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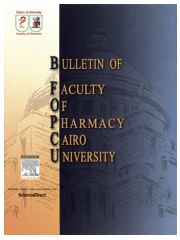

ORIGINAL ARTICLE

Chiral separation of (d)- and (l)-enantiomers of doxylamine succinate in rat plasma

Tadiboyina Sirisha *, **Bannimath Gurupadayya, Bharathkumar Inturi**

Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Mysuru, Karnataka 570015, India

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Wavelength;
Liquid chromatography

Abstract A selective chiral ultra fast liquid chromatography (UFLC-DAD) method was developed and validated to separate and quantify the (d)- and (l)-enantiomers of doxylamine in rat plasma. After extraction of the plasma samples with acetonitrile, the separation of doxylamine succinate enantiomers and internal standard (I.S., diphenhydramine hydrochloride) was achieved on a cellulose Tris (4-chloro,3-methylphenylcarbamate) column with a mobile phase of 20 mM ammonium bicarbonate buffer–acetonitrile (65:35 v/v) with 0.15% diethylamine in the buffer at a flow rate of 1.0 mL/min. The diode array (DAD) detection wavelength was set at 220 nm. The peaks obtained were identified as (d) and (l) by injecting the pure (d) form into the liquid chromatography and comparing the chromatograms. The effect of column oven temperature on the retention of doxylamine and mobile phase variables which have an effect on the enantiomers separation like ionic strength, type and concentration of organic modifier was studied. Linear calibration curves were obtained over the range of 100–1400 ng/mL in plasma for both enantiomers ($R^2 > 0.995$). The mean extraction recoveries were 94.5–104.7% of rat plasma. The mean relative standard deviation (RSD%) of accuracy and intra-day and inter-day precision for both enantiomers were $\leqslant$10%. The method can be further applied to determine the pharmacokinetics of (d)- and (l)-enantiomers in rat plasma.

1. Introduction

Antihistamine drugs can be broadly classified into H_1 receptor antagonists, H_2 receptor antagonists and H_3 receptor antagonists. H_1 receptors present in smooth muscles are blocked by classical antihistamines. H_2 receptors present in the stomach, stimulate gastric acid secretion. H_3 receptors primarily found in the brain, trigger the release of excitatory neurotransmitters. H_1 receptor antagonists can be classified into seven groups based on their chemical structure. Doxylamine succinate (Fig. 1a) is one of the first generation H_1 receptor antagonists of the ethanolamine group.[1] Diphenhydramine hydrochloride (Fig. 1b) is anti-histamine and used as internal standard.

Doxylamine succinate is used as an anti-allergic and it is also used in the treatment of hay fever. Doxylamine because of its sedative action acts as a sleeping aid.[2] Doxylamine succinate is a basic drug with the *p*Ka 8.8 and its IUPAC name is (*RS*)-*N*,*N*-dimethyl-2-(1-phenyl-1-pyridin-2-yl-ethoxy)-etha namine. It has one chiral carbon center in the phenyl ethoxy

* Corresponding author. Tel.: +91 8884699984.
E-mail address: tadiboyina.sirisha@gmail.com (T. Sirisha).
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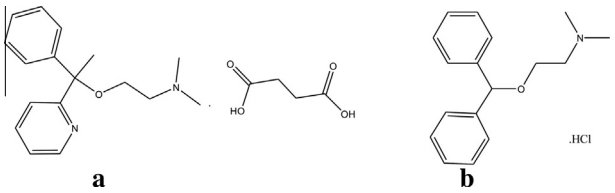

Figure 1 (a) Structure of doxylamine succinate, (b) structure of diphenhydramine hydrochloride.

chain to which pyridine-2-yl was attached. It is commercially available as the succinate salt. Investigations have shown that doxylamine was used as a racemic mixture clinically. But according to the patent published on 25th December 2013,[3] (d)-doxylamine is more efficient in resisting allergy and improving sleep when compared with the racemic form. Hence there is a need for the separation of enantiomers.

Examination of the literature reveals that few methods like derivative spectrophotometry,[4] FT-IR,[5] UV,[6] HPLC,[7] LC–MS[8,9] have been reported for the quantification of racemic (±) doxylamine in pharmaceutical formulations and in biological fluids. Also few methods have been reported for the resolution of doxylamine enantiomers in human plasma by using capillary electrophoresis[10] and high performance liquid chromatography.[11,12] However the reported methods have their own drawbacks like individual enantiomers (d)- and (l)-doxylamine were not identified, the methods were developed in the normal phase mode and also the stability of the enantiomers was not determined.

