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HPLC–atmospheric pressure chemical ionization mass spectrometric method for enantioselective determination of *R*,*S*-propranolol and *R*,*S*-hyoscyamine in human plasma

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Abstract

A method for the simultaneous determination of *R*- and *S*-propranolol and *R*- and *S*-hyoscyamine in human plasma was developed, validated and applied to the analysis of samples from a clinical study. Sample preparation was performed by solid-phase extraction of 2 ml of human plasma using Oasis MCX cartridges and the enantioselective separations were achieved using a Chirobiotic V chiral stationary phase. The chromatography was carried out using gradient elution with a mobile phase composed of methanol:acetic acid:triethylamine which was varied from 100:0.05:0.04 to 100:0.05:0.1 (v/v/v) over 30 min and delivered at a flow rate 1 ml/min. The internal standard was *R*,*S*-propranolol-d_7 and the analytes were quantified using a single quadrupole mass spectrometer employing APCI interface operated in the positive ion mode with single ion monitoring. The enantioselective separation factors, α, were 1.15 and 1.07 for *S*- and *R*-propranolol and *R*- and *S*-hyoscyamine, respectively. The standard curves were linear for all target compounds with coefficients of determination (r^2) ranging from 0.9977 to 0.9999. The intra- and inter-day precision and accuracy were $\leq$13.2% and $\leq$10.2%, respectively. The assay was used to analyze plasma samples from seven subjects who had received i.v. infusions of *R*,*S*-propranolol and *R*,*S*-hyoscyamine. The initial data indicate that *R*-propranolol was eliminated faster than *S*-propranolol (CL/*f* = 2.34 ± 0.13 L/kg min vs. 2.07 ± 0.22 L/kg min) and that *R*-propranolol had a larger volume of distribution at steady-state (V_{ss}/f = 705 ± 165 L/kg vs. 589 ± 130 L/kg). In the case of *R*,*S*-hyoscyamine, *S*-hyoscyamine was eliminated faster than *R*-hyoscyamine (CL/*f* = 0.0537 ± 0.0073 L/kg min vs. 0.0439 ± 0.0086 L/kg min), while the volumes of distribution at steady-state were similar for the hyoscyamine enantiomers (V_{ss}/f = 7.82 ± 2.66 L/kg vs. 7.73 ± 1.39 L/kg).
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Keywords: Atropine; Propranolol; Chirobiotic V chiral stationary phase; LC–APCI-MS

1. Introduction

Analysis of beat-to-beat heart rate variability (HRV) has been shown to be a useful non-invasive marker of sympathetic and parasympathetic autonomic nervous system activity [1–3]. One approach to the analysis of HRV data is to wavelength transform analysis which has been shown to work after the separate administration of hyoscyamine (atropine, Hyosc) and propranolol (Prop). However, plasma drug concentrations were not measured during these studies and a quantitative relationship between drug concentration and effect could not be determined [4,5]. The assay developed in this study will be used in a clinical investigation to investigate the quantitative relationship between the plasma concentrations of Prop enantiomers and Hyosc enantiomers and the observed effects on HRV, when *R*,*S*-Prop is administered alone or in conjunction with *R*,*S*-Hyosc (NIA/NIH protocol # 2003-121).

Prop (Fig. 1A) is a non-selective beta-receptor antagonist primarily used in the treatment of cardiovascular diseases like hypertension or angina pectoris and in prevention of conditions

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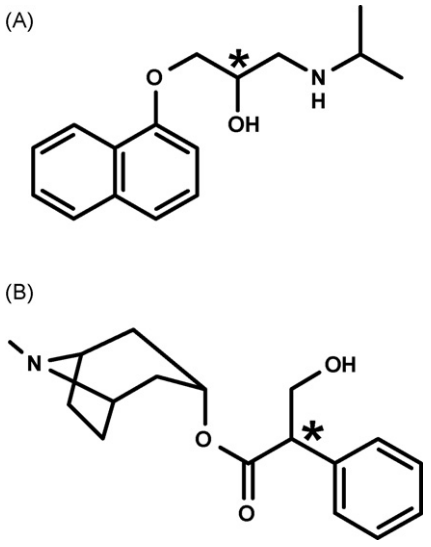

Fig. 1. Chemical structures of *R*,*S*-propranolol (A) and *R*,*S*-hyoscyamine (B). Asterisk "*" indicates position of a chiral carbon.

like migraine [6]. It is a chiral compound (*R*,*S*-Prop) and the β-blocking activity is produced by the *S*-enantiomer while both enantiomers possess equal membrane stabilizing effect [6]. Numerous achiral methods have been published for the determination of Prop in human fluids [7–12] as well as several enantioselective assays using chiral stationary phases (CSPs). The measurement of *R*- and *S*-Prop in human serum has been accomplished using a (*R*-*N*-(3,5-dinitrobenzoyl)-phenylglycine) CSP [13] and a cellulose-tris(3,5-dimethylphenylcarbamate) CSP [14], the latter method was applied to a pharmacokinetic study of Prop enantiomers in healthy male volunteers [15]. *R*- and *S*-Prop and analogues have also been resolved using a β-cyclodextrin CSP [16], an α_1-acid glycoprotein CSP employing micellar mobile phases and aliphatic, anionic additives [17], a teicoplanin-based CSP (Chirobiotic T) [18] and a vancomycine-based CSP (Chirobiotic V) [19,20].

