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Bio-analytical chiral chromatography method for the enantioselective separation of carbinoxamine maleate in human plasma

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Abstract

Background: A selective chiral high-performance liquid chromatography (HPLC) method was developed and validated to separate and quantify the (d) and (l) carbinoxamine enantiomers in human plasma.

Methods: Plasma samples were extracted by liquid-liquid extraction. The separation of carbinoxamine enantiomers and internal standard (IS, pargeverine hydrochloride) was achieved on an amylose tris(5-chloro-2-methylphenylcarbamate) column with a mobile phase of n-Hexane/isopropanol/ethanol/diethyl amine (850:75:75:0.1, *v/v/v/v*) at a flow rate of 0.8 mL/min. The ultraviolet (UV) detection wavelength was set at 220 nm.

Results: Baseline separation of carbinoxamine enantiomers and IS, free from endogenous interferences, was achieved in less than 15 min. Ratio of peak area of each enantiomer to IS was used for quantification of plasma samples. Linear calibration curves were obtained over the range of 20–7500 g/mL in plasma for both enantiomers ($R^2 > 0.99$). The mean extraction recoveries were 103.8 ± 1.5 and 94.5 ± 1.8 % for (d) and (l) enantiomers of carbinoxamine enantiomers and 96.35 % for IS from human plasma. The mean relative error (RE %) of accuracy and the mean relative standard deviation (RSD %) of intra-day and inter-day precision for both enantiomers were <10 %.

Conclusions: The method was validated with accuracy, precision, recovery, and stability and can be used to determine the pharmacokinetics of carbinoxamine enantiomers in human plasma.

Keywords: Chiral chromatography, Carbinoxamine enantiomers and human plasma

Background

In the pharmaceutical industry, enantiomeric separation has become an important area of study as the single-enantiomer drugs are found to be safer than racemic drugs (Pharma Book 2009). Carbinoxamine (CA) is an oral antihistamine agent that acts primarily by competing with the histamine in binding to H_1 receptor. It is used as monotherapy or in combination with pseudoephedrine (or) paracetamol in the management of hay fever and allergic conjunctivitis. It is also has anticholinergic properties, and because of which, it is used in the treatment of Parkinson's disease. The chemical structure of CA was shown in Fig. 1, with one chiral center attached with pyridyl ring and chlorphenyl ring. It is commercially available as the maleic acid salt (River's Edge 2011). Investigations have shown that CA was used as the racemic mixture clinically.

Ethanolamine class of H_1 antihistamines were proven to show stereoselectivity in binding to the receptors (Casy et al. 1992). Doxylamine which is a similar kind of molecule to that of carbinoxamine showed that its (d) or (+) form has more binding affinity compared to its antipode (Tadiboyina et al. 2015). It is currently unclear whether CA enantiomers also possess different pharmacodynamics, toxicological, or pharmacokinetic properties. To determine the pharmacokinetic properties of CA enantiomers in vivo, it is necessary to establish a reliable method for the detection of CA enantiomers in biological samples. Table 1 shows some methods for determination of racemic CA in biological fluids and dosage forms. So

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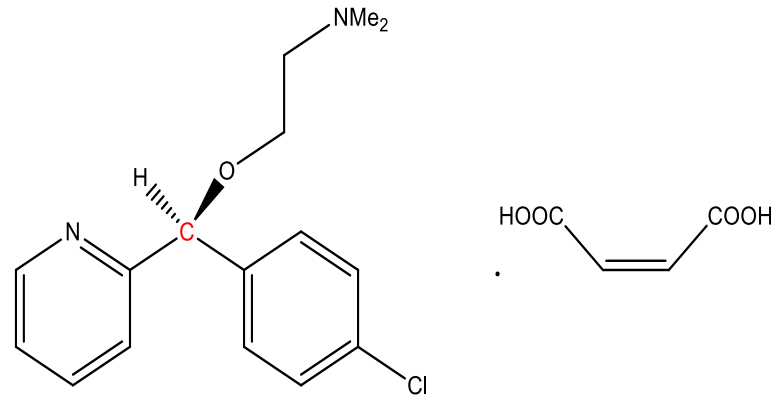