The objective of the present study is to develop a method for the resolution of enantiomers in a reverse phase mode, which is more reproducible when compared to the normal phase, identifying the (d)- and (l)-doxylamine enantiomeric peaks and also carrying out the full validated bio-analytical procedure including the stability study for the enantiomers in rat plasma.

2. Experimental

2.1. Materials and methods

An Analytically pure reference standard of doxylamine succinate was supplied by R L Fine Chem (Bangalore, India), and was used as such without further purification. The chemicals like ammonium bicarbonate (HPLC grade, Sigma–Aldrich, India), acetonitrile (HPLC grade, Merck Specialities Pvt. Ltd., Mumbai, India), and diethylamine (HPLC grade, Spectrochem Pvt. Ltd., Mumbai, India) were used in the analysis. Blank plasma was obtained from JSS Medical College and Hospital, Mysuru, India.

2.2. Instrumentation

The ultra fast liquid chromatography (UFLC) used to be of Shimadzu Prominence LC-20AD equipped with a 1260 binary pump VL (35 MPa), Prominence SIL-20ACHT Autosampler, and Prominence SPD-M20A Diode array detector. All weighings for analysis were performed on a Shimadzu electronic analytical balance AY-220 (Shimadzu). Mixing was performed on a REMI Cyclomixer followed by sonication on an ultrasonic bath (Mark ultrasonic sonicator). Centrifugation was done using REMI cooling centrifuge model number 412 LAG (REMI instruments division, Vasai, India). Pipetting was performed by using 20–200 µL and 500–5000 µL adjustable micropipettes (Phenomenex). Bio-samples were stored at −80 °C in an Ultra low, deep freezer (REMI). Plasma samples were filtered using 0.45 µm filters from Phenomenex. Water used for analysis was prepared from Millipak Express 20 filter unit. The blank plasma has been procured in its original form, from the animal house of JSS College of Pharmacy, Mysuru, India.

2.3. Chromatography conditions

The chromatographic separation of diphenhydramine hydrochloride (IS) and doxylamine enantiomers was accomplished using a 250 × 4.6 mm cellulose Tris (4-chloro,3-methyl phenylcarbamate) column attached to a pre-column holder for Cartridge containing cellulose Tris (4-chloro,3-methylphe nylcarbamate) (4 × 2.0 mm) guard column. The UV wavelength was set at 220 nm. Chromatography data were collected and compiled by use of LC solutions software. The mobile phase used was a 65:35 (v/v) mixture of 20 mM ammonium bicarbonate buffer (pH 6.6) and acetonitrile to which 0.15% diethylamine was added. The mobile phase was pumped at a flow rate of 1.0 mL/min.

2.4. Preparation of standard drug solutions

Doxylamine succinate stock solution was prepared by dissolving 10 mg of pure drug in 100 mL of Milli pore water (100 µg/mL). The single enantiomer stock solution was prepared by dissolving 10 mg of pure (d)-form of doxylamine in 100 mL of Millipore water (100 µg/mL). Diphenhydramine hydrochloride (internal standard) stock solution was prepared by dissolving 10 mg of pure drug in 100 mL of Milli pore water (100 µg/mL). The solutions were sonicated for 5 min and allowed to equilibrate at room temperature. The working standard drug solutions (1–14 µg/mL) were prepared by serial dilution of the stock drug solution. The working IS solution of 5 µg/mL was prepared by appropriate dilution of the IS stock solution. All solutions were stored at 2–8 °C.

2.5. Preparation of standard plasma samples

Standard plasma samples ranged from 100 to 1400 ng/mL of the drug with 500 ng/mL of IS prepared by spiking 500 µL blank rat plasma with appropriate working standards of drug and IS.

2.6. Preparation of quality control samples

Three quality control (QC) plasma samples at concentrations of 200, 600, and 1000 ng/mL and one lower limit of quantification (LLOQ) sample at a concentration of 100 ng/mL were prepared by spiking 500 µL blank plasma with appropriate drug and IS solutions.

2.7. Plasma sample treatment

A simple protein precipitation method was followed for the extraction of doxylamine from rat plasma. To an aliquot of 500 µL plasma sample, IS solution (200 µL of 5 µg/mL) was added and mixed for 45 s on a Cyclomixer (Remi Instruments, Mumbai, India) then doxylamine standard solution (200 µL of 1–14 µg/mL) was added and mixed for 45 s on a Cyclomixer. The sample solution was made up to 2 mL with acetonitrile followed by centrifugation for 10 min at 10,000 rpm on a Remi Centrifuge (Eppendorf, Germany) at 4 °C. The supernatant layer was transferred into another tube and filtered through a 0.45 µm syringe filter. A 10 µL of the filtrate was injected onto the HPLC column.