Hyosc (Fig. 1B) is a tropane alkaloid extracted from the deadly nightshade (*Atropa belladonna*) and other plants of the Solanaceae family. Like Prop, it is a mixture of optical isomers, with *S*-Hyosc (also know as *l*-hyoscyamine) being responsible for the observed pharmacological effects. Hyosc belongs to an anticholinergic group of therapeutics and is mainly used in general anesthesia, in the treatment of toxicities caused by cholinesterase inhibitors, and in the treatment of cardiac disorders [21].

A number of achiral assays have also been reported for the determination of S-Hyosc in plant extracts and pharmaceuticals [22–26] and in human serum [27,28]. Hyosc has been enantioselectively resolved on the α_1-acid glycoprotein-CSP [29,30], on the human serum albumine-triazine CSP [31] and the teicoplanin-based Chirobiotic T CSP [32]. The chromatographic separation and determination of tropane and related alkaloids has been recently reviewed by Dräger [33]. Although several methods for enantioselective determination of Hyosc have been published, they were mainly used to determine purity of *S*-Hyosc in plant extracts. To date there have been no papers published for the simultaneous HPLC determination of hyosc enantiomers in human plasma for use in pharmacokinetic/pharmacodynamic studies.

The present paper describes the first LC–APCI-MS method for a simultaneous enantioselective determination of *R*,*S*-Prop and *R*,*S*-Hyosc in human plasma. The method employs a Chirobiotic V CSP with gradient elution and the assay has been validated and applied to plasma samples obtained from volunteers enrolled in a clinical study.

2. Experimental

2.1. Chemicals and reagents

R,*S*-Hyosc (atropine) (>99% TLC), *S*-Hyosc (>99% TLC), *R*,*S*-Prop (>99% TLC), triethylamine, 96% formic acid, 28% ammonium hydroxide solution were supplied by Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol and acetic acid glacial were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deuterium labeled *R*,*S*-propranolol-d_7 (rac-Prop-d_7) was purchased from CDN Isotopes (Quebec, Canada). *R*- and *S*-Prop were kindly provided by Dr. H.Y. Aboul-Enein (King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia). Purified water was prepared using a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Sample preparation

2.2.1. Collection of plasma from human samples

Human blank plasma collected from whole blood in sodium heparin used for method development and validation purposes was purchased from Valley Biomedical Inc. (Winchester, VA, USA). Plasma samples were obtained from participants enrolled in the clinical study "Wavelet Transform and Pharmacodynamic Analysis of Atropine and Propranolol Induced Changes in Human Heart Rate Variability" (NIA/NIH protocol # 2003-121). After signing informed consent according to the Declaration of Helsinki the subjects received a 20-min infusion of Prop at 0.8 mg/kg h during which the maximum dose did not exceed 20 mg. At the end of the initial infusion, the subjects received a second 30-min infusion of either saline (0.25 ml/min) or Hyosc sulfate (20 μg/min h). The subjects who were given Hyosc also received an additional i.v. bolus of this drug (10 μg/kg) at the end of the 30-min infusion for a total dose of 20 μg/kg. Blood samples (10-ml) were drawn into tubes containing sodium heparin (Becton–Dickinson, Franklin Lakes, NJ, USA) at 20, 30, 45, 55, 65, 70, 75, 90 min and 2, 3, 4, 5 and 6 h after dosing. Plasma was obtained by centrifugation and stored in −80 °C until the day of analysis.

2.2.2. Extraction procedure

A 20 μl aliquot of a 10 μg/ml solution of the internal standard (rac-Prop-d_7) was added to 2 ml of plasma in a borosilicate glass tube (13 mm × 100 mm), vortex-mixed for 30 s, acidified with 1 ml of 2% formic acid and vortex-mixed for 30 s. The analytes were extracted using solid-phase extraction with Oasis Mixed-Mode Cation Exchange (MCX) 3 ml cartridges (Waters,

Milford, MA, USA) and the extractions were performed using a 24-port vacuum manifold from Fisher Scientific (Fair Lawn, NJ, USA). The MCX cartridges were conditioned with 3 ml of methanol, equilibrated with 3 ml of 2% formic acid, the plasma samples were loaded onto the cartridges and sequentially washed with 3 ml of 2% formic acid, 3 ml of methanol, 3 ml of a mixture 30% methanol in water and ammonium hydroxide solution (28%) 95:5. The analytes were eluted with 4 ml of 2.25% ammonium hydroxide in methanol into plastic tubes (12 mm × 75 mm), the eluents were evaporated to dryness in a Speed Vac (Thermo Savant, NY, USA) for 2 h at 80 °C and the residue was resuspended in 100 μl of mobile phase composed of methanol:acetic acid:triethylamine (100:0.05:0.04, v/v/v), vortex-mixed for 30 s, transferred into vials and 50 μl was injected into the HPLC system for analysis. All ammonium solutions were prepared each day of analysis.

2.3. Chromatographic conditions

The chromatography was carried out using an Agilent Technologies system (Palo Alto, CA, USA) 1100 LC/MSD Series (liquid chromatography-mass selective detector) composed of a vacuum degasser (G1379 A), a quaternary pump (1311A), a thermostated autosampler (G1329 A) and a thermostated column compartment (G1316A). The mass selective detector (MSD Quad SL, G1956B) was used with atmospheric pressure chemical ionization interface (APCI, G1947A) and on-line nitrogen generation system (Parker, Haverhill, MA, USA). The data was acquired by ChemStation software, Rev. A.10.02 [1757] (Agilent Technologies, Palo Alto, CA, USA). The chiral separations were achieved on a Chirobiotic V column (250 × 4.6 mm I.D., 5 um particle size) connected to a Chirobiotic V guard column (2 cm × 4.0 mm I.D., 5 um particle size) (Astec, Advanced Separation Technologies Inc., Whippany, NJ, USA).

