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HPLC separation technique for analysis of bufuralol enantiomers in plasma and pharmaceutical formulations using a vancomycin chiral stationary phase and UV detection

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Abstract

A sensitive and selective high-performance liquid chromatographic (HPLC) method has been developed for the simultaneous determination of bufuralol enantiomers in plasma and pharmaceutical formulations. Enantiomeric resolution was achieved on a vancomycin macrocyclic antibiotic chiral stationary phase (CSP) known as Chirobiotic V with UV detection set at 254 nm. The polar ionic mobile phase (PIM) consisting of methanol–glacial acetic acid–triethylamine (100:0.015:0.010, v/v/v) has been used at a flow rate of 0.5 ml/min. The method is highly specific where other coformulated compounds did not interfere. The stability of bufuralol enantiomers under different degrees of temperature was also studied. The results showed that the drug is stable for at least 7 days at 70 °C. The method was validated for its linearity, accuracy, precision and robustness. An experimental design was used during validation to evaluate method robustness. The calibration curves in plasma were linear over the range of 5–500 ng/ml for each enantiomer with detection limit of 2 ng/ml. The mean relative standard deviation (RSD) of the results of within-day precision and accuracy of the drug were ≤10%. There was no significant difference ($p > 0.05$) between inter- and intra-day studies for each enantiomer which confirmed the reproducibility of the assay method. The mean extraction efficiency for *S*-(−)- and *R*-(+)-bufuralol from plasma was in the range 97–102% at 15–400 ng/ml level for each enantiomer. The overall recoveries of bufuralol enantiomers from pharmaceutical formulations was in the range 99.6–102.2% with %RSD ranging from 1.06 to 1.16%. The assay method proved to be suitable as chiral quality control for bufuralol formulations by HPLC and for therapeutic drug monitoring.

Keywords: Bufuralol enantiomers; Vancomycin chiral stationary phase; Plasma and pharmaceutical formulations

1. Introduction

Drug stereoisomerism is a recognized issue having research, clinical and regulatory implications as differences are observed in the pharmacodynamic and pharmacokinetic properties of the enantiomers of chiral molecules [1–3]. Differences between the biological activity of enantiomers arise because of differences between protein binding and transport, mechanism of action, rate of metabolism, rate of clearance and persistence in the environment [4–6]. Despite this, many compounds such as drugs, agrochemicals and food additives have been marketed as racemic mixture.

On the other hand, the enantioselective character of pharmacokinetic processes leads to different plasma concentration–time profiles for the constituent enantiomers [7]. For this reason, the evaluation of the disposition of a drug employed as a racemic mixture based on data achieved from the non-selective assay is not only seriously limited but can also be highly misleading, particularly when attempting to relate plasma concentration to a pharmacological effect or therapeutic benefit [8]. This is true for both newly developed drugs and agents with long-term use in clinical practice, but now subject to reevaluation in this area. The investigation of enantioselectivity represents a great challenge for clinical pharmacology in terms of the control of individual variability of clinical responses. The clinical relevance of the phenomenon is particularly important for drugs of low therapeutic index administered as racemates.

The beta-blockers comprise a group of drugs that are mostly used to treat cardiovascular disorders. Each of these drugs

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possesses at least one chiral center, and an inherent high degree of enantioselectivity in binding to the β-adrenergic receptors. For beta-blockers with a single chiral center, the (−)-enantiomer possesses much greater affinity for binding to the β-adrenergic receptors than antipode [9]. Except for timolol, all of these drugs are administered clinically as the racemate. As a class, the beta-blockers are quite diverse from a pharmacokinetic perspective, as they display a high range of values in plasma protein binding and percent of drug elimination by metabolism or remain unchanged in urine and in hepatic extraction ratio. With respect to plasma concentration, in most cases the enantiomers of the beta-blockers show only a modest degree of stereoselectivity. However, the relative magnitude of the concentrations of the enantiomers in plasma is not constant in all situations and varies from drug to drug [9]. With respect to bufuralol enantiomers, plasma level and area under plasma curve of *S*-(−)-bufuralol were higher than those of the *R*-(+)-isomer. The elimination half life of *S*-(−)-bufuralol was shorter than that of *R*-(+)-bufuralol. Both isomers were cleared almost entirely by metabolism [10].

A prerequisite for progress in acquiring knowledge of stereospecific pharmacodynamics and pharmacokinetics of optical isomers is the development of an enantioselective analytical methodology.