2.8. Validation procedure

2.8.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples from at least six different lots to investigate the potential interferences at the LC peak region for enantiomers and IS. The acceptance criterion for the experiment was that at least four out of six lots should have response less than five times the lower limit of quantitation (LLOQ) level response in the same matrix.

2.8.2. Recovery

The efficiency of Doxylamine enantiomers and IS extracted from rat plasma was determined by comparing the responses of the analyte extracted from replicating QC samples ($n = 6$) with the response of analyte from neat standards at equivalent concentrations by the protein precipitation process. Recoveries of Doxylamine enantiomers were determined at QC low and QC high concentrations that is, 200 and 1000 ng/mL. whereas the recovery of the IS was determined at a single concentration of 500 ng/mL

$$\text{Extraction efficiency} = C_{ex}/C_0 \quad (1)$$

where C_{ex} = concentration of doxylamine enantiomer from spiked plasma sample. C_0 = concentration of doxylamine enantiomer obtained after direct injection.

2.8.3. Calibration curve

The six point calibration curve (100, 200, 600, 800, 1000 and 1400 ng/mL) was constructed by plotting the peak area ratio of Doxylamine enantiomer: IS against the nominal concentration of calibration standards in blank rat plasma. Following the evaluation of different dilution factors, the results were fitted to linear regression analysis. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. For each back-calculated standard concentration the acceptance criteria were ±15% deviation from the actual value except at LLOQ, which was set at ±20%.

2.8.4. Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing doxylamine at four different QC levels, that is, 100, 200, 600 and 1000 ng/mL. The inter-assay precision was determined by analyzing the four levels of QC samples on four different runs. The criteria for acceptability of the data include, accuracy within 85–115% of the actual values and a precision of within ±15% relative standard deviation (RSD) except for LLOQ, where it should be within 80–120% for accuracy and <20% of RSD.

2.8.5. Stability experiments

Stability tests were conducted to evaluate the stability of Doxylamine enantiomers in plasma samples under different conditions. Stock solution stability (6 h) was done by keeping the original stock solution, spiked stock solution and IS stock solution at room temperature for 6 h. then processed, analyzed and compared with the freshly prepared solutions, In-injector stability (24 h), bench-top stability (12 h), freeze–thaw stability (three cycles) in which three aliquots of LQC (200 ng/mL) and HQC (1000 ng/mL) samples were kept in the freezer (−20 °C) for about 24 h and then thawed at room temperature. This cycle is repeated for two more times and the samples were analyzed after the third cycle. Freezer stability (−20° ± 10 °C for 25 days) was tested at LQC (200 ng/ml) and HQC (1000 ng/mL) levels using three replicates at each level. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. 85–115% of fresh samples) and precision (i.e. ±15% RSD).

3. Results

3.1. Optimization of chromatographic conditions

The method was optimized to separate the two enantiomers of doxylamine succinate. The main target of the chromatographic method is to get the separation between the closely eluting enantiomers which have the same physical–chemical properties. The structure and nature of the chiral stationary phase (CSP) play a very important role in the resolution of enantiomers. Nowadays most of the racemic drugs are being resolved by using polysaccharide chiral columns. Polysaccharide based chiral columns contain amylose or cellulose as a back bone forming a helical structure in the coating. When compared with amylose, the cellulose helical structure was coiled loosely which accommodates the enantiomer interaction more readily. Cellulose Tris (4-chloro-3-methylphenylc arbamte)[13] is the chlorinated cellulose derivative column which offers a unique chiral recognition abilities. The separation was tried on this column using basic buffers (the enantiomeric resolution of doxylamine is pH sensitive. And as the drug is a basic one, the method development was started with the basic pH buffers. Finally, both the enantiomers of doxylamine were resolved properly at pH 6.6.) and organic solvents like acetonitrile, methanol and isopropanol with different proportions. The effect of alcoholic modifiers on the resolution of enantiomers is depicted in Fig 2. As it is seen the retention increased in the order acetonitrile < isopropanol < methanol. Hence acetonitrile has been optimized as an alcoholic modifier. In order to improve the resolution factor between the enantiomers different organic modifiers like tri-ethylamine and diethylamine were used in the mobile phase. The effect of buffer strength and % organic modifier on the resolution of enantiomers has been studied and represented graphically (Fig. 3a,b). From this study it is concluded that as the buffer strength increases the resolution between the enantiomers