Fig. 1 Chemical structure of carbinoxamine maleate

far, very few studies were carried out to separate carbinoxamine into its individual enantiomers. Yi-Fen et al. (2000) developed a quantitative chiral analysis of carbinoxamine, doxylamine, and orphenadrine by capillary zone electrophoresis, but the method is not validated and not applied for bio-analytical studies. Therefore, it is unknown whether this method provides sufficient sensitivity and selectivity for bio-analytical studies. To our knowledge, no enantioselective methods have been developed for the determination of CA enantiomers in biological samples using a polysaccharide-based chiral column. In this paper, we describe the development of a stereoselective method for CA in the presence of internal standard pargeverine hydrochloride (PGV) (Fig. 2) by the normal phase method. Stationary phase was selected on the basis of functional groups present in the compound; mobile phase composition was systemically studied and finally optimized the method conditions by using eco-friendly solvents.

Table 1 Methods for the determination of CA in biological fluids and dosage forms

Method	Conditions	Detection system	Matrices	References
LC-MS/MS	BDS Hypersil C_8 (100 × 4.6) column; mobile phase: acetonitrile:buffer (80:20 *v/v*); buffer (25 mM ammonium formate)	MRM mode, electrospray-positive ionization	Human plasma	(GeetaBhavani et al. 2014)
LC-MS/MS	Waters PVA-Sil HPLC (50 × 4.0) mm column; acetonitrile:ethylacetate:water:methanol: formic acid:morpholine (500:200:100:60:0.2:0.025 *v/v/v/v*)	Sciex API 4000, triple quadrupole mass spectrometer	Human plasma	(Michael et al. 2008)
Ion exchange chromatography	Column bed:carboxymethylcellulose	Absorbance at 264 mμ	Pharmaceutical formulations (syrups, drops, and tablets)	(Ramadan and Mandil 2006)
Spectrophotometry and HPLC	ACE C_{18} (250 × 4.6 mm) column; mobile phase: gradient elution program Acetonitrile, 0.01 sodium perchlorate (pH 3)	DAD detection, absorbance at 210–300 nm range	Pharmaceutical formulations (capsule)	(Ismail and Feyyaz 2012)
DPP and DCP	Mobile phase: phosphate buffer (pH 1.69) using dropping mercury electrode (DME) vs. Ag/Agcl	Diffusion currents (id) and peak currents (ip)	Pharmaceutical formulations (tablets, capsules, syrups, and oral drops)	(Abdul et al. 2009)
Ion pair reverse phase high-performance liquid chromatography	C18 (300 × 3.9 mm); mobile phase: methanol: monobasic phosphate buffer (60:40 *v/v*) with 1 ml phosphoric acid, 0.5 ml TEA and 0.25 g sodium lauryl sulfate	UV detection 300 nm	Pharmaceutical formulations (tablets)	(de Carina and Marcone 2009)
Perchloric acid titration	Titrate: 0.1 N perchloric acid with crystal violet TS as indicator	Blue-green end point	Bulk drug	(USP29-NF24 2007)
Capillary zone electrophoresis	Pre-column dervetization using sulfated beta-cyclodextrin as a chiral selector; mobile phase: tris buffer (100 mM pH 4.6)	UV detection 200 nm (anode at detection side)	Bulk drug	(Yi-Fen et al. 2000)
HPLC	Amylose-based chiral column (250 × 4.6 mm); mobile phase: hexane:ethanol (90:10 *v/v*)	UV detection 220 nm (22 °C)	Bulk drug	(Phenomenex 2013)

DPP differenetial pulse polarography, *DCP* differential current polarography

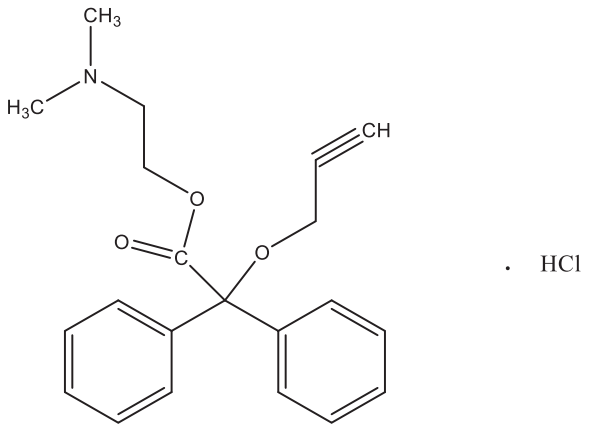

Fig. 2 Chemical structure of the internal standard pargeverine hydrochloride

Methods

Chemicals and materials

Carbinoxamine maleate and pargeverine hydrochloride standards were gifted by RL Fine Chemicals, Bengaluru, India. n-Hexane, 2-proponol, ethanol, and diethylamine were procured from Merck, Mumbai, India. High-performance liquid chromatography (HPLC)-grade water was obtained from a Milli-Q unit (Millipore, Milford, USA). Blank human plasma was obtained from JSS Medical College and Hospital, JSS University, Mysuru, India.