Separation of enantiomers can be achieved using different chromatographic techniques such as gas chromatography [11], liquid chromatography [12,13], supercritical fluid chromatography [14] and capillary electrochromatography [15]. Among the previous methods, high-performance liquid chromatography (HPLC) is well recognized as a powerful, fast, selective and highly efficient technique, successfully employed for separation and determination of enantiomers of drugs. In this technique, chiral selectors can be used as mobile phase additives or as part of the stationary phase. The most common HPLC approach for resolving enantiomers involves the use of chiral stationary phases (CSPs) [16]. Many various CSPs have been introduced and search for new ones continues [17]. In the past decade, the macrocyclic antibiotic CSPs became a very important tool for separation of a wide range of structurally different chiral compounds [18]. At present, the most useful and popular among them are based on teicoplanin and vancomycin chiral selectors [19]. The enantioselectivity of these chiral selectors due to several reasons: (i) they are amphoteric (i.e. contain acidic and basic ionizable groups); (ii) they have the necessary geometry and functionalities that accentuate chiral recognition in solution; and (iii) they contain both hydrophilic and hydrophobic moieties [20].

A simplified approach has proven very effective for the resolution of a broad spectrum of racemate analytes. The first condsideration in this direction is the structure of the analytes. If the compound has more than one functional group capable of interacting with the stationary phase and at least one of those groups is at or near the stereogenic center, then the first mobile phase choice would be the polar organic phase. Due to the strong polar groups present in the macrocyclic peptides, it was possible to convert the original mobile phase concept to 100% methanol with an acid/base added to enhance selectivity. The key factor in obtaining complete resolution is still the ratio of acid to base [21]. The importance and superiority of macrocyclic antibiotics as chiral selector, in comparison with other chiral selectors, is because they can be used in normal and reversed phases with greater stability and capacity [22].

Bufuralol, 1-(7-ethylbezofuran-2-yl)-2-tert-butylamino-1-hydroxyethane [23], is a potent non-selective β-adrenoseptor antagonist [24] with a β_2 partial agonist properties [25,26] and administered as a racemic mixture. The β-blocking potency of *S*-(−)-bufuralol is approximately 100 times greater than that of the *R*-(+)-enantiomer [27]. A search on the Internet indicates that only one published method for the determination of optically active bufuralol and its metabolites in human plasma by HPLC using Ultron ES-OVM column and UV detection has been developed by Ikegawa et al. [28].

The importance of the present work is the ability of the selected vancomycin macrocyclic antibiotic CSP to separate and determine bufuralol enantiomers in plasma and in pharmaceutical formulations, whereas the complementary chiral selector teicoplanin macrocyclic antibiotic CSP failed to separate these enantiomers. Also, in this study, we used mobile phase (methanol) which is considered less toxic than other solvents used in normal mode. Moreover, the method is linear in the range of 5–500 ng/ml and provides the required sensitivity for monitoring the blood level of bufuralol enantiomers. However, this method should be used as chiral formulations quality control of bufuralol to check the purity of *S*-(−)-isomer, as well as for plasma analysis. With the present broad range of available CSPs and advances in column technology, the present enantioselective HPLC technique can be considered as the method of choice.

2. Experimental

2.1. Materials

(±)-Bufuralol, *S*-(−)-, *R*-(+)-bufuralol and levobunolol were obtained from RBI (Natick, MA, USA). HPLC-grade methanol, analytical-grade triethylamine, glacial acetic acid and chloroform were purchased from BDH Chemicals (Poole, UK). Analytical-grade sodium hydroxide was purchased from WINLAB (UK). Human plasma was obtained from King Khalid University hospital (Riyadh, KSA), and was kept frozen until use after gentle thawing. Deionized water was used throughout the experiments.

2.2. Instrumentation

The HPLC instrument (Jasco, Japan) is equipped with a pump (model PU-980), a UV/VIS detector (model UV-975) and an injection valve with 20 μl sample loop. Signal acquisition and data handling were performed with LG computer connected to the instrument. The CSP used in this study was the macrolide-type antibiotic vancomycin, known as Chirobiotic V (150 mm × 4.6 mm i.d.), purchased from Advanced Separation Technologies (Whippany, NJ, USA). The mobile phase was methanol–glacial acetic acid–triethylamine (100:0.015:0.010, v/v/v). The mobile phase was filtered through a Millipore mem-

brane filter (0.2 μm) from Nihon, Millipore (Yonezawa, Japan), and was degassed before use. The flow rate was 0.5 ml/min and the detection wavelength was set at 254 nm.