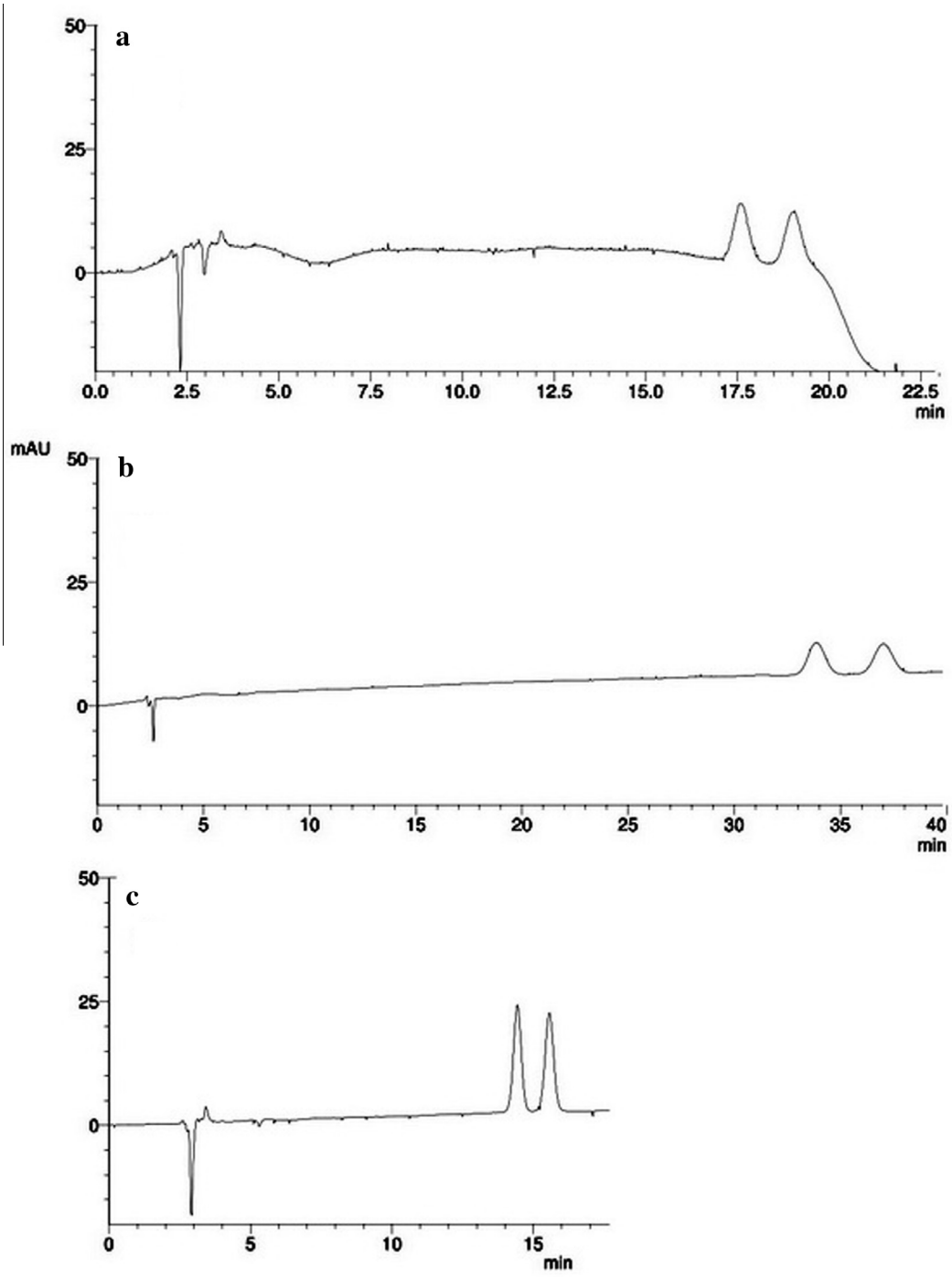

Figure 2 Effect of alcoholic modifiers on the retention of enantiomers (a) methanol, (b) isopropanol, (c) acetonitrile.

decreases and as the % organic modifier increases the resolution between the enantiomers increases. Hence, from the study the buffer strength and % organic modifier were optimized to 20 mM and 0.15% respectively. The effect of column oven temperature on the resolution of doxylamine enantiomers is represented in Fig. 3c which says that the increase in column oven temperature increases the solubility of the compound in the mobile phase, hence better resolution is obtained between the enantiomers. Thus the mixture of 20 mM ammonium bicarbonate buffer, acetonitrile and diethylamine (65:35:0.15 v/v/v) as a mobile phase with 1.0 mL/min flow rate through Cellulose column (250 mm × 4.6 mm, 5 μm) at 40 °C column oven temperature and 220 nm wavelength was successful in separating both the enantiomers and the internal standard (Table 1).