The analyses were performed using a mobile phase composed of methanol:acetic acid:triethylamine and gradient elution in which the mobile phase composition was changed from 100:0.05:0.04 to 100:0.05:0.1 (v/v/v) within 25 min, after which the mobile phase was changed back to the initial composition for an additional 5 min. The assay took 30 min and was performed at 30 °C with a flow rate of 1 ml/min and a 10:1 split ratio.

2.4. Optimization of the mass selective detector (MSD) parameters

An atmospheric pressure chemical ionization interface (APCI) was used with the MSD operating in the positive mode. The optimized conditions for *R*- and *S*-Prop and *R*- and *S*-Hyosc were as follows: fragmentor voltage 60 V, 70 V, gain 4 and 20, respectively, drying gas flow 6 l/min, nebulizer pressure 60 psig, drying gas temperature 350 °C, vaporizer temperature 210 °C, capillary voltage 4000 V and corona needle current 4.0 μA. Target compounds were quantified in the single ion monitoring mode (SIM). *R*- and *S*-Prop was monitored at *m/z* 260.2, *R*- and *S*-Hyosc at *m/z* 290.3 and *R*- and *S*-Prop-d_7 at *m/z* 267.2.

2.5. Preparation of standard solutions

Stock solutions of Hyosc (1 mg/ml as free base), Prop (1 mg/ml as free base) and deuterated rac-Prop-d_7 (10 μg/ml as free base) were prepared individually in methanol and were kept at −20 °C. Working solutions for both compounds were prepared weekly in two levels 1 μg/ml and 25 μg/ml and were kept at +4 °C.

2.6. Preparation of calibration curves and QC samples

Calibration and quality control samples were prepared daily by spiking 2 ml plasma samples with working solutions. Calibration curves for each enantiomer of Hyosc ranged over 0.5, 1.25, 2.5, 5, 10, 25 to 50.0 ng/ml and for each enantiomer of Prop 0.25, 0.75, 2.5, 10, 25, 100–250 ng/ml using internal standard in the final constant concentration of 100 ng/ml. The linearity of obtained standard curves was tested by calculating a relative error (RE) percentage using Microsoft Office Excel 2003 operating on a PC. Quality control concentrations for *R*- and *S*-Hyosc and *R*- and *S*-Prop were as follows 1.0, 12.0, 50.0 ng/ml and 1.0, 50.0 and 200.0 ng/ml, respectively.

2.7. Validation

2.7.1. Matrix effect (ME), recovery (RE) and process efficiency (PE)

Matrix effect studies were performed according to Matuszewski et al. [34]. ME, RE and PE were studied at four levels for *R*- and *S*-Prop 1.0, 20.0, 50.0 and 200.0 ng/ml and at three levels for *R*- and *S*-Hyosc 1.0, 20.0 and 50.0 ng/ml. In order to perform these studies three sets of samples were prepared: set A—standards in mobile phase composed of methanol:acetic acid:triethylamine (100:0.05:0.04, v/v/v), set B—plasma extracts spiked with standards after extraction; set C—plasma extracts spiked with standards before extraction. The assay was performed with plasma from five different sources. Both absolute (using peak heights) and relative (using peak height ratios of compound/IS) ME, RE and PE were examined. The formulas used were as follows [35]:

$$\mathrm{ME}\,(\%) = \frac{\mathrm{B}}{\mathrm{A}} \times 100 \quad (1)$$

$$\mathrm{RE}\,(\%) = \frac{\mathrm{C}}{\mathrm{B}} \times 100 \quad (2)$$

$$\mathrm{PE}\,(\%) = \frac{\mathrm{C}}{\mathrm{A}} \times 100 \quad (3)$$

2.7.2. Precision and accuracy

Precision and accuracy were determined by analyzing concentrations for five samples of each quality control level (LQC, MQC, UQC). The study was repeated three times, every second day. Precision and accuracy was also determined for LLOQ in quintuplicate. The acceptance criteria for accuracy and precision for QCs and LLOQ were followed according to FDA guidance in which the mean accuracy values for QCs should be within

15% of the actual value except for LLOQ—20%, and the precision determined at each QC level should not exceed 15% of the coefficient of variation (CV) and 20% for LLOQ [36].

2.7.3. Stability

The resident time in the autosampler of the processed compounds as well as freeze and thaw stability studies were determined for three QC levels in triplicates.

2.8. Pharmacokinetic data analysis

Individual profiles of Hyosc and Prop enantiomers were characterized using a standard linear two-compartment model [37]:

$$\frac{dA_p}{dt} = K_0 - (k_{el} + k_{12})A_p + k_{21}A_t \quad (4)$$

$$\frac{dA_t}{dt} = k_{12}A_p - k_{21}A_t \quad (5)$$

where A_p and A_t are the amounts of drug in the central and peripheral compartments, k_{el} is the first-order elimination rate constant, and k_{12} and k_{21} represent first-order rate constants controlling drug transfer between the central and peripheral compartments. Plasma drug concentrations (C_p) were set equal to: $C_p = A_p/(V_c/f)$, where V_c is the volume of the central compartment corrected by the fraction of the administered dose present in the *R*- or *S*-conformation. The initial conditions of Eqs. (4) and (5) were set equal to zero, and the zero-order constant infusion rate (K_0) was set equal to the rates of drug infusion for time, t, less than the duration of the infusion (otherwise set to zero). The bolus intravenous injection of Hyosc administered at the end of the infusion was modeled using the Dirac delta function as implemented in ADAPT (Biomedical Simulations Resource, University of Southern California, Los Angeles, CA, USA). Model parameters were estimated using the maximum likelihood estimator in ADAPT, and the variance model was defined as:

$$\mathrm{Var}(y) = (\sigma_1 + \sigma_2 y)^2 \quad (6)$$

with the estimated parameters, σ_i (σ_1 was fixed to zero), and y represents the model predicted outcome. The primary overall pharmacokinetic parameters, total systemic clearance (CL/*f*) and steady-state volume of distribution (V_{ss}/f), were calculated according to the following equations:

$$\frac{CL}{f} = \frac{k_{el}V_c}{f} \quad (7)$$

$$\frac{V_{ss}}{f} = \frac{V_c}{f}\left(\frac{1 + k_{12}}{k_{21}}\right) \quad (8)$$