2.3. Preparation of stock and standard solutions

Stock solutions containing 1 mg/ml of individual *S*-(−)- and *R*-(+)-bufuralol hydrochloride were prepared in methanol on a free-base basis and corrected for salt and purity. Its purity was found to be 99.8 ± 0.31 by spectrophotometric measurement at 254 nm in 0.1 N HCl. Working standards solution (10 μg/ml) were prepared by dilution of individual aliquot of stock solution with the same solvent. The internal standard levobunolol was prepared in methanol to give a concentration of 0.1 mg/ml and was further diluted with methanol to get the working solution of 40 μg/ml. The solutions were stable for at least 7 days if kept in the refrigerator. Appropriate dilutions of the individual working solutions of bufuralol were made and used for constructing the calibration curves and spiking plasma.

2.4. Preparation of plasma quality control samples

The quality control (QC) samples at three concentration levels, i.e. 15, 200 and 400 ng/ml, were prepared by spiking the drug-free plasma with appropriate volumes of individual *S*-(−)- and *R*-(+)-bufuralol and were stored frozen until analysis. Before spiking, the drug free plasma was tested to make sure that there was no endogenous interference at retention time of *S*-(−)- and *R*-(+)-bufuralol and the internal standard. The QC samples were extracted with the calibration standards and the percentage biological content of the stored QC samples were found in the accepted range (98–99.6%).

2.5. Plasma samples extraction procedure

A human plasma sample (0.5 ml) was placed in 1.5 ml Eppendrof tubes, and accurately measured aliquots of 1.5, 20,and 40 μl of the individual working standard *S*-(−)- and *R*-(+)-bufuralol solutions (10 μg/ml) were added. Then 100 μl of the internal working standard solution (40 μg/ml) was added to each tube and diluted to 900 μl with deionized water and mixed well to give final concentrations of 15, 200 and 400 ng/ml of each bufuralol enantiomer. The mixture was treated with 100 μl of 0.1 M sodium hydroxide and vortexed vigorously for 60 s and then centrifuged at 3000 rpm for 5 min. The aqueous phase was extracted with 3 × 5 ml chloroform and centrifuged at 3000 rpm for 5 min. The organic phase was evaporated to dryness using a Savant speed vac concentrator (Farmingdale, NJ, USA). The residue was recognized in 100 μl methanol, and 20 μl was injected into HPLC system. Blank human plasma samples were processed in the same manner using deionized water instead of bufuralol enantiomers.

2.6. Preparation of tablet solutions

Ten prepared tablets were powdered by use mortar apparatus. An accurately weighed portion equivalent to 10 mg bufuralol was transferred to 100 ml volumetric flask diluted to the mark with methanol. The solution was sonicated for 15 min, centrifuged at 3000 rpm for 10 min. Accurately measured aliquots of the supernatant were transferred to 5-ml volumetric flasks containing 100 μl of the internal standard and diluted to 5 ml with methanol to give final concentration of 125, 250 and 375 ng/ml of bufuralol.

2.7. Stability of standard solution and plasma samples

ICH guidelines [29] and Baskshi and Singh [30] recommended stability studies testing on standard solution of the drug. This means there is no suggestion of conducting stress studies directly on formulations. Therefore, the stability of standard solutions under different temperatures was tested by the proposed HPLC method over a period of 7 days. The freshly prepared solutions at room temperature and the 7-day-stored samples in thermostatic oven at 50 and 70 °C were analyzed by the optimized proposed HPLC method. The concentrations of the stored samples were calculated and compared to that of the freshly prepared samples. Moreover, the stability of bufuralol enantiomers in human plasma at room temperature for 4 h was evaluated using QC samples in triplicates. Three freeze–thaw cycles (−80 °C/room temperature) were applied to QC samples to assess the stability of the analytes. Freezing stability of *S*-(−)- and *R*-(+)-bufuralol in human plasma was assessed by analyzing QC samples stored at −80 °C for 1 month. The in-autosampler (4 °C) stability of bufuralol enantiomers in the reconstitute solvent was evaluated by reinjecting QC samples 48 h after the initial injection. The peak area of *S*-(−)- and *R*-(+)-bufuralol in different QC levels at initial condition was used as reference to determine the relative stability of *S*-(−)- and *R*-(+)-bufuralol in the experiments described above.

2.8. Selectivity

The selectivity of the assay was checked by analyzing six independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analytes. Moreover, the selectivity of the assay was checked by analyzing 10 placebo tablet samples. The chromatograms of these placebo tablets samples were compared with chromatograms obtained by analyzing prepared tablets containing the drug.