3.2. Elution order of enantiomers

The elution order of the doxylamine enantiomers, was determined by injecting the freshly prepared (d)-doxylamine stock

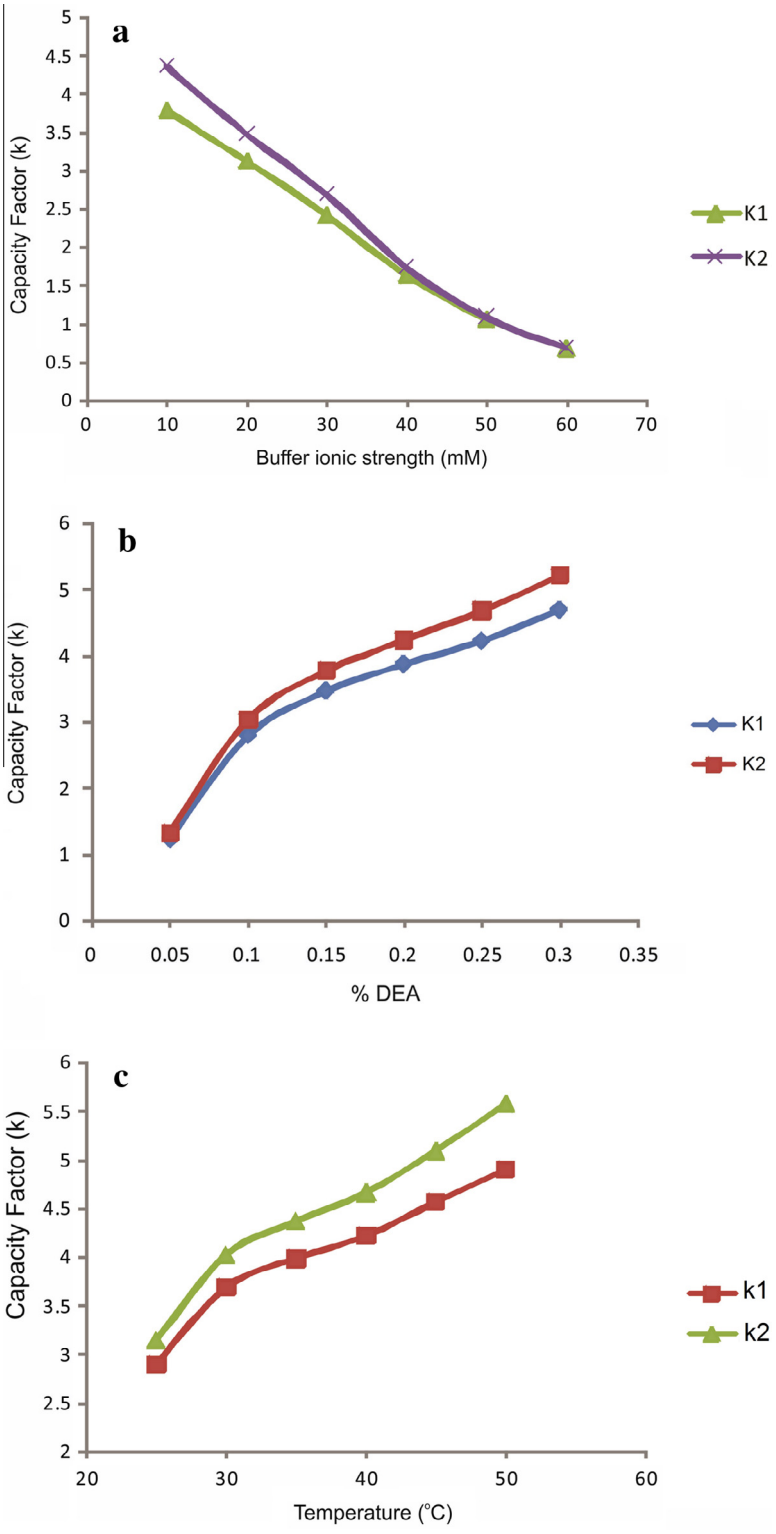

Figure 3 (a) Effect of buffer strength of the capacity factor of 'l' enantiomer (k_1) and capacity factor of 'd' enantiomer (k_2), (b) effect of % diethylamine (DEA) on the capacity factor of 'l' enantiomer (k_1) and capacity factor of 'd' enantiomer (k_2), (c) effect of column oven temperature on the capacity factor of 'l' enantiomer (k_1) and capacity factor of 'd' enantiomer (k_2).

solution onto the HPLC (Fig. 4) and comparing the peak with the racemic mixture peaks. It was found that the diastereomeric complex formed by the (d)-doxylamine has more binding affinity toward the stationary phase compared to (l)-doxylamine. Hence it was confirmed that the (l)-form of doxylamine elutes earlier than (d)-doxylamine.

