–ESI–MS studies with a narrowbore β-CD column. The post-column split of 1:8 was used for connection to the ESI–MS detector. The ESI–MS detector was operated in the single ion monitoring mode and alternately recorded at separate channels, m/z 302 $(THP + H)^+$ and m/z 310 $(I.S. + H)^+$, with the dwell time of 0.5 s and five replicates for each data point. Interscan delay was 0.02 s. The ESI ion source was operated at the temperature of 70°C, with the cone voltage at 40 V, high voltage lens at 0.50 kV, skimmer offset at 5 V, skimmer at 1 V and RF lens at 0.6 V. The ESI probe capillary was held at 3.00 kV.

The identification of the elution order of THP enantiomers was performed by the injection of enantiomerically pure (R)-(−)-THP.HCl and (S)-(+)-THP.HCl (UV detection at 258 nm, both 0.3 mg/ml in MP).

The repeatability was evaluated by a semi-automated approach. The multiple serum samples (spiked with THP and the I.S.) were extracted by the SPE procedure and then analyzed by the optimized LC method with MS detection. The serum concentrations of THP enantiomers and the I.S. used for this study were 31.0 and 8.9 ng/ml, respectively. The analytical process except the SPE was fully automated using the HP1100 autosampler. The intra-assay repeatability was determined by the replicate measurements of the same sample. The inter-assay repeatability was determined by the same analytical procedure with identically prepared samples. The injection volume was set to 10 μl for all the repeatability studies.

3. Results and discussion

3.1. Chiral separation of trihexyphenidyl

A native β-CD stationary phase column was selected for this study due to its broad applicability and compatibility with the structural features of THP. THP complies with the structural requirements for analysis on β-CD column because it contains (a) aromatic system, and (b) hydrogen-bonding group (hydrogen donor) on the stereogenic center, and (c) another hydrogen bonding group (hydrogen acceptor) within a short distance from the stereogenic center. This provides an opportunity for intramolecular hydrogen bonding between the hydroxyl hydrogen and the heterocyclic nitrogen atom. Such bonding produces some degree of rigidity at the chiral center due to the formation of an energetically favorable transient six-member ring and maximizes the differences between the three-dimensional structures of THP enentiomers. The presence of the aforementioned hydrogen bond was proven by Camerman et al. [15] in their X-ray difractometric studies of THP. Although the separation mechanism of chiral molecules on CD-based stationary phases is not yet completely known, some hypotheses were made by using model chiral molecules, such as β-blockers [14,16]. It is believed that the separation of enantiomers is based on hydrogen bonding of the molecules to the CD structure [14,16]. However, the hydrogen bonding alone is not sufficient for an enantiomeric resolution. The 'cone-like' structure of the CD molecule suggests the formation of inclusion complexes between the analyte molecule and the CD cavity. Both interactions may cause the formation of transient diastereoisomeric complexes between the analyte and the CD stationary phase. Since the mobile phase affects the extent of these interactions directly, its composition must be optimized for maximal resolution of the THP enantiomers.

The elution order of THP enantiomers by this method is (S)-(+)-THP followed by (R)-(−)-THP (chromatograms not shown). The peak assignment was done by comparing the retention times of enantiomerically pure THP standards with those of racemate.

3.1.1. Effect of CH_3OH on trihexyphenidyl separation

It has been reported in the literature that a small amount of CH_3OH is needed if someone wants to achieve any separation at all and bring the retention times to the acceptable levels [14]. The CH_3OH influence on the THP separation was therefore investigated by the procedure described in Section 2.4.

As the CH_3OH content in the MP increased, the enantioselectivity and the resolution of THP enantiomers decreased ($R_s = 1.54$ at CH_3OH volume ratio of 1, $R_s = 0.78$ at CH_3OH volume ratio of 5). Also, the retention times of THP enantiomers decreased gradually with the increase of CH_3OH content (data not shown).

These results indicate that the role of CH_3OH in the MP seems to be to compete with the chiral analytes for the hydrogen bonding sites on the β-CD stationary phase. When the smaller amount of CH_3OH is used, the drug enantiomers can interact to a larger extent with the hydrogen bonding sites on the cone edge of β-CD. This results in stronger interactions of drug enantiomers with the stationary phase, which lengthen their retention times and improve their resolution. CH_3OH is also capable of hydrogen-bonding with THP nitrogen, which prevents the formation of an intramolecular hydrogen bond within the THP molecule. Consequently, the resolution decreases due to the decrease of spatial rigidity on the chiral center of the THP molecules.