2.9. Linearity

Weighted ($1/x^2$) least-squares linear regression with ratio of the area of the analyte to that of internal standard versus concentration was used for calibration. Calibration plots for the *S*-(−)- and *R*-(+)-bufuralol enantiomers in plasma were prepared daily by diluting stock solutions with pooled human plasma to yield seven concentrations level, i.e. 5, 20, 50, 100, 200, 300 and 500 ng/ml for each enantiomer, respectively. Calibration standards at each concentration were extracted and analyzed in six replicates. Calibration curves of bufuralol enantiomers were

constructed using the observed analyte peak area over internal standard peak area versus nominal concentrations of the analytes. Least-squares linear regression analysis of the data gave slope, intercept and correlation coefficient data. From this data, a first order polymonial model was selected for each analyte.

2.10. Precision and accuracy

The within-run and between-run accuracy and precision of the assays in plasma were determined by assaying three QC samples in five replicates over a period of 3 days. The concentrations represented the entire range of the calibration curves. The lowest level was at 3 times the expected limit of quantitation (LOQ) for each enantiomer. The second level was near the mid-point of the calibration curves and the third level was 80% of the upper concentration of the calibration curves. Calibration curves were prepared and analyzed daily and linear models were used to determine concentrations in the QC samples. Precision was reported as %relative standard deviation (%RSD) = (SD/mean) × 100. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Percent accuracy was reported as %error = (measured − nominal)/nominal × 100.

2.11. Limit of detection and lower limit of quantification

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined as 3 and 10 times the baseline noise, respectively, following the United States Pharmacopoeia [31]. The results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear squares treatment of the results along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of the residuals ($S_{y/x}$), were obtained.

2.12. Recovery

The absolute recoveries of each enantiomer from plasma was calculated by comparing drug peak area of the spiked analyte samples to unextracted analyte of stock solution that has been injected directly into an HPLC system. The assay absolute recovery for each compound, at each concentration, was computed using the following equation: absolute recovery = (peak area of extract/mean peak area of direct injection) × 100.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The chemical structures of *S*-(−)-bufuralol, *R*-(+)-bufuralol and levobunolol (IS) are shown in Fig. 1 [23]. In recent years, the direct separation of enantiomers by chiral chromatography has been the target of intense research. Enantioselectivity is usually achieved by the appropriate choice of the chiral stationary phase and the mobile phase composition.

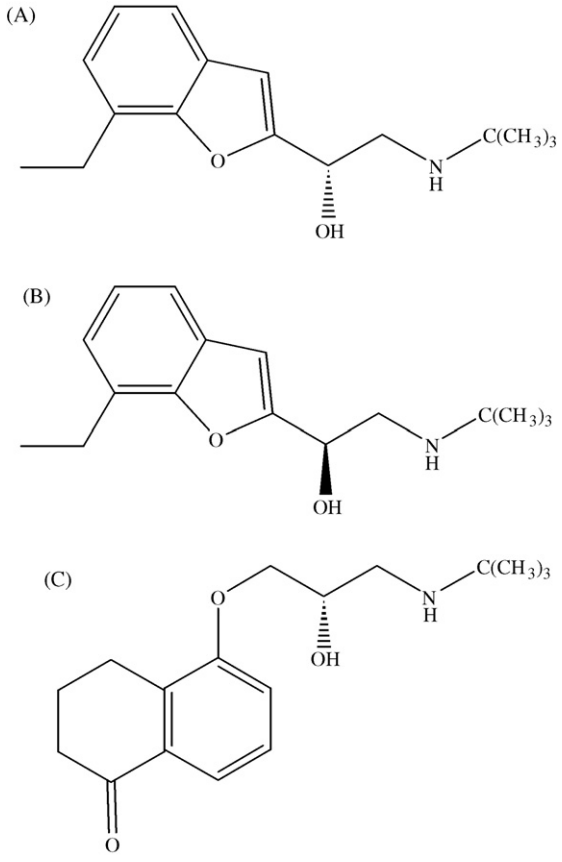

Fig. 1. The chemical structures of (A) *S*-(−)-bufuralol; (B) *R*-(+)-bufuralol; and (C) levobunolol (IS).

Vancomycin chiral stationary phase has been widely used for enantiomeric resolution because it very effectively recognizes the enantiomers of anionic compounds.