Table 1 Chromatography data for enantioseparation of doxylamine on chiral column.

S. No.	Chromatographic parameter	Formula	Values obtained
1	Capacity factor of first eluted enantiomer (K_1)	$K_1 = \frac{t_1 - t_0}{t_0}$	1.62
2	Capacity factor of second eluted enantiomer (K_2)	$K_2 = \frac{t_2 - t_0}{t_0}$	1.82
3	Selectivity factor	$\alpha = \frac{K_2}{K_1}$	1.12
4	Resolution factor	$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$	2.7
5	Theoretical plates	$N = 16\left(\frac{t_r}{w_b}\right)^2$	6320

Where t_0 = retention time of unretained compound.
t_1 = retention time of earlier eluted compound.
t_2 = retention time of later eluted compound.
k_1 = capacity factor of earlier eluted compound.
k_2 = capacity factor of the later eluted compound.
w_1 = peak width of earlier eluted compound.
w_2 = peak width of later eluted compound.
t_r = retention time of peak.
w_b = the peak width at a given peak height.

3.3. Validation of bio-analytical method

A full validation according to the USFDA[14] was performed for the estimation of doxylamine enantiomers in rat plasma. The proposed method was validated with respect to selectivity, linearity, accuracy, precision, recovery, and stability.

3.3.1. Specificity and selectivity

Selectivity is the capability of the developed method to differentiate the analyte with the other endogenous components in the sample. Selectivity is ensured at LLOQ level (Fig. 5). Aqueous samples of LLOQ were injected six times. Spiked samples of LLOQ were injected six times along with six injections of blank plasma sample. All the chromatograms were analyzed and found that the doxylamine enantiomers and the internal standard peaks did not interfere with any endogenous components. There was also a good resolution between the two enantiomer peaks. Sensitivity is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision (i.e., LLOQ) and from the method the LLOQ was found to be 100 ng/mL.

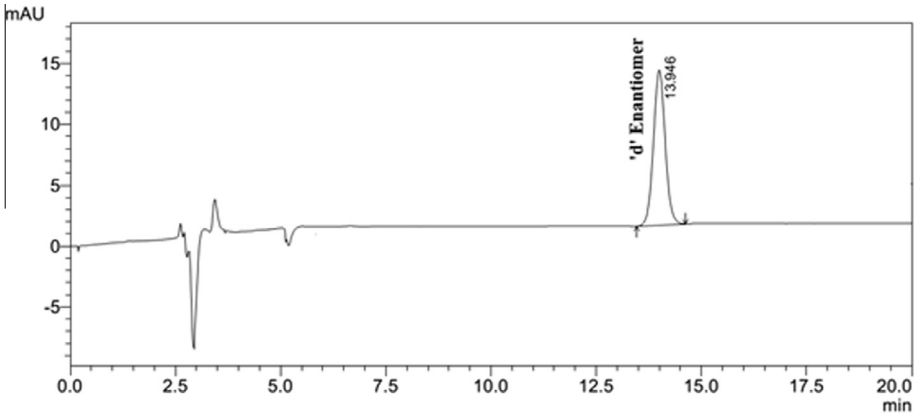

Figure 4 Chromatogram of (d)-doxylamine.

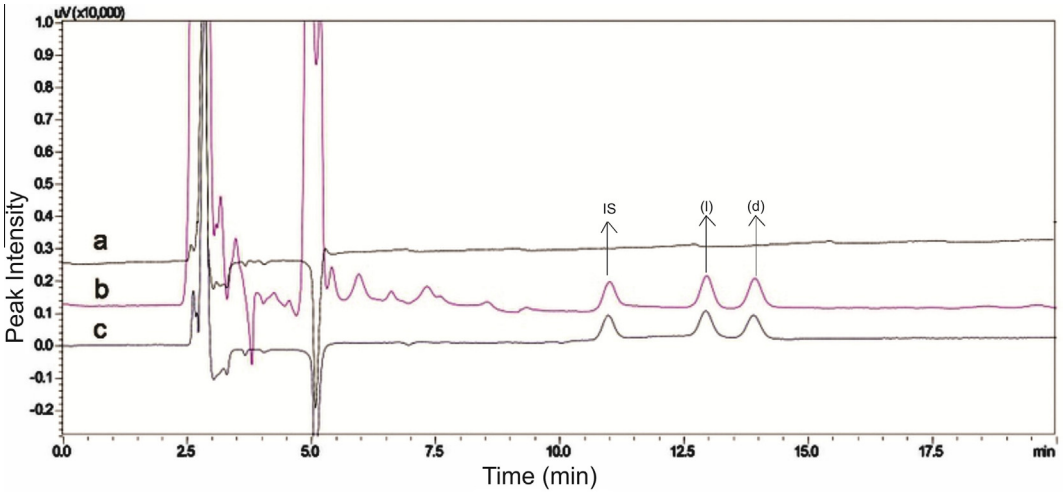

Figure 5 (a) Chromatograms of blank plasma sample, (b) aqueous LLOQ sample, (c) spiked LLOQ sample.