3.1.2. Effect of HAc and triethylamine on trihexyphenidyl separation

The effect of the two remaining organic modifiers (HAc and TEA) on the resolution of THP was investigated in a similar manner to that of CH_3OH while keeping in mind that both their total amount (HAc + TEA) as well as their ratio (HAc/TEA) will have effect on THP resolution. First, the total amount of HAc + TEA was varied while keeping their ratio constant. Then the ratio was varied while keeping the total amount constant.

The total amount of HAc + TEA apparently affects the resolution of THP enantiomers, even though it does not affect their retention times in

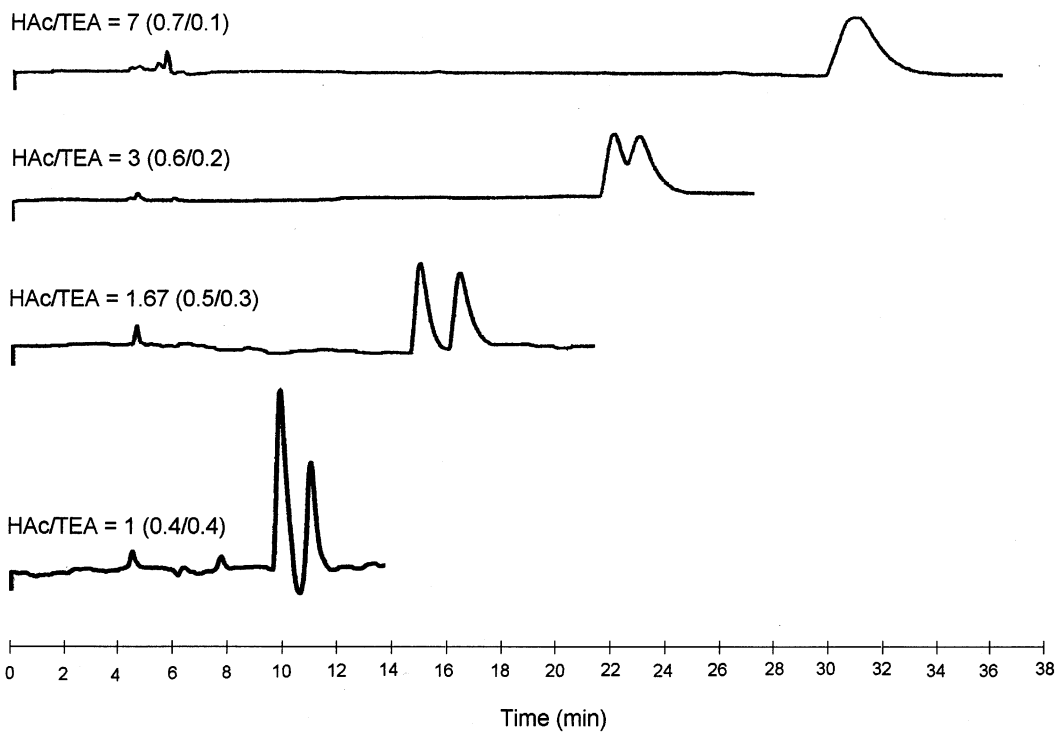

Fig. 2. The effect of volume ratio of HAc/TEA in the mobile phase on THP separation on β-CD column (250 × 4.6 mm, MP: 95/1/x/y, v/v/v/v-CH_3CN/CH_3OH/HAc/TEA, x and y represent the relative contents corresponding to HAc and TEA, respectively, HAc + TEA, v + v is constant, 0.8). Flow rate, 0.8 ml/min; injection volume, 25 μl.

any significant way. Our studies reveal that the resolution increases as the total amount of HAc + TEA increases, and reaches its maximum between 0.7 and 0.9. The resolution decreases as the total amount of HAc + TEA increases further (data not shown). Moreover, the ratio of HAc/TEA has a dramatic effect on both the resolution and the retention times as can be seen in Fig. 2. The enantioselectivity totally disappeared when HAc/TEA = 7 and improved as HAc/TEA decreased (Fig. 2).