3. Results and discussion

3.1. Chromatographic conditions

In the screening process the Cyclobond I 2000 DNP, Chirobiotic T and Chirobiotic V CSPS were examined. On Cyclobond I 2000 DNP CSP only partial enantioselective resolutions for *R*- and *S*-Prop were achieved with α values of around 1.05 and R_S values ranging from 0.22 to 0.58. Hyosc was not resolved on this CSP (Table 1). A greater enantioselective resolution of *R*- and *S*-Prop was achieved on the Chirobiotic T CSP (Table 1). The resolution was improved by eliminating acetonitrile from the mobile phase and by changing the ratio of acetic acid to triethy-

Table 1
The observed retention times (t_R) for the first eluting enantiomers of propranolol (Prop) and hyoscyamine (Hyosc) and the enantioselectivity (α) and resolution (R_S) features

HPLC CSP	Mobile phase composition (v/v/v)				t_R (min)		α		R_S	
	ACN	MeOH	HOAc	TEA	Prop	Hyosc	Prop	Hyosc	Prop	Hyosc
Cyclobond I 2000 DNP	95	5	0.2	0.15	16.2	22.7	1.04	NR	0.37	NR
	95	5	0.3	0.2	18.8	26	1.04	NR	0.46	NR
	90	10	0.3	0.2	12.1	16.1	1.02	NR	0.25	NR
	90	10	0.45	0.3	10.4	12.8	1.02	NR	0.22	NR
	100	–	0.3	0.2	31.6	51.2	1.05	NR	0.58	NR
Chirobiotic T	90	10	0.3	0.2	15.1	28.7	1.07	NR	0.98	NR
	95	5	0.3	0.2	19.5	35.0	1.09	NR	1.19	NR
	–	100	0.2	0.1	6.5	14.4	1.11	NR	1.37	NR
	–	100	0.2	0.2	11.0[a]	23.6[a]	1.11	NR	1.16	NR
	–	100	0.2	0.2	5.5	11.7	1.11	NR	1.02	NR
	–	100	0.1	0.1	7.9	18.2	1.11	NR	1.34	NR
	–	100	0.05	0.05	12.0	29.3	1.11	NR	1.69	NR
	–	100	0.05	0.1	9.5	22.0	1.11	NR	1.49	NR
	–	100	0.1	0.05	10.2	24.8	1.11	NR	1.51	NR
	–	100	0.05	0.025	15.4	38.7	1.11	NR	1.59	NR
	–	100	0.05	0.05	15.0[b]	–	1.11	NR	1.79	NR
	–	100	0.05	0.04	13.7	34.4	1.11	NR	1.79	NR
Chirobiotic V	–	100	0.05	0.04	10.5	25.2	1.15	1.07	1.69	0.81

The experiments were carried out at a flow rate of 1.0 ml/min, unless otherwise indicated, using the experimental approach described in the text. NR: not resolved
[a] Flow rate 0.5 ml/min.
[b] Flow rate 0.8 ml/min.

lamine (Table 1). Further optimization of the chiral resolution of Prop on the Chirobiotic T CSP was achieved using an approach proposed by Ruiz-Angel et al. [38]. For method optimization an equation based on eight independent chromatographic runs was determined, expressed as following:

$$R_S = 2.2833 - 2.3715x_1 - 1.9712x_2 - 0.4454x_3 \quad (9)$$

where x_1 is the volume of acetic acid added per 100 ml of methanol (ml); x_2 the volume of triethylamine added per 100 ml of methanol (ml); and x_3 is flow rate (ml/min).

The optimization was accomplished with the use of STATISTICA Software v. 6.0 (StatSoft, OK, USA).

Based on this equation the best ratio of HOAc to TEA predicting R_S of over 1.7 was 0.05–0.04 in 100 parts of methanol with flow rate 1 ml/min. These conditions were used in subsequent experiments.

Despite the optimized conditions for the enantioselective separation *R,S*-Prop, *R,S*-Hyosc was not resolved and the retention times were greater than 30 min. Using the observation of "Complementary stereoselectivity" as proposed by Advanced Separation Technologies Inc. [39] the Chirobiotic T was replaced by the Chirobiotic V. According to the Chirobiotic handbook, the use of term "complementary" describes the condition where an increase in selectivity is obtained in the exact same mobile phase conditions on a different Chirobiotic phase. The reasons for this phenomenon arise from the subtle differences in the enantioselective binding sites between the phases [39]. An enantioselective separation was obtained for *R* and *S*-Prop and *R* and *S*-Hyosc with R_S values 1.69 and 0.81, respectively (Table 1). In order to improve peak shape and to shorten analysis time, a gradient of triethylamine was used, in which the relative mobile phase concentration (v/v/v) of triethylamine was increased from 0.04 to 0.1 within the first 25 min of the run.