Table 2 Recovery of doxylamine enantiomers ($n = 6$).

Quality control sample (ng/mL)	Mean recovery % (SD)	
	(l)-doxylamine	(d)-doxylamine
LQC (200)	102.2 (2)	103 (7.60)
MQC (600)	96.4 (3)	101.5 (2.7)
HQC (1000)	102.4 (6)	94.5 (8.9)

Table 3 Intra-day and inter-day accuracy and precision data ($n = 6$).

Validation parameter	(l)-doxylamine	(d)-doxylamine
Concentration (ng/mL)	*Intra-day repeatability CV (%)*	
LLOQ (100)	7.1	10.5
LQC (200)	5.1	3.3
MQC (600)	4.1	2.0
HQC (1000)	4.5	1.7
Inter-day repeatability CV (%)		
LLOQ (100)	6.5	6.6
LQC (200)	4.6	3.1
MQC (600)	6.4	3.7
HQC (1000)	4.2	1.7
Accuracy (%) (SD)		
LLOQ (100)	93.4 (6.6)	101.7 (10.7)
LQC (200)	106.9 (5.7)	93.7 (3.1)
MQC (600)	106.4 (4.4)	99.8 (2.0)
HQC (1000)	103 (4.6)	104.4 (1.8)
Linearity range (ng/mL)	100–1400	100–1400
y-Intercept	0.0195	0.0118
Slope	0.3416	0.3269
R^2	0.998	0.996

3.3.2. Recovery

The efficiency of extraction procedure employed was determined by comparing the peak area of the analyte extracted from replicate QC samples ($n = 6$) by a protein precipitation process with the peak area of analyte from neat standards at equivalent concentrations. Recoveries of doxylamine succinate were determined at low (LQC), medium (MQC) and high (HQC) quality control concentrations i.e., 200, 600 and 1000 ng/mL. Mean recoveries for each enantiomer are mentioned in Table 2. It was found that the recoveries were ranged from 96.4 to 102.4% for (l)-doxylamine and 94.5–103% for (d)-doxylamine enantiomers.

3.3.3. Calibration curve

The six point calibration curve (100, 200, 600, 800, 1000 and 1400 ng/mL) was constructed by plotting the peak area ratio of doxylamine succinate: IS (500 ng/mL) against the nominal concentration of calibration standards in blank rat plasma (Fig. 6). Excellent linearity with a high correlation coefficient (0.995–0.996) was observed.

3.3.4. Precision and accuracy

The intra-day and inter-day precision and accuracy were estimated by analyzing six replicates containing doxylamine succinate at the lower limit of quantification (LLOQ) and three different QC levels, that is, 100, 200, 600 and 1000 ng/mL respectively. The six replicates of each quality control sample were analyzed on the same day and also on three non consecutive days to determine the intra-day and inter-day precision and the accuracy. The results of accuracy and precision are summarized in Table 3. The precisions of (l)- and (d)-doxylamine enantiomers were <7.1 and 10.5%, respectively. The accuracy, expressed as a percentage of the measured concentration to the theoretical concentration was ranged from 93.4 to 106.9% for (l)-enantiomer and 93.7–104.4% for (d)-doxylamine enantiomer.

3.3.5. Stability experiments

Stability tests were conducted to evaluate the stability of doxylamine enantiomers in plasma samples under different conditions at low (200 ng/mL) and high (1000 ng/mL)