Instrumentation

The instrumentation consisted of a Shimadzu Prominence LC-20 AD ultrafast liquid chromatography (UFLC) equipped with a 1260 binary pump VL (35 MPa), Prominence SIL-20ACHT Auto sampler, and Prominence SPD-M20A Diode array detector. All weighings for analysis were performed on a Shimadzu electronic analytical balance AY-220 (Shimadzu). Plasma samples were sonicated on a Mark ultrasonic sonicator and vertexed on a Remi cyclomixer; centrifugation was done using REMI centrifuge model number 412 LAG (REMI Instruments Division, Vasai, India). Water used for analysis was prepared from Millipak Express 20 filter unit. Microsoft Excel 2007 was used for analysis of validation results.

Chromatographic conditions

The chromatographic separations were carried out on amylose tris(5-chloro-2-methylphenylcarbamate) column. The mobile phase was a mixture of n-Hexane/isopropanolthanol/diethyl amine (850:75:75:0.1, *v*/*v*/*v*/*v*) with 0.8 mL/min of flow rate, and detection was performed by a photodiode array detector (PDA) at 220 nm.

Preparation of stock and standard solutions

Stock solutions of CA and PGV in isopropanol were prepared separately at a concentration of 10 μg/mL. A series of standard mixture solutions were prepared by the appropriate dilution of the stock standard solutions with isopropanol to a concentration range of 0.4–1000 μg/mL for CA and 40 μg/mL for PGV.

Preparation of samples

The plasma samples were thawed at room temperature. The samples were vortexed adequately before pipetting. To a 200-μL aliquot of plasma, 100 μL of racemic CA stock solution (0.4–1000 μg/mL), 100 μL of IS solution (40 μg/mL), and 100 μL of 1 M NaOH were added and vortexed for 1 min in a 2-mL Eppendorf tube. The mixed samples were then extracted with 1.5 mL of dichloromethane: n-Hexane (1:2), by vortex mixing for 2 min. After centrifugation at 5000 rpm for 15 min, 1.0 mL of the upper organic layer was transferred to another tube. Extracts were concentrated to dryness at the 40 °C under a gentle stream of nitrogen and reconstituted with 150 μL of ethanol. A 25-μL aliquot of the solution was injected into the UFLC system for the analysis.

Bio-analytical method validation

Calibration curve

The plasma calibration curve was constructed using a blank sample (matrix sample processed without analyte or internal standard), a zero sample (matrix sample processed without analyte but with internal standard), and six non-zero samples (matrix samples processed with analyte and internal standard) covering the expected range including lower limit of quantification (LLOQ), 20–7500 ng/mL with 2000 ng/mL of IS concentration.

Extraction recovery

The efficiency of carbinoxamine enantiomers and IS extraction from human plasma was determined by comparing the responses of the analyte extracted from six reproduce QC samples with the response of analyte from neat standards at equivalent concentrations by a liquid-liquid extraction process. Recoveries of CA were determined at the LLOQ, QC low, QC medium, and QC high concentrations that is, 20, 300, 1200, and 5000 ng/mL. Whereas the recovery of the IS was determined at a single concentration of 2000 ng/mL.

Accuracy and precision

The intra-assay accuracy and precision were estimated by analyzing six replicates containing CA at four different QC levels, that is, 20, 300, 1200, and 5000 ng/mL. The inter-assay precision was estimated by studying the quality control samples on four different runs. The criteria for acceptability of accuracy data within 85–115 % of the actual values and ±15 % relative standard deviation (RSD) except for LLOQ for precision.