Based on these results, the role of HAc and TEA in the MP seems to be to affect the acid-base equilibrium of the drug. It can be seen from structure of THP (Fig. 1), that it is a Lewis base. Under acidic conditions, the THP molecule can be partially protonated. Hence, the extent of intramolecular hydrogen bonding within the THP molecule will be reduced. The resulting semi-rigid arrangement at the chiral center will adversely affect the resolution as explained earlier in the study of CH_3OH effect on the THP separation. Under more basic conditions, the differences between the spatial arrangement of THP enantiomers are maximized by the formation of transient six-member rings via intramolecular hydrogen bonding, resulting in more rigid structures. Consequently, the resolution is improved. The long retention times of THP under the acidic conditions may be attributed to the hydrogen bonding of the resulting THP acetate to β-CD on the column. The best resolution has been achieved at HAc/TEA = 1.67. Since the optimal amount of HAc + TEA was established under other than the optimal ratio of HAc/TEA, subsequent experiments were performed to insure that the optimal total amount of HAc + TEA (= 0.8) established previously, would still produce the maximal resolution at the optimal ratio of HAc/TEA (= 1.67). Our studies showed that the resolution did not

Table 1
Recoveries of THP enantiomers and diphenidol (I.S.) from human serum by the SPE procedure

Diphenidol (I.S., n = 3)			
Concentration	Recovery (%)		Detection method
22.4 μg/ml	102.1 ± 2.5		UV at 258 nm
8.9 ng/ml	96.1 ± 3.3		ESI–MS
THP enantiomers (n = 3)			
Concentration	(S)-(+)-THP recovery (%)	(R)-(−)-THP recovery (%)	Detection method
45.5 μg/ml	102.7 ± 2.0	101.8 ± 1.9	UV at 258 nm
11.4 ng/ml	95.8 ± 4.1	96.6 ± 3.2	ESI–MS

improve significantly under other amounts of HAc + TEA at the ratio HAc/TEA of 1.67. Therefore, the MP composition of 95/1/0.5/0.3 (v/v/v/v) — CH_3CN/CH_3OH/HAc/TEA was used for all subsequent separations.

3.2. Liquid–liquid extraction vs. solid-phase extraction comparison

The LLE method developed by Knitz [2] (see Section 2.3) was first considered for sample preparation in this study. It was re-evaluated in terms of recovery of both THP enantiomers and diphenidol (I.S.). Even though the extraction procedure was modified to achieve better recovery (4 × extraction instead of single extraction reported previously), the recovery was found to be inconsistent and remarkably low. The inconsistent recovery could be corrected for by the introduction of I.S. assuming the recoveries for THP and the I.S. were the same. This was true only for serum samples with high concentrations of THP and the I.S. (45.5 μg/ml THP enantiomers, 22.4 μg/ml diphenidol in serum). The recoveries of THP enantiomers and the I.S. were found to be only 67%. The recoveries were quantitative when the extraction was done from H_2O instead of serum. The situation was more complicated at low, therapeutic concentrations of THP enantiomers (11.4 ng/ml THP, 8.9 ng/ml diphenidol). The recoveries were inconsistent and different for THP and the I.S. The best recoveries were 32% for THP enantiomers and 51% for diphenidol. The low recoveries may be attributed to the formation of a 'sponge-like' texture from denatured serum proteins at the interface of the phases during the extraction, which made the quantitative removal of cyclohexane very difficult, and resulted in the loss of analytes. This was also the reason for the increase of number of extraction steps to four. The binding of the drugs to serum proteins and their incomplete release during the extraction may also contribute to the low and inconsistent recoveries. The low and inconsistent recoveries are unacceptable and the further increase of number of extraction steps impractical for rapid clinical analysis.

In this work, SPE procedure was developed using Dual-Zone cartridges from Diazem. According to the manufacturer, the cartridge contains one of so called 'restricted-access media' with hydrophobic C_8 chain inside the pores of the packing material and hydrophilic polymer on the surface of the packing material. This design combines the size exclusion and the electrostatic repulsion mechanism to prevent the access of large protein molecules to the pores. As a result, the protein molecules pass through the column unretained within its void volume. On the other hand, the small drug molecules can reach the hydrophobic phase inside the pores of the packing material and interact via reverse-phase retention mechanism. The secondary hydrophilic interaction with the external phase can also play a role in the overall retention mechanism.