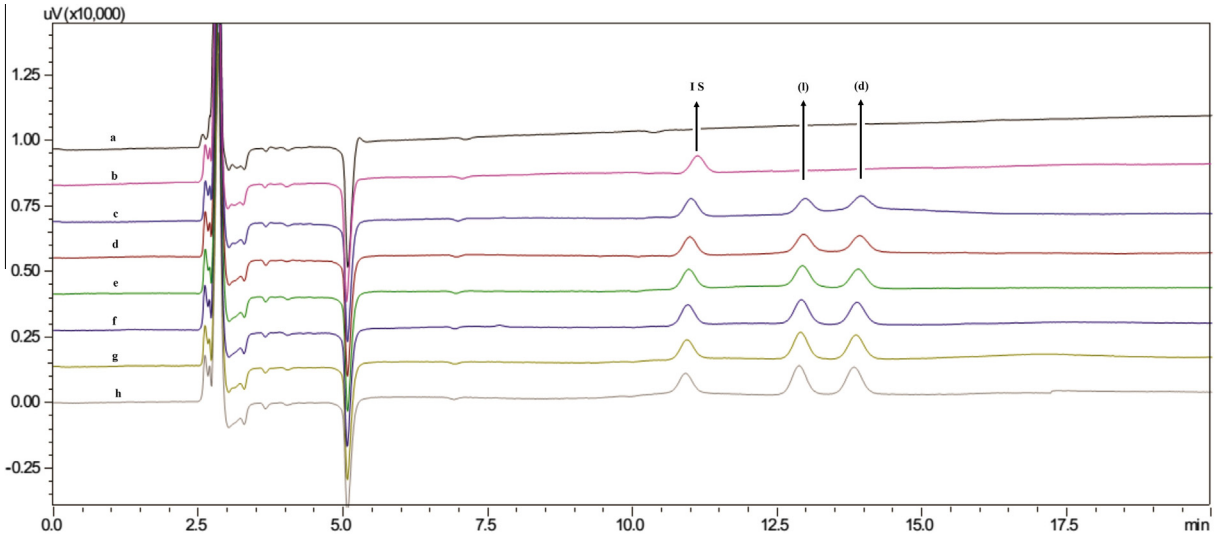

Figure 6 Chromatograms of (a) blank plasma, (b) plasma spiked with only IS, (c) plasma spiked with different concentrations of doxylamine 100 ng/mL, (d) 200 ng/mL, (e) 600 ng/mL, (f) 800 ng/mL, (g) 1000 ng/mL, (h) 1400 ng/mL.

Table 4 Stability of doxylamine enantiomers in plasma (n = 3).

Concentration added (ng/mL)	Concentration found (ng/mL)		Accuracy (%) (SD)	
	(l)-doxylamine	(d)-doxylamine	(l)-doxylamine	(d)-doxylamine
In-injector stability (24 h)				
LQC (200)	202.8	197	101.4 (4.0)	98.5 (3.4)
HQC (1000)	977	987	97.7 (4.8)	98.7 (4.7)
Freeze–thaw stability (three cycles)				
LQC (200)	191.6	193.6	95.8 (5.6)	96.8 (4.3)
HQC (1000)	1016	1025	101.6 (2.8)	102.5 (2.0)
Freezer stability (25 days)				
LQC (200)	190.8	190.2	95.4 (2.4)	95.1 (3.1)
HQC (1000)	976	964	97.6 (1.9)	96.4 (1.9)
Bench top stability (12 h)				
LQC (200)	200.8	206	100.4 (3.6)	103 (2.7)
HQC (1000)	987	1018	98.7 (2.3)	101.8 (0.9)

concentrations. Stock solution stability (6 h) In-injector stability (24 h), bench-top stability (12 h), freeze–thaw stability (three cycles) and freezer stability (−20 ° ± 10 °C for 25 days) were tested. As shown in Table 4, the results for stability of the (d)- and (l)-enantiomers were found to be stable in rat plasma.

4. Discussion

The method resolved the (d) and (l) enantiomers of doxylamine in rat plasma. The concentration of organic modifier and the buffer strength played an important role to achieve better retention and resolution between the enantiomers. The selectivity of the enantiomers on the cellulose column was good and no endogenous peaks from plasma interfered with the enantiomer peaks. Doxylamine enantiomers were extracted efficiently from rat plasma by a simple protein precipitating process using acetonitrile as a protein precipitating agent. The chromatographic parameters like capacity factor (k), separation factor (α) were found to be better than the reported methods. The sensitivity of the method was found to be very low compared with reported methods. The method was developed in the reverse phase mode in which the results will be more reproducible compared with the existing normal phase methods (9, 10). The enantiomers were found to be precise, accurate to within the runs, and between the runs also found to be stable in different stability conditions.

5. Conclusion

In summary, we have developed and validated a sensitive, specific and reproducible UFLC assay to quantitate doxylamine enantiomers in rat plasma. The method involved sample preparation with adequate recovery by simple one-step protein precipitation. This method can be further explored for the quantification of doxylamine enantiomers in clinical drug monitoring and in studying the pharmacokinetics profile.

6. Conflict of interest

The authors confirm that this article content has no conflict of interest.

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