The selectivity towards these CSP is because of the presence of amine groups in the chiral selector and, in fact, better recognition is obtained at acidic buffer pH below or close to the isoelectric point of the antibiotic. The polar ionic mode (PIM) has been described as a novel method to obtain difficult enantioselective separation with macrocyclic antibiotic-based chiral stationary phases [32]. This approach uses a non-aqueous polar component (methanol) with glacial acetic acid or trifluoroacetic acid and an amine such as triethylamine which are necessary to achieve enantioseparation. In this study, baseline separation of the bufuralol enantiomers was achieved on the vancomycin CSP with a polar ionic mobile phase consisting of methanol–glacial acetic acid–triethylamine (100:0.015:0.010, v/v/v) (Table 1). No enantiomeric separation were observed in the absence of triethylamine when the mobile phase consisted of methanol–acetic acid (100:0.015, v/v). This could be explained on the basis of strong repulsive effects between the protonated amino groups of the analyte molecules and of the CSP. An increase in the triethylamine concentration in the mobile phase (to about 0.1%) decreases the retention factors of the studied analytes. Increasing the concentration of acetic acid in the mobile phase (to about 0.1%) also decreased the retention factors of the studied analytes. This demonstrates

Table 1
Chromatographic parameter data for bufuralol enantiomers and internal standard

Analyte	R_S[a]	α[b]	K[c]	T_R[c]
S-(−)-bufuralol	1.81	1.17	1.61 ± 0.02	10.56 ± 0.07
R-(+)-bufuralol	5.86	1.65	1.89 ± 0.01	11.71 ± 0.05
Levobunolol	[d]	[d]	3.14 ± 0.02	16.74 ± 0.10

[a] $R_S = (t_2 - t_1)/0.5\ (w_2 + w_1)$. Where t_2 and t_1 are the retention of the second and first peaks and w_{b2} and w_{b1} are the half peak width of the second and first peaks.
[b] Separation factor, calculated as k_2/k_1.
[c] T_R: retention time. Mean ± SD, $n = 3$.
[d] Not calculated.

that it is the concentration of acetic acid and triethylamine in mobile phase that has a substantial influence on the retention factors and not the ionic strength of the mobile phase that was constant.

Aboul-Enein and Ali [33] reviewed the possible bonding between the enantiomers and the macrocyclic glycopeptide antibiotics CSPs. The most important bondings involved are π–π complexation, hydrogen bonding, inclusion complexation, dipole interactions, steric interactions, and anionic and cationic bindings. These bondings are a result of the complex structures of this CSP which consists of sugar moieties, phenyl rings, along with several chiral centers, inclusion baskets, hydrogen donor and acceptor sites. It has been reported that these bonding sites are responsible for the surprising chiral selectivities of these antibiotics [34]. The studied enantiomers of bufuralol (Fig. 1) contain nitrogen and oxygen atoms, along with benzene ring, which interact with the complimentary groups on the chiral selectors (antibiotics). The inclusion baskets and the other functional moieties provide the chiral sites in which the enantiomers fit stereogenically in different fashion which results in the chiral discrimination between the bufuralol enantiomers. Besides, the steric effect also plays an important role for the chiral resolution of the studied drug on this CSP [35].

3.2. Applications to plasma samples

The extraction procedure used in this study afforded percentage recovery range of 93–96% for linearity in the range 5–500 ng/ml for *S*-(−)- and *R*-(+)-bufuralol. The addition of 0.1 M sodium hydroxide was necessary to obtain such recoveries. Otherwise, use of di-sodium tetraborate buffer pH 9 or carbonate buffer pH 10 gave percentage recovery range of 69–80%. The high percent recoveries of bufuralol enantiomers from plasma was due to the pH of plasma matrix after addition of sodium hydroxide which was found 12.5, and which is higher than the pK_a of bufuralol (8.9) [23]. Assuming that the unionized analyte is more predominant than ionized bufuralol when the pH of plasma matrix is adjusted with 0.1 M sodium hydroxide. Fig. 2A and B show chromatograms of a blank plasma sample and a calibration sample, respectively. The retention times for *S*- and *R*-bufuralol were 10.56 ± 0.07 and 11.71 ± 0.05 min, respectively.

3.3. Linearity

The linear regression analysis of *S*-(−)- and *R*-(+)-bufuralol was constructed by plotting the peak area ratio of each enantiomer to the internal standard (y) versus analyte concentration (ng/ml) in spiked plasma samples (x). The calibration curves were linear in the range of 5–500 ng/ml for *S*- and *R*-bufuralol, with correlation coefficient (r) of more than 0.998. A typical calibration curve has the regression equation of $y = 0.0086x + 0.0010$ for *S*-(−)-bufuralol and $y = 0.0075x - 0.0010$ for *R*-(+)-bufuralol.