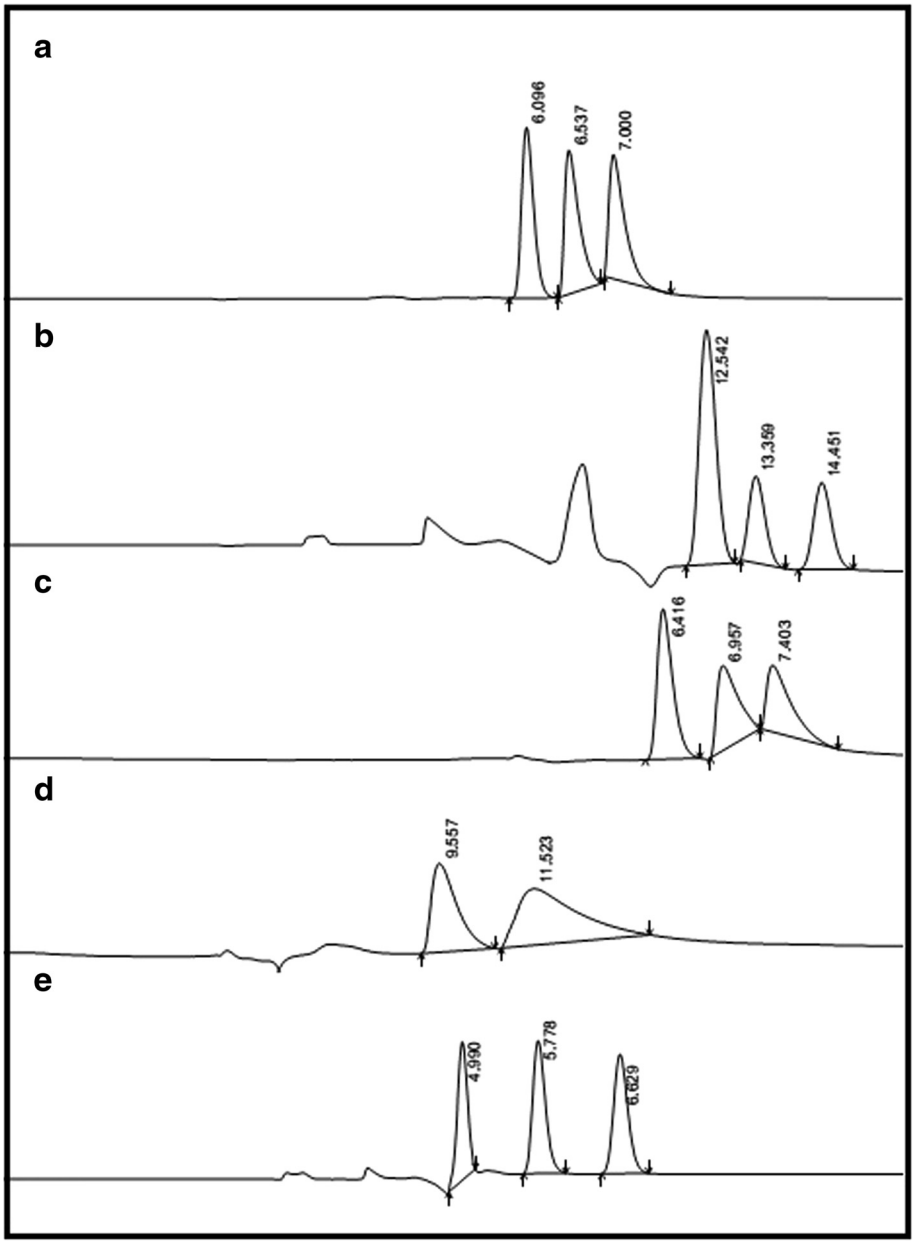

Fig. 3 Effect of organic modifiers on the resolution of CA enantiomers **a** methanol, **b** ethanol, **c** isopropanol, **d** n-butanol, **e** ethanol:isopropanol (1:1)

Table 2 Screening of alcoholic modifiers

Trail no.	Alcoholic modifiers	Alcoholic modifier (×) per 100 mL of mobile phase (mL)	Observations
1	Methanol	15	Poor resolution between the enantiomers, tailing of peaks observed
2	Ethanol	15	Enantiomers eluted very lately and the peak shape is broad.
3	Isopropanol	15	Enantiomers eluted early but not separated completely; no resolution between the peaks.
4	n-Butanol	15	No proper separation of enantiomers
5	Ethanol:isoproponol	7.5:.5	Enantiomers eluted early with good resolution and capacity factor.