Under chromatographic conditions used in this assay the observed enantioselective separation factors, α, were 1.15 and 1.07 for *R*- and *S*-Prop and *R*- and *S*-Hyosc, respectively, and the separation ions were achieved in less than 30 min (Fig. 2A and B). The elution order for Prop enantiomers was determined by a comparison of retention times for *R*- and *S*-Prop reference substances, with *S*-Prop eluting before *R*-Prop (t_R 12.1 min vs. 13.3 min). The elution order for *R*- and *S*-Hyosc was determined based on a reference retention time (*S*-Hyosc), with *R*-Hyosc eluting before *S*-Hyosc (t_R 24.1 min vs. 25.5 min).

Analysis of five different plasma pools at the target *m/z* values proved to be free of interferences, a representative chromatogram is presented in Fig. 2C.

3.2. Calibration curves, limits of detection and QC samples

The calculated standard curves were linear with high coefficients of determination ranging from 0.9977 to 0.9999 for all target compounds. After examining the linearity of the curves it was determined that despite high values of coefficients the relative error for small concentrations of all target compounds exceeded 15%. Therefore "low range concentration" standard

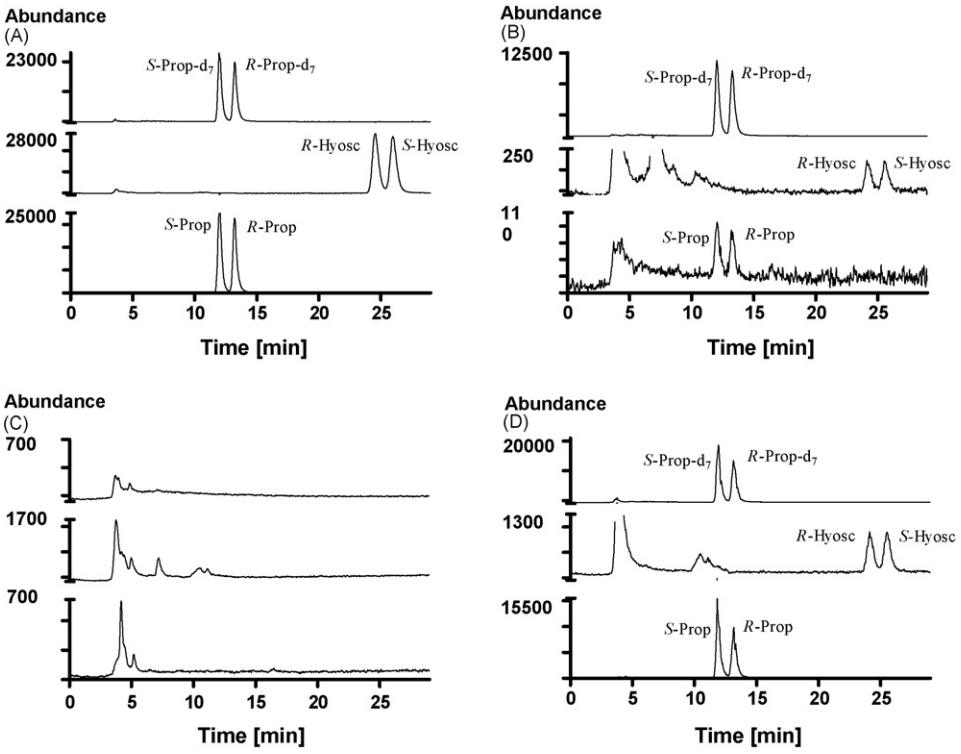

Fig. 2. HPLC chromatogram of 50 μl injection of an extract of (A) control plasma spiked with *R,S*-propranolol, *R,S*-hyoscyamine (50 ng/mL) and IS, (B) control plasma spiked with *R,S*-propranolol, *R,S*-hyoscyamine at LLOQ (0.25, 0.5 ng/mL, respectively) and IS, (C) blank plasma and (D) patient's plasma extract at 45 min after administration; the calculated drugs' concentrations for *R*-propranolol, *S*-propranolol, *R*-hyoscyamine and *S*-hyoscyamine were 33.3, 38.1, 1.9 and 1.9 ng/mL, respectively.

Table 2
Calibration equations for *R*,*S*-propranolol and *R*,*S*-hyoscyamine with the use of internal standard, where y = peak height ratio, x = concentration (ng/ml), b = slope, a = intercept

Compound	Concentration range (ng/ml)	Standard curve equation, $y = bx + a$		r^2
		Slope	Intercept	
S-propranolol	0.25–2.5	0.0182 ± 0.0001	0.0033 ± 0.0001	0.9999
	0.25–200.0	0.0212 ± 0.0001	−0.0026 ± 0.0133	0.9998
R-propranolol	0.25–2.5	0.0183 ± 0.0009	0.0031 ± 0.0013	0.9977
	0.25–200.0	0.0203 ± 0.0002	0.0193 ± 0.0181	0.9996
R-hyoscyamine	0.5–2.5	0.0207 ± 0.0004	0.0020 ± 0.0010	0.9994
	0.5–50.0	0.0234 ± 0.0002	−0.0058 ± 0.0055	0.9994
S-hyoscyamine	0.5–2.5	0.0202 ± 0.0001	0.0006 ± 0.0005	0.9999
	0.5–50.0	0.0219 ± 0.0001	−0.0037 ± 0.0015	0.9999

curves were constructed for the analytes. For *R*- and *S*-Prop the low range was between 0.25 and 2.5 ng/ml and for *R*- and *S*-Hyosc the low ranges were from 0.5 to 2.5 ng/ml. All standard curve equations used along with slopes, intercepts and standard deviations are presented in Table 2.