Using the SPE sample preparation protocol described in Section 2.3, we were able to achieve excellent recoveries for both THP enantiomers

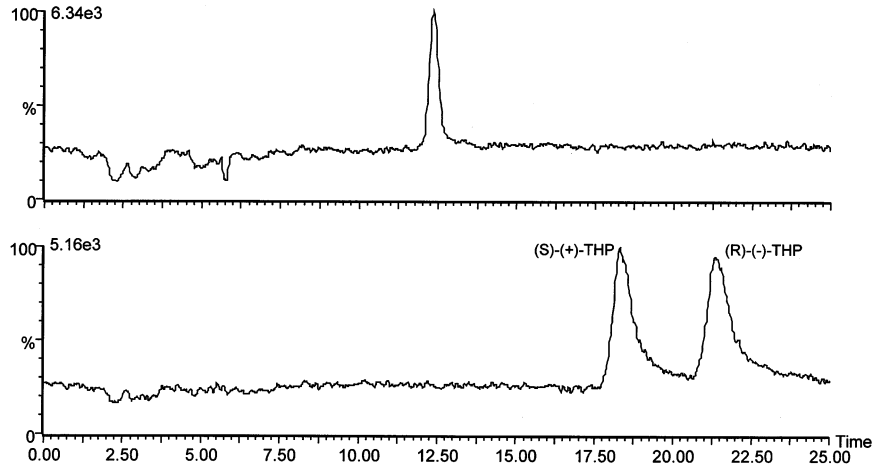

Fig. 3. LC–ESI–MS chromatograms of THP enantiomers (lower trace, m/z 302, 11.4 ng/ml) and diphenidol (internal standard, upper trace, m/z 310, 8.9 ng/ml) in human serum after SPE. LC conditions: β-CD column (250 × 2.0 mm); flow rate, 0.25 ml/min; injection volume, 10 μl; post-column split, 1:8; MP, 95/1/0.5/0.3, v/v/v/v-CH_3CN/CH_3OH/HAc/TEA.

and the I.S. at the two concentration levels used in the LLE sample preparation. The results are summarized in Table 1. The recovery values of 95% or higher indicate that there was no significant loss of analytes during the process despite the possibility of their binding to the serum proteins. If any portion of the drugs bound to the serum proteins, it was probably either released from the proteins in the sample dilution step prior to the SPE, or during the SPE process. In the sample dilution step, the concentrations of unbound drugs decrease, which shift the equilibria between the bound and unbound drugs in favor of unbound drugs. In addition, the conformation of serum proteins may change, which may lead to the release of the drugs as well.

The quantitative recoveries at 4000 times of the therapeutic concentrations (enantiomer concentrations up to 0.15 mg/ml were tested) show excellent preconcentration features of this SPE method. This feature makes it suitable for the chiral determinations of THP at very low concentrations where ample preconcentration is needed for analytical procedures.

3.3. Analytical performance

The internal calibration was used for the quantitation of THP enantiomers with diphenidol as the internal standard. Diphenidol is an achiral, anti-emetic drug structurally similar to THP. It was spiked to the serum sample prior to the SPE treatment and LC–ESI–MS analysis. A typical chromatogram of serum THP by SPE and ESI–MS detection is shown in Fig. 3. The regression parameters of the calibration curves are summarized in Table 2. According to Desage [10], the maximal therapeutic plasma concentration of THP for a single 15-mg dose was 55 ng/ml. As judged from the correlation coefficients, our method shows excellent linearity. In fact, the calibration curves still showed excellent linearity for THP concentrations at 48 times higher than that of maximal therapeutic plasma concentration.

Table 2
Regression parameters of THP enantiomer internal calibration with ESI–MS detection[a]

	Regression equation	Correlation coefficient
(S)-(+)-THP	$y = 0.0308$ (± 0.0002) $x + 0.005$ (± 0.015)	0.9997
(R)-(−)-THP	$y = 0.0291$ (± 0.0002) $x + 0.001$ (± 0.015)	0.9997

[a] $n = 5$, confidence level 95%, enantiomer concentration range 1.3–132.3 ng/ml.