Table 3 System suitability parameters

Name	Retention time (tR)(min)	Theoretical plates (*N*)	Tailing factor (*T*)	Resolution (Rs)	Capacity factor (*K*)
Pargeverine HCl (IS)	4.9	3784	1.12	–	–
(d)-form	5.8	5387	1.38	3.95	1.15
(l)-form	6.79	5821	1.32	3.01	1.28

Stability experiments

Stability tests were conducted to evaluate the stability of CA enantiomers in plasma samples under different conditions. In-injector stability (24 h), bench-top stability (12 h), freeze-thaw stability (3 cycles), and freezer stability (80 ± 10° C for 25 days) were tested at LQC (300 ng/mL) and HQC (5000 ng/mL) levels using six replicates at each level. Samples were said to be stable if assay values were within the acceptable limits (i.e., 85–115 % accuracy and ±15 % RSD from fresh samples).

Results and discussion

Method development and optimization

Selection of chiral stationary phase

The finest chiral stationary phases (CSP) for the separation of aromatic compounds with functional groups such as carbonyl, alcohol, and amine (like carbinoxamine) are amylose-based polysaccharide stationary phases (LCGC 2008). Immobilized polysaccharide columns are compatible with a wide range of solvents; hence, they can be operated in both reverse phase and normal phase which increased the range of applications (Othman et al. 2012). Hence, the coated amylose-based polysaccharide column was selected for screening.

The CSP is amylose tris(5-chloro-2-methylphenylcarbamate) bonded to silica gel. The separation of enantiomers may be attributed to hydrogen bonding interactions between the amine group of solute and the hydrophilic carbamate group on the CSP. Steadying effect on the solute-CSP complex will be there for the solutes having aromatic functional groups (Irving and Rose 1987). This type of steadying effect (or) stabilization effect may also exist in CA owing to the presence of aromaticity.

Selection and optimization of additives

As polysaccharide-based CSPs generally show a higher success rate in resolving enantiomers under normal-phase elution (Brian 2010); hence, this mode was explored first. The selection of mobile phases was initiated with traditional alkane/alcohol mixtures (Chiral Technologies 2004). n-Hexane was given first preference as it is comparatively green (Paul et al. 2012). For separation of most of the analytes containing basic and/or acidic functional groups, the additive plays a major role in increasing the chromatographic efficiency (Zhang et al. 2012). The additives were used in the present word in