The limit of detection (LOD) calculated as a signal to noise ratio (S/N) equal to 3 was 0.03 ng/ml for each of Prop enantiomer and 0.1 ng/ml for each of Hyosc enantiomer.

3.3. Matrix effect (ME), recovery (RE) and process efficiency (PE)

The calculated according to the Eq. (1) relative ME was in the range of 119.9 to 139.5% for both analytes. Average RE for *S*- and *R*-Prop were 86.8 and 85.4%, respectively, and for *S*- and *R*-Hyosc, 83.0 and 81.3%, respectively. The mean PE for *S*- and *R*-Prop was 102% and for *S*- and *R*-Hyosc 111% (Table 3).

During method development it was determined that the detection signal of *R*-Prop was decreased due to ME by 60–70% (data not presented). Since the target sensitivity was 1 ng/ml several changes were made to eliminate the ME. The electrospray (ES) interface used in the preliminary studies was replaced by atmospheric pressure chemical ionization interface (APCI), as had been previously suggested [35]. The suppression and high variability due to ME was also addressed by the use of a rac-Prop-d_7 as the internal standard.

The sample preparation procedure was also optimized, and SPE cartridges with different packing materials were investigated including C18 (Bond Elut, Varian, Harbor City, CA, USA) and Oasis cartridges: hydrophilic–lipophilic balance (HLB), mixed-mode cation exchange (MCX) and weak cation exchange (WCX) (Waters, Milford, MA, USA). The highest recovery with the smallest ME was observed using the MCX cartridges. An extra washing step was added to the SPE procedure using a mixture of 30% methanol in water and ammonium hydroxide in the ratio 95:5 (v/v).

A ME was also observed for *S*-Prop, *R*- and *S*-Hyosc. Unlike *R*-Prop, the ME led to peak enhancement for these analytes. The validation studies demonstrated that the ME effect on *S*-Prop, *R*- and *S*-Hyosc did not interfere with the assay and the FDA guidance criteria [36] were met (Table 4).

3.4. Precision and accuracy

The intra-day and inter-day precision data are presented in Table 4. Both precision and accuracy were within the limits proposed in FDA guidance [36].

3.5. Stability

Prop and Hyosc standards were tested in freeze–thaw studies on three levels of concentrations for each enantiomer 1, 20

Table 3
Matrix effect (ME), recovery (RE) and process efficiency (PE) results for propranolol (Prop) and hyoscyamine (Hyosc) investigated in five different control human plasma lots (calculated from mean peak heights ratios—compound/IS)

	Matrix effect (ME) (%)				Recovery (RE) (%)				Process efficiency (PE) (%)			
	Prop		Hyosc		Prop		Hyosc		Prop		Hyosc	
	S	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*
1 ng/mL	123.1	114.2	127.3	134.7	77.2	77.6	80.2	78.4	95.1	88.6	102.0	105.5
20 ng/mL	126.1	125.0	154.5	153.5	88.8	88.9	78.2	78.1	112.0	111.1	120.8	119.9
50 ng/mL	101.2	106.2	128.6	130.2	95.2	94.9	86.0	83.2	96.3	100.8	110.6	108.3
200 ng/mL	134.3	134.1	–	–	77.4	80.8	–	–	104.0	108.3	–	–
Mean	121.2	119.9	136.8	139.5	84.6	85.5	81.5	79.9	101.8	102.2	111.1	111.2
S.D.	14.1	12.2	15.3	12.4	8.9	7.8	4.1	2.9	7.8	10.1	9.4	7.6
CV (%)	11.7	10.2	11.2	8.9	10.5	9.2	5.0	3.6	7.7	9.8	8.5	6.9

Table 4
Precision and accuracy data in determination of *R*,*S*-propranolol and *R*,*S*-hyoscyamine in human plasma extracts

Compound	Nominal concentration (ng/ml)	Precision		Accuracy	
		Intra-day, CV (%) $n=5$	Inter-day, CV (%) $15 \leq n \leq 30$	Concentration calculated $n=5$	Average (%) $n=5$
S-propranolol	0.25 (LLOQ)	12.0	ND	0.3	110.2
	1.0 (LQC)	8.3	9.8	1.0	108.3
	50.0 (MQC)	5.5	10.2	51.4	102.8
	200.0 (UQC)	2.5	7.6	207.2	103.6
R-propranolol	0.25 (LLOQ)	10.7	ND	0.26	102.7
	1.0 (LQC)	5.7	11.7	0.9	97.0
	50.0 (MQC)	5.1	9.4	50.3	100.7
	200.0 (UQC)	2.2	7.5	217.1	108.5
R-hyoscyamine	0.5 (LLOQ)	13.2	ND	0.5	105.2
	1.0 (LQC)	4.1	10.3	0.9	91.8
	12.0 (MQC)	9.1	12.1	12.1	100.6
	50.0 (UQC)	6.9	7.6	51.8	103.6
S-hyoscyamine	0.5 (LLOQ)	12.3	ND	0.5	104.6
	1.0 (LQC)	6.6	7.7	0.9	90.9
	12.0 (MQC)	5.9	12.4	12.6	105.4
	50.0 (UQC)	6.8	7.0	52.0	104.1

ND: not determined.

and 50 ng/ml. No observable degradation of the compounds was noticed after three freeze–thaw cycles. The stability of the analytes at three different concentrations for *R*- and *S*-Prop (1, 50 and 200 ng/ml) and two for *R*- and *S*-Hyosc (1 and 50 ng/ml) in the autosampler was also investigated. Spiked plasma extracts were stable in the autosampler up to 24 h of investigation (Table 5).