3.4. Precision and accuracy

A summary of the accuracy and precision results is given in Tables 2 and 3. The acceptance criteria (within-run and between-run %RSD of <15% and an accuracy between 85 and 115%) were met in all cases. The precision and accuracy of the method

Table 2
Accuracy and precision data for bufuralol enantiomers in standard solution

Analyte	Actual concentration (ng/ml)	Experimental concentration (ng/ml)	Accuracy (%)	RSD (%)[b]	Error (%)[c]
S-(−)-bufuralol[a]	5	4.96 ± 0.12	99.2	2.4	−0.8
	20	19.90 ± 0.38	99.5	1.9	−0.5
	50	49.50 ± 0.85	99.0	1.7	−1.0
	100	98.50 ± 1.38	98.5	1.4	−1.5
	200	197.60 ± 3.16	98.8	1.6	−1.2
	300	298.80 ± 6.57	99.6	2.2	−0.4
	500	491.50 ± 12.42	98.3	2.5	−1.7
R-(+)-bufuralol[a]	5	4.96 ± 0.13	99.3	2.6	−0.8
	20	19.92 ± 0.39	99.6	1.9	−0.5
	50	49.45 ± 0.88	98.9	1.8	−1.1
	100	98.70 ± 1.41	98.7	1.4	−1.3
	200	197.40 ± 3.35	98.7	1.7	−1.3
	300	298.50 ± 6.86	99.5	2.3	−0.5
	500	490.50 ± 12.88	98.1	2.6	−1.9

[a] Mean ± SD based on $n = 6$.
[b] Expressed as %RSD: (SD/mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

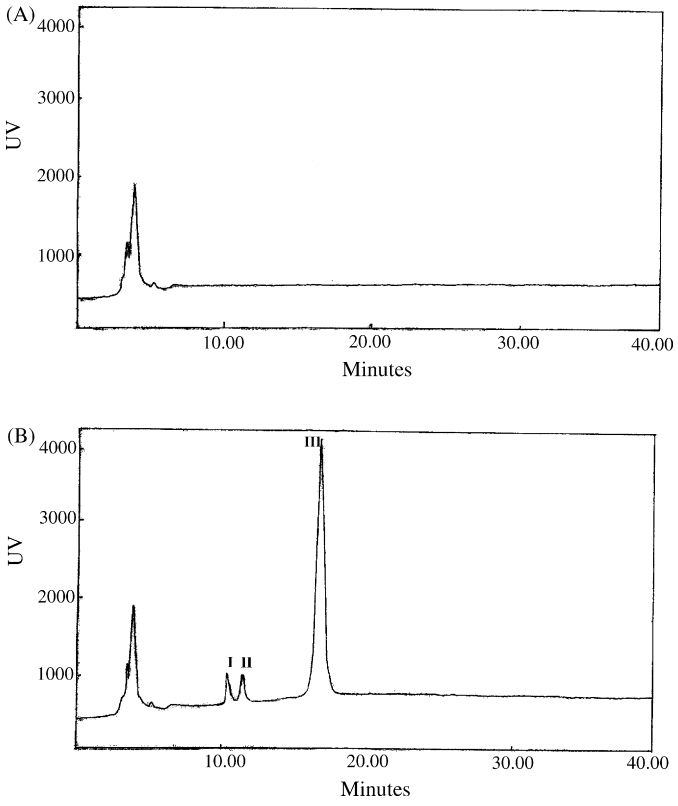

Fig. 2. Chromatograms of (A) blank human plasma and (B) spiked with 15 ng/ml of *S*-(−)-bufuralol (I), *R*-(+)-bufuralol (II) and 4000 ng/ml levobunolol (III).

were determined by using plasma samples spiked at three levels (Table 3). The data indicate that within-run precision and accuracy ($n = 6$) as expressed by %RSD and %error were 2.1–4.4% and 1.6–2.4%, respectively, for *S*-(−)-bufuralol and 2.2–4.2% and 1.7–2.2% for *R*-(+)-bufuralol, respectively. The between-run precision and accuracy ($n = 6$) expressed by %RSD and %error were 2.5–4.9% and 0.8–1.6% for *S*-(−)-bufuralol and 2.6–4.9% and 0.6–1.8% for *R*-(+)-bufuralol, respectively. The detailed analytical data are shown in Table 3.