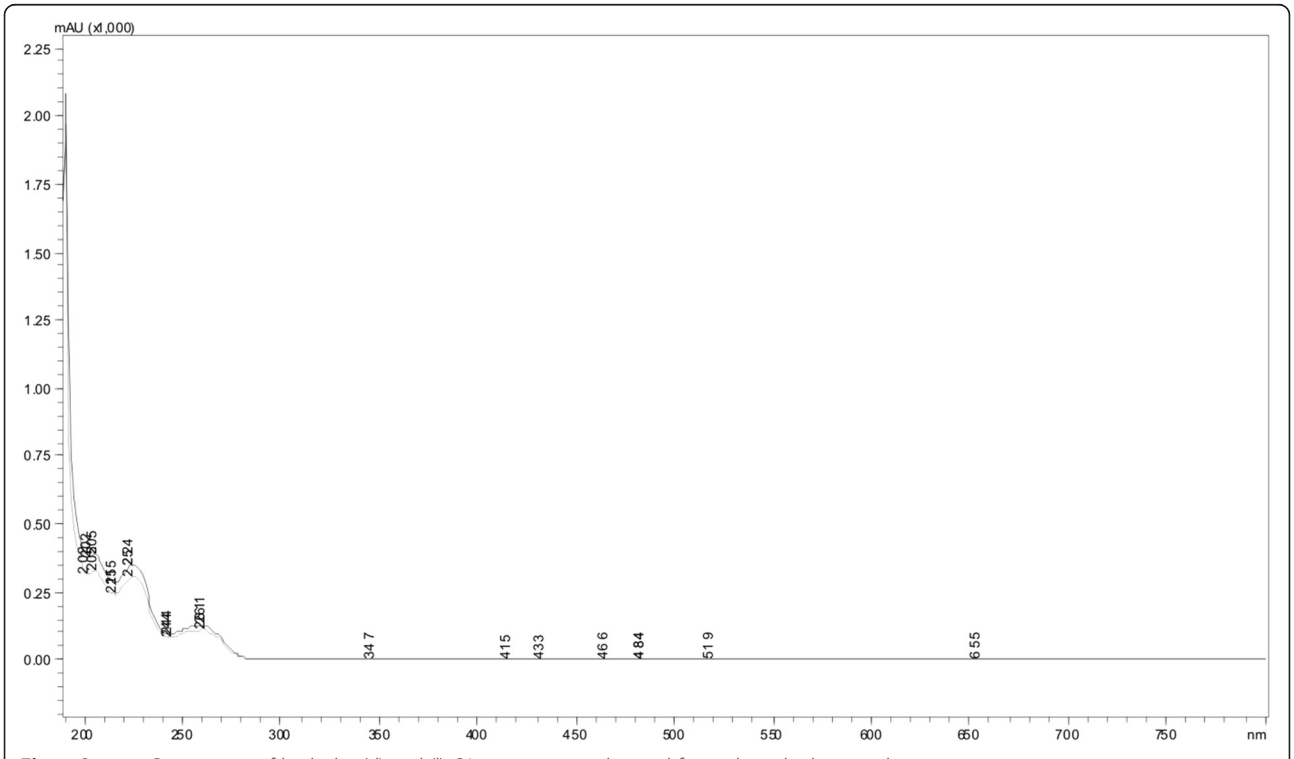

Fig. 4 Spectra Comparison of both the (d) and (l) CA enantiomers obtained from photodiode array detector