In this work, with ESI–MS detection, a narrowbore LC column (2.0 mm i.d.) was used for the separation. Due to its lower void volume, the sample dilution within the column was reduced to yield relatively higher analyte concentrations at the exit of the column, and consequently resulted in higher detection sensitivity and resolution. Besides, the MP flow rate was also reduced (compared to the standard 4.6-mm i.d. column), which allowed easy connection to the ESI–MS detector (28 μl/min MP flow rate to ESI–MS) and substantial solvent savings. The improvement of sensitivity (along with more solvent savings) may be achieved by the use of a microbore column with an i.d. of 1.0 mm or less. These columns are directly compatible with most ESI–MS systems. However, it is worth noticing that the loading capacity of a microbore column is lower than that of a regular-bore column and smaller amounts or lower concentrations of analytes should be used in its applications.

The detection limits of LC–ESI–MS for the THP standards were 1.3 ng/ml for both THP enantiomers (at $S/N = 3$). The overall detection limits of the method (including the SPE procedure) were found to be 0.1 ng/ml for both THP enantiomers. Fig. 4 shows representative chromatograms of a blank serum, a blank serum spiked with the I.S., and a serum spiked with the I.S. and the THP enantiomers at the detection limits. The detection limits may be improved by further preconcentration with the SPE and the use of a microbore column for LC separation.

The intra-assay and inter-assay repeatibilities were also evaluated. The results are summarized in Table 3. The %CVs were lower than 4% in both cases. These results show excellent repeatability of experimental and instrumental conditions.

In the method developed, the entire analytical procedure can be automated if needed. Therefore, the sample throughput can be improved for the routine analyses. Moreover, the method can be applied to other drugs with minor or no modifications.

Table 3
Intra-assay and inter-assay repeatability data[a]

	Intra-assay		Inter-assay	
	THP/IS	%CV	THP/IS	%CV
(S)-(+)-THP	3.06 ± 0.07[b]	2.20	3.13 ± 0.11	3.62
(R)-(−)-THP	3.07 ± 0.06	2.00	3.16 ± 0.09	2.96

[a] $n = 6$, concentrations in serum: THP enantiomers 31.0 ng/ml; I.S. 8.9 ng/ml.
[b] $n = 5$.

4. Conclusions

A simple and quantitative LC–MS method for the determination of THP enantiomers in human serum has been developed. The method shows excellent analytical performance and is applicable to clinical analysis. The SPE sample preparation procedure proved to be user-friendly and reproducible with good preconcentration features. Our studies show good recoveries (higher than 95% for both THP enantiomers and the I.S.), which indicates that no significant drug binding to serum proteins occurred or that it was disrupted during the sample preparation step. The substantial improvement of detection limits (0.1 ng/ml) was achieved by the combined use of SPE with LC–ESI–MS. This was previously impossible due to poor UV absorption properties of THP. Possible co-eluting interferences were easily eliminated by the mass selectivity of ESI–MS detection. The recent advances in MS technology (i.e. more user-friendly operating procedures, easier maintenance, reduced instrumental and operating cost) make the use of MS for detection affordable and feasible for many clinical applications.

Fig. 4. LC–ESI–MS chromatograms of A, blank serum; B, blank serum spiked only with diphenidol internal standard (8.9 ng/ml); C, serum spiked with diphenidol internal standard (8.9 ng/ml) and THP enantiomers at the detection limit concentration level (0.1 ng/ml). All samples are after SPE. Upper traces in A, B, C correspond to m/z of 310 (diphenidol) and lower traces correspond to m/z of 302 (THP). LC conditions: β-CD column (250 × 2.0 mm); flow rate, 0.25 ml/min; injection volume, 10 μl; post-column split, 1:8; MP, 95/1/0.5/0.3, v/v/v/v-CH_3CN/CH_3OH/HAc/TEA.