3.5. Limit of detection and limit of quantitation

The LOD as defined in the experimental section were 2 ng/ml for *S*-(−)- and *R*-(+)-bufuralol. The LOQ of each calibration graph was 5 ng/ml for each enantiomer. The good linearity of the calibration graphs and the negligible scatter of experimental points are evident by the values of the correlation coefficient and standard deviation [36]. The robustness of the method was demonstrated by the versatility of the experimental factors that affect the peak area.

3.6. Selectivity

The analytical figures of merit for this method are shown in Table 1. *S*-(−)- and *R*-(+)-bufuralol enantiomers were well separated under the HPLC conditions applied. Retention times were 10.56 and 11.71 for *S*-(−)- and *R*-(+)-bufuralol enantiomers, respectively. No interference was observed in drug-free human plasma samples (Fig. 2A and B). Otherwise, there is no peaks detected at the retention time of individual bufuralol enantiomer and of internal standard at the level of LLOQ or less. Excipients commonly coformulated with the studied drug such as magnesium stearate, cellulose, starch, calcium hydrogen phosphate, colloidal silicon dioxide and coloring agents also did not interfere with the determination of bufuralol enantiomers, indicating the high selectivity of the method (Fig. 3).

3.7. Application to pharmaceutical formulations

The validity of the method developed here was applied to various concentrations taken from the pharmaceutical formulations for determining their content of bufuralol enantiomers. The values of the overall drug percentage recoveries and the %RSD value of *S*-(−)- and *R*-(+)-bufuralol are presented in Table 4, indicating that these values are acceptable and the method is accurate and precise.

Table 3
Accuracy and precision data for bufuralol enantiomers in spiked human plasma

Analyte	Actual concentration (ng/ml)	Experimental concentration (ng/ml)	Accuracy (%)	RSD (%)[b]	Error (%)[c]
Within-day [a] *S*-(−)-bufuralol	15	14.64 ± 0.65	97.6	4.4	−2.4
	200	196.80 ± 4.18	98.4	2.1	−1.6
	400	406.40 ± 10.65	101.6	2.6	1.6
R-(+)-bufuralol	15	14.67 ± 0.61	97.8	4.2	−2.2
	200	196.40 ± 4.22	98.2	2.2	−1.8
	400	406.80 ± 11.25	101.7	2.7	1.7
Between-day [a] *S*-(−)-bufuralol	15	14.76 ± 0.73	98.4	4.9	−1.6
	200	198.40 ± 5.05	99.2	2.5	−0.80
	400	403.60 ± 12.57	100.9	3.1	0.9
R-(+)-bufuralol	15	14.74 ± 0.72	98.3	4.9	−1.7
	200	198.80 ± 5.21	99.4	2.6	−0.6
	400	407.20 ± 12.65	101.8	3.2	1.8

[a] Mean ± SD based on $n = 6$.
[b] Expressed as %RSD: (SD/mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

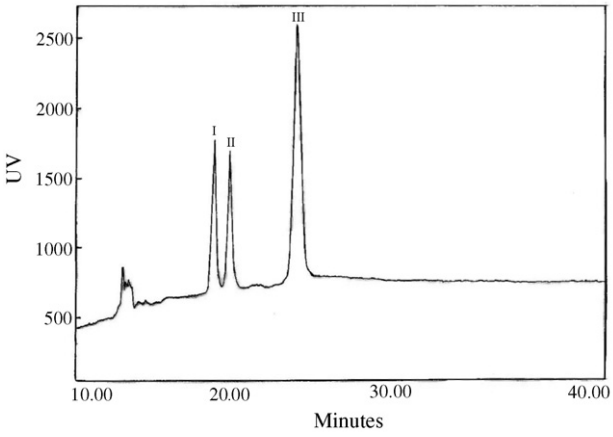

Fig. 3. Chromatogram of 250 ng/ml of *S*-(−)-bufuralol (I), *R*-(+)-bufuralol (II) and 5000 ng/ml levobunolol (III) recovered from bufuralol tablets.

3.8. Stability of standard solutions and plasma samples

The stability of standard solutions under different temperature degrees was tested by the proposed HPLC method over a period of 7 days. The freshly prepared solutions at room temperature and the 7-day-stored samples at 50 and 70 °C were analyzed by the proposed HPLC method. The concentrations of bufuralol enantiomers in the stored samples were calculated and compared to that present in the freshly prepared samples (Fig. 4). From these results, we can conclude that there are no degradation products at elevated temperature and the drug is stable at 70 °C for 7 days, indicating the possibility of using bufuralol samples over a period of 7 days at 70 °C without degradations. The stability study results of plasma samples under various conditions were summarized in Table 5. Bufuralol enantiomers at all QC levels were stable in human plasma for 4 h at ambient temperature, after three freeze-thaw cycles, as well as after storage at −80 °C for 1 month. Bufuralol enantiomers was also stable in the reconstituted solvent for 48 h in the autosampler at 4 °C. The high stable property of *S*-(−)- and *R*-(+)-bufuralol in human plasma suggested that no special care was needed during sample preparation. The high stability of bufuralol enantiomers in reconstituted solvent at 4 °C also suggested that a large batch