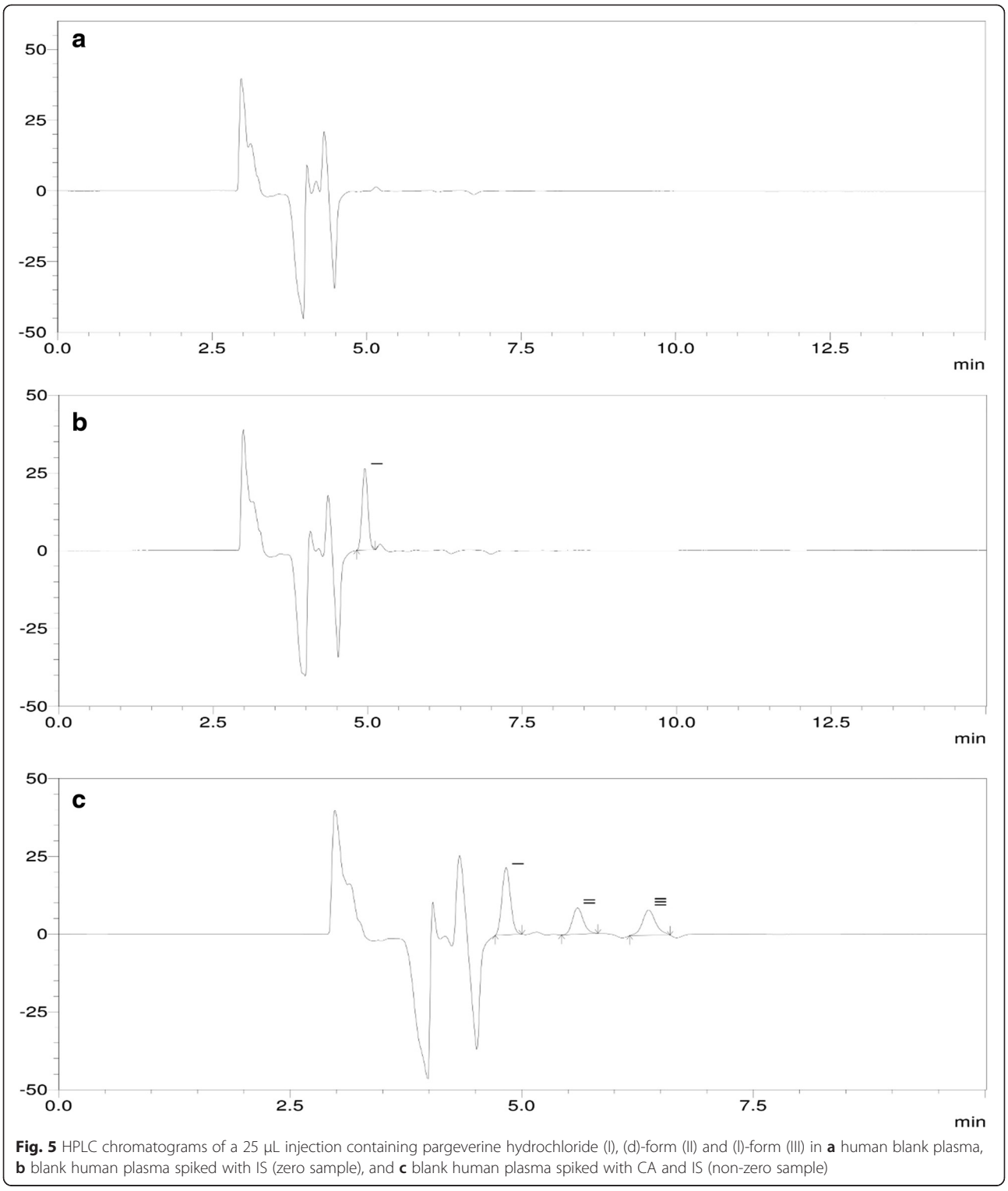

Fig. 5 HPLC chromatograms of a 25 μL injection containing pargeverine hydrochloride (I), (d)-form (II) and (l)-form (III) in **a** human blank plasma, **b** blank human plasma spiked with IS (zero sample), and **c** blank human plasma spiked with CA and IS (non-zero sample)

order to improve the peak shape. All chromatographic parameters were kept constant (flow rate, 0.8 mL/min, ambient column oven temperature; injection volume, 25 μL; wavelength, 220 nm) except the % additive added. Diethylamine and triethylamine were often used as a modifier for basic drugs with amine groups to ensure elution from the column and to obtain good peak shape (Toussaint et al. 2000). Hence 0.01 % diethylamine was

Table 4 Recovery of Carbinoxamine enantiomers ($n = 6$)

	Mean recovery % ± SD	
Quality control sample (ng/mL)	(l)-carbinoxamine	(d)-carbinoxamine
LQC ± 300	103.1 ± 2	101.7 ± 7.60
MQC ± 1200	94.4 ± 3	104.5 ± 2.7
HQC ± 5000	97.4 ± 6	94.1 ± 8.9

introduced into the mobile phase, CA enantiomers were eluted at around 10 min, and the peaks were highly asymmetric. Further increase in diethylamine content (0.1 %) has significant impact on the peak shape, whereas the mobile phase without diethylamine significantly distorted the peak shape.

Selection of alcoholic modifiers

In chiral separations, the alcohols play a major role in creating a selectivity difference (Tang 1996). Hence, the ratio of the mobile phase components n-Hexane/alcohol/diethylamine was fixed at 85:15:0.01 (*v*/*v*/*v*) and the effect of different alcohols on selectivity was studied (Fig. 3). The details of different alcohols screened (trials 1–5) and their results are given in Table 2. The results of trials 3 and 4 appeared to be complementary. Ethanol alone as an alcoholic component in trial 2 produces broad peaks with slow elution. Isopropanol alone eluted the enantiomers very early, and because of which, the resolution is not proper. Hence, to achieve the complementary effects of trials 2 and 3, ethanol and isopropanol was tested together (trial 5). Since trial 5 showed a significant improvement in the retention and resolution, an equal ratio of ethanol and isopropanol were used for the analysis. Hence, at the optimized chromatographic conditions, the internal standard and the enantiomers were eluted at 4.9, 5.8, and 6.79 min, respectively, passing all the chromatographic parameters (Table 3).

Optical rotation of CA enantiomers

The order of elution of ('d') or (+) and ('l') or (−) CA enantiomers was determined by collecting eluent fractions from repeated injections (Dalia et al. 2008). There were ~45 injections made into the UFLC, with 150 μg racemate per injection. Figure 4 depicts the spectral comparison of both the enantiomers, which shows that both the peaks represent the same drug. After compiling the eluent fractions corresponding to each enantiomer, they were dried by passing the nitrogen gas and reconstituted in methanol and the optical rotation was determined using a Jasco P-2000 (JASCO Analytical instruments, USA).

Assay validation

The bio-analytical validation was performed according to FDA guidelines (FDA guideline 2001).

Calibration curve

The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was constructed by determining the best fit of peak area ratios (peak area analyte/peak area IS) vs. concentration and fitted to the $y = mx + c$. The average regression ($n = 3$) was found to be ≥0.996 for both the (d) and (l) enantiomers of CA. The lowest concentration with the RSD <20 % was taken as the LLOQ and was found to be 20 ng/mL.