3.6. Assay application

The validated method was applied to the measurement of *R*- and *S*-Prop and *R*- and *S*-Hyosc in plasma of patients who had received i.v. doses of both drugs. A representative chromatogram from the analyses is presented in Fig. 2D. Mean plasma concentration-time curves for *R*- and *S*-Prop and *R*- and *S*-Hyosc were determined for the initial 7 subjects in the study, Fig. 3A and B, respectively. Our results appear to suggest that *R*-Prop is eliminated faster than *S*-Prop ($CL/f = 2.34 \pm 0.13$ L/kg min vs. 2.07 ± 0.22 L/kg min), which is in agreement with previously published enantioselective pharmacokinetic data [40,41]. Differences between the volumes of distribution at steady-state were also observed with a larger volume exhibited by the *R*-enantiomer ($V_{ss}/f = 705 \pm 165$ L/kg vs. 589 ± 130 L/kg).

In the case of *R*- and *S*-Hyosc, the data suggest that *S*-Hyosc was eliminated faster than *R*-Hysoc ($CL/f = 0.0537 \pm 0.0073$ L/kg min vs. 0.0439 ± 0.0086 L/kg min). In contrast to Prop, the volumes of distribution at steady-state for Hyosc enantiomers were similar ($V_{ss}/f = 7.82 \pm 2.66$ L/kg vs. 7.73 ± 1.39 L/kg). These trends are consistent with the pharmacokinetic study reported by Aaltonen and co-workers, where either *S*-Hyosc or Hyosc were measured with radioreceptor assay or radioimmunoassay, respectively [42]. They reported stereospecific differences in pharmacokinetics of *R*- and *S*-Hyosc with more than three times smaller AUC for *S*-Hyosc in comparison to Hyosc. However, this assay did not record simultaneous determination the *R*- and *S*-Hyosc enantiomers. A complete pharmacokinetic analysis will be reported at the conclusion of the clinical study.

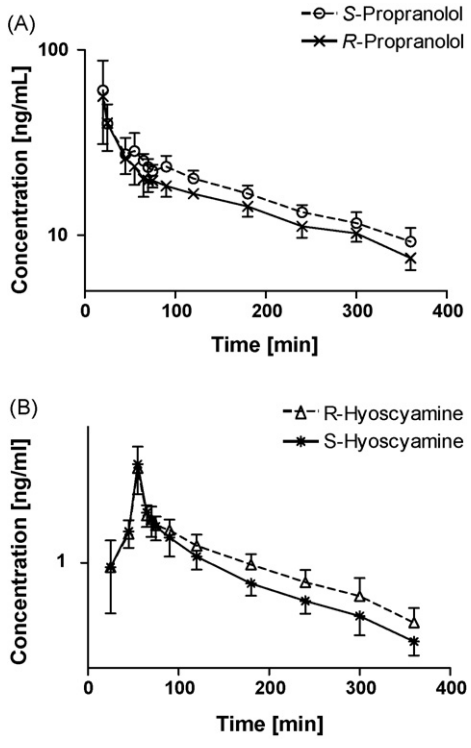

Fig. 3. Time-dependent mean concentrations of *R*,*S*-propranolol and *R*,*S*-hyoscyamine determined in plasma of seven clinical study participants (±S.D.).

Table 5
Stability of analytes in the freeze–thaw and post-preparative tests

Nominal concentration (ng/ml)	Compounds									
	S-propranolol			*R*-propranolol			*R*-hyoscyamine		*S*-hyoscyamine		
	Measured conc. (ng/ml)	CV (%)	Accuracy (%)	Measured conc. (ng/ml)	CV (%)	Accuracy (%)	Measured conc. (ng/ml)	CV (%)	Accuracy (%)	Measured conc. (ng/ml)	CV (%)	Accuracy (%)
Freeze and thaw studies												
1.0 ng/ml	1.2	8.8	108.1	1.1	2.4	109.6	1.0	15.2	100.9	1.0	10.6	103.3
20.0 ng/ml	18.9	13.3	94.4	18.4	11.8	92.1	22.0	3.3	110.2	22.6	4.1	113.0
50.0 ng/ml	50.9	4.0	101.7	53.3	4.3	106.6	55.3	2.9	110.6	54.7	0.9	109.4
Post-preparative stability (up to 24 h)												
1.0 ng/ml	1.1	6.3	112.2	1.1	4.1	106.7	0.9	4.0	91.9	0.9	3.9	89.6
50.0 ng/ml	51.6	5.5	103.2	52.6	7.7	105.3	50.5	4.3	101.1	51.4	3.7	102.8
200.0 ng/ml	213.0	1.5	106.5	213.0	1.7	106.5		ND			ND	

ND: not determined

4. Conclusions

The results demonstrate that the developed method is sensitive, precise, accurate and specific and can be applied to the simultaneous determination of *R*- and *S*-Prop and *R*- and *S*-Hyosc enantiomers. This is the first reported assay for the direct, simultaneous determination of *R*- and *S*-Hyosc in human plasma.