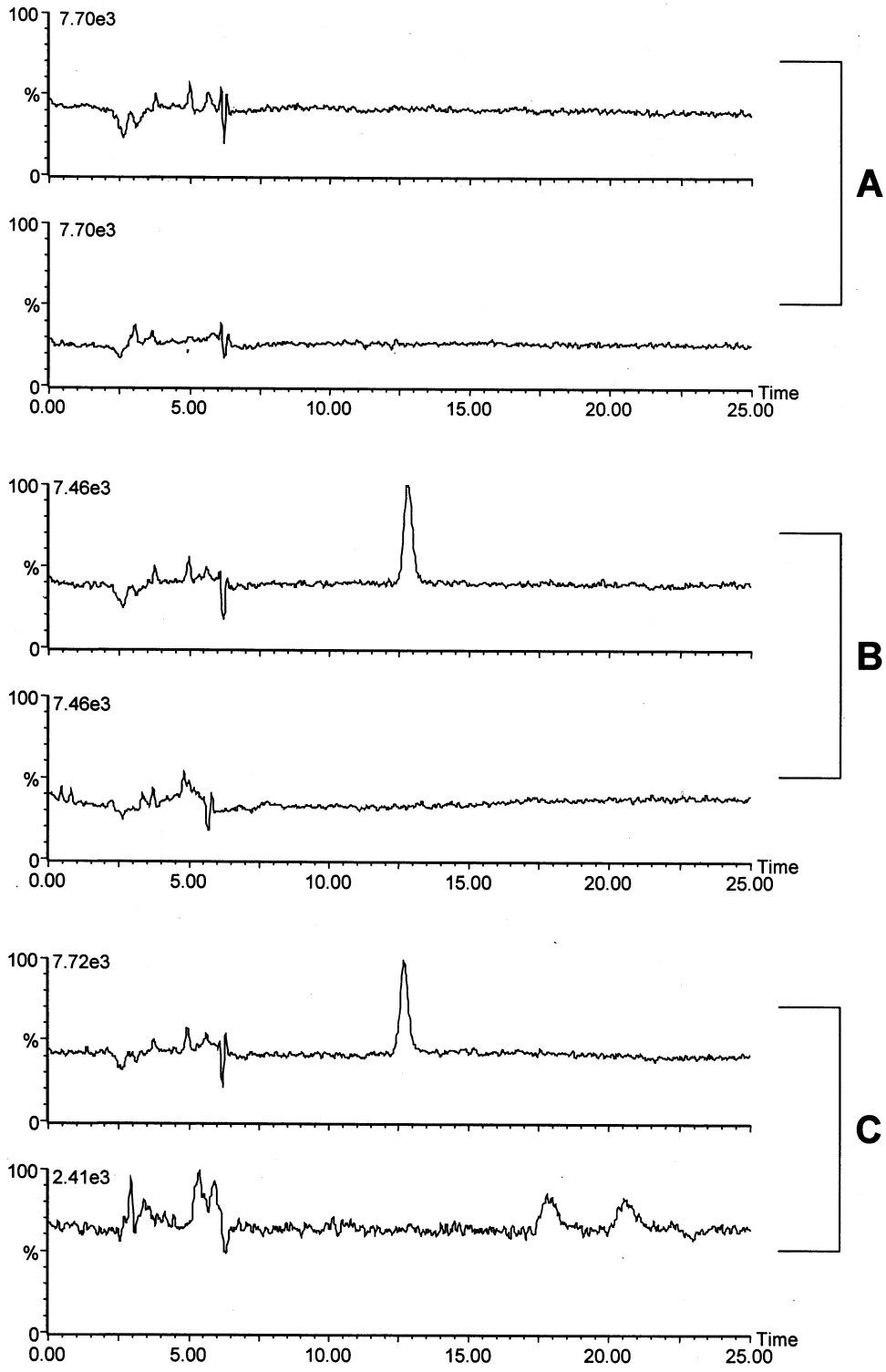
100
7.70e3
%
0
100
7.70e3
%
0
Time
0.00
5.00
10.00
15.00
20.00
25.00
A
100
7.46e3
%
0
100
7.46e3
%
0
Time
0.00
5.00
10.00
15.00
20.00
25.00
B
100
7.72e3
%
0
100
2.41e3
%
0
Time
0.00
5.00
10.00
15.00
20.00
25.00
C

Fig. 4.

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