Extraction recovery

One-step liquid-liquid extraction technique gave adequate recovery and cleaner samples (Fig. 5). The results of the comparison of neat standards vs. human plasma-extracted standards were estimated for CA enantiomers at low (300 ng/mL), medium (1200 ng/mL), and high

Table 5 Intra- and inter-day precision of determination of CA in human plasma

Theoretical concentration (ng/mL)	Measured concentration (ng/mL), ($n = 6$)					
	Mean ± SD		RSD		Accuracy (%)	
	(d)-form	(l)-form	(d)-form	(l)-form	(d)-form	(l)-form
Intra-day variation						
20	20.1 ± 0.85	19.4 ± 0.35	4.2	1.8	102	97
300	320 ± 5.28	307 ± 6.35	1.65	2.06	107	102
1200	1228 ± 10.4	1261 ± 9.37	0.84	0.74	102	105
5000	4734 ± 24.5	4675 ± 20.15	0.51	0.43	95	94
Inter-day variation						
20	20.5 ± 0.17	20.6 ± 0.74	0.82	3.5	103	103
300	314 ± 2.38	324 ± 7.12	0.75	2.19	105	108
1200	1274 ± 11.3	1257 ± 18.74	0.88	1.49	106	105
5000	4735 ± 70.1	4823 ± 88.45	1.48	1.83	95	96

Table 6 Stability data CA quality controls in human plasma

Nominal con. (ng/mL)	Stability	Mean ± SD		Accuracy (%)		Precision (% CV)	
		(d)-form	(l)-form	(d)-form	(l)-form	(d)-form	(l)-form
300	0 h	317 ± 9.07	286 ± 8.34	106	95	2.86	2.91
	12 h (bench-top)	323 ± 5.01	257 ± 2.76	108	86	1.55	1.07
	24 h (in-injector)	326 ± 9.72	317 ± 5.2	109	106	2.98	1.64
	Freeze-thaw	340 ± 12	296 ± 10	113	99	3.52	3.4
	25 days at −80 °C	338 ± 1.47	318 ± 3.4	113	106	0.43	1.0
5000	0 h	4681 ± 54.3	4478 ± 28.9	93	90	1.16	0.64
	12 h (bench-top)	4734 ± 47.4	5183 ± 38.5	95	104	1	0.74
	24 h (in-injector)	4697 ± 35.1	5013 ± 60.7	94	100	0.74	1.21
	Freeze-thaw	4737 ± 70.2	4826 ± 51.2	95	97	1.48	1.05
	25 days at −80 °C	4705 ± 35.4	4478 ± 32.7	94	90	0.76	0.73

(5000 ng/mL) levels, and the mean recovery for (d) and (l) enantiomers of CA was shown in Table 4. The recovery of internal standard (2000 ng/mL) was 96.35 %.

Accuracy and precision

The results of accuracy and precision throughout the standard curve for (d) and (l) enantiomers of CA are presented in Table 5. The RSD values of intra-day and inter-day precision for two enantiomers were less than 4.2 %. All the values of accuracy and precision including the LLOQ fell within the limits which were considered as acceptable.

Stability studies

The concentrations of CA at low (300 ng/mL) and high (5000 ng/mL) level samples deviated within ±15 % of the nominal concentrations in stability tests like in-injector (24 h), bench-top (12 h), three repeated freeze-thaw cycles, and freezer stability (−80 ± 10 °C for 25 days) (Table 6). The results were found to be within the assay variability limits during the entire process.

Conclusions

The developed method was found to be sensitive, specific, and robust for quantification of both the (d) and (l) enantiomers of carbinoxamine in human plasma. The method involved sample preparation with adequate recovery by liquid-liquid extraction. Method parameters like % alcoholic modifier, % basic additive, and flow rate were successfully adjusted. The method ensured as a robust one from the responses obtained. Even for injection volume 25 μL the method is quite sensitive. The method is user-friendly because of using green solvents n-Hexane and ethanol. It is concluded that the method is suitable for the routine quantification of (d) and (l) enantiomers of CA in human plasma.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ST has carried out the major research work presented in this article. BMG, acted as guide for research work and helped in giving the inputs for various topics carried out in this research work. BKI, helped in preparing and drafting the manuscript

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