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References

[1] S. Akselrod, D. Gordon, F.A. Ubel, D.C. Shannon, A.C. Berger, R.J. Cohen, Science 213 (1981) 220.
[2] M. Pagani, F. Lombardi, S. Guzzetti, O. Rimoldi, R. Furlan, P. Pizzinelli, G. Sandrone, G. Malfatto, S. Dell'Orto, E. Piccaluga, et al., Circ. Res. 59 (1986) 178.
[3] N. Craft, J.B. Schwartz, Am. J. Physiol. 268 (1995) H1441.
[4] V. Pichot, J.M. Gaspoz, S. Molliex, A. Antoniadis, T. Busso, F. Roche, F. Costes, L. Quintin, J.R. Lacour, J.C. Barthelemy, J. Appl. Physiol. 86 (1999) 1081.
[5] A. Wellstein, H.F. Pitschner, Naunyn Schmiedebergs Arch. Pharmacol. 338 (1988) 19.
[6] B.B. Hoffman, in: J.G. Hardman, L.E. Limbird, A. Goodman Gilman (Eds.), Goodman & Gilman's the Pharmacological Basis of Therapeutics, McGraw-Hill, New York, 2001, p. 215.
[7] H.H. Maurer, O. Tenberken, C. Kratzsch, A.A. Weber, F.T. Peters, J. Chromatogr. A 1058 (2004) 169.
[8] T.W. Wilson, W.B. Firor, G.E. Johnson, G.I. Holmes, M.C. Tsianco, P.B. Huber, R.O. Davies, Clin. Pharmacol. Ther. 32 (1982) 676.
[9] M. Gergov, J.N. Robson, E. Duchoslav, I. Ojanpera, J. Mass Spectrom. 35 (2000) 912.
[10] M. Josefsson, A. Sabanovic, J. Chromatogr. A 1120 (2006) 1.
[11] R.D. Johnson, R.J. Lewis, Forensic Sci. Int. 156 (2006) 106.
[12] H. Sanbe, J. Haginaka, Analyst 128 (2003) 593.
[13] I.W. Wainer, T.D. Doyle, K.H. Donn, J.R. Powell, J. Chromatogr. 306 (1984) 405.
[14] R.J. Straka, R.L. Lalonde, I.W. Wainer, Pharm. Res. 5 (1988) 187.
[15] R.L. Lalonde, M.B. Bottorff, R.J. Straka, D.M. Tenero, J.A. Pieper, I.W. Wainer, Br. J. Clin. Pharmacol. 26 (1988) 100.
[16] M.W. Matchett, S.K. Branch, T.M. Jefferies, Chirality 8 (1996) 126.
[17] C.P.D. Haupt, D. Westerlund, Chirality 5 (1993) 224.
[18] Y.Q. Xia, R. Bakhtiar, R.B. Franklin, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 788 (2003) 317.
[19] G. D'Orazio, Z. Aturki, M. Cristalli, M.G. Quaglia, S. Fanali, J. Chromatogr. A 1081 (2005) 105.
[20] L.N. Nikolai, E.L. McClure, S.L. Macleod, C.S. Wong, J. Chromatogr. A 1131 (2006) 103.
[21] J. Heller Brown, P. Taylor, in: J.G. Hardman, L.E. Limbird, A. Goodman Gilman (Eds.), Goodman & Gilman's the Pharmacological Basis of Therapeutics, McGraw-Hill, New York, 2001, p. 155.
[22] C. Kirchhoff, Y. Bitar, S. Ebel, U. Holzgrabe, J. Chromatogr. A 1046 (2004) 115.
[23] A. Djilani, B. Legseir, Fitoterapia 76 (2005) 148.
[24] T. Ceyhan, M. Kartal, M.L. Altun, F. Tulemis, S. Cevheroglu, J. Pharm. Biomed. Anal. 25 (2001) 399.
[25] L. Kursinszki, H. Hank, I. Laszlo, E. Szoke, J. Chromatogr. A 1091 (2005) 32.
[26] I.N. Papadoyannis, V.F. Samanidou, G.A. Theodoridis, G.S. Vasilikiotis, G.J.M. van Kempen, G.M. Beelen, J. Liq. Chromatogr. 16 (1993) 975.
[27] P.A. Boermans, H.S. Go, A.M. Wessels, D.R. Uges, Ther. Drug Monit. 28 (2006) 295.

[28] P.A. Steenkamp, N.M. Harding, F.R. van Heerden, B.E. van Wyk, Forensic Sci. Int. 145 (2004) 31.
[29] E. Arvidsson, S.O. Jansson, G. Schill, J. Chromatogr. 506 (1990) 579.
[30] D. Breton, D. Buret, P. Clair, M. Lafosse, J. Chromatogr. A 1088 (2005) 104.
[31] Q. Zhang, H. Zou, X. Chen, H. Wang, Q. Luo, J. Ni, Chirality 12 (2000) 714.
[32] U.R. Cieri, J. AOAC Int. 88 (2005) 1.
[33] B. Dräger, J. Chromatogr. A 978 (2002) 1.
[34] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[35] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[36] Food and Drug Administration, in: Guidance for Industry, Bioanalytical Method Validation, May 2001.
[37] M. Gibaldi, D. Perrier, Pharmacokinetics, Marcel Dekker, New York, 1982.
[38] M.J. Ruiz-Angel, J.R. Torres-Lapasio, S. Carda-Broch, M.C. Garcia-Alvarez-Coque, J. Chromatogr. Sci. 41 (2003) 350.
[39] Chirobiotic Handbook, A guide to using macrocyclic glycopeptide bonded phases for chiral LC separations, Advanced Separation Technologies Inc., 2004, p. 1.
[40] L.S. Olanoff, T. Walle, U.K. Walle, T.D. Cowart, T.E. Gaffney, Clin. Pharmacol. Ther. 35 (1984) 755.
[41] G. Egginger, W. Lindner, G. Brunner, K. Stoschitzky, J. Pharm. Biomed. Anal. 12 (1994) 1537.
[42] L. Aaltonen, J. Kanto, E. Iisalo, K. Pihlajamaki, Eur. J. Clin. Pharmacol. 26 (1984) 613.