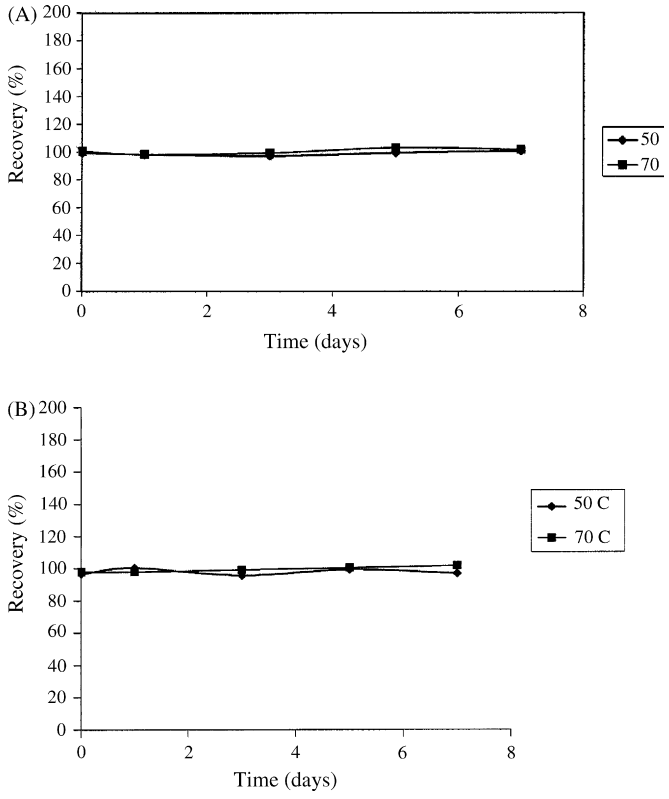

Fig. 4. Unaltered concentration recovery vs. time of (A) *S*-bufuralol and (B) *R*-bufuralol at 50 and 70 °C.

of samples could be processed at one time within 48 h, which would compensate for the shortcoming of relative long analysis time of this assay.

3.9. Robustness

The optimum HPLC conditions set for this method have been slightly modified for samples of bufuralol as a means to evaluate the method robustness. The small changes made include the flow rate, detection wavelength, time (day), temperature and new CSP (Table 5). It was found that the percent recoveries of bufuralol enantiomers were good under most conditions and remain unaffected by small but deliberate changes in experi-

Table 4
Determination of bufuralol enantiomers in tablets

Pharmaceutical preparation	Enantiomer	Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Recovery (%)
Bufuralol tablet[a]	*S*-(−)-	125	127.75	102.2
		250	255.00	102.0
		375	373.99	99.6
Overall recovery (±SD)				101.3 ± 1.18
RSD (%)				1.16
	R-(+)-	125	127.62	102.1
		250	254.50	101.8
		375	373.87	99.7
Overall recovery (±SD)				101.2 ± 1.07
RSD (%)				1.06

[a] Prepared tablets in our lab.

Table 5
Stability of bufuralol enantiomers at various experimental condition

Analyte	QC sample (ng/ml)	Stability condition	%Remaining ± SD
S-(−)-bufuralol	15	4 h at room temperature	96.7 ± 1.4
		3 freeze–thaw cycles	98.4 ± 2.2
		30 days storage at −80 °C	102.2 ± 2.6
		48 h in autosampler at 4 °C	100.3 ± 2.1
	200	4 h at room temperature	98.6 ± 1.2
		3 freeze–thaw cycles	99.4 ± 2.4
		30 days storage at −80 °C	104.2 ± 3.1
		48 h in autosampler at 4 °C	100.5 ± 1.6
	400	4 h at room temperature	97.8 ± 2.2
		3 freeze–thaw cycles	99.2 ± 2.4
		30 days storage at −80 °C	105.6 ± 4.3
		48 h in autosampler at 4 °C	98.8 ± 2.5
R-(+)-bufuralol	15	4 h at room temperature	97.1 ± 1.3
		3 freeze–thaw cycles	98.5 ± 1.9
		30 days storage at −80 °C	102.6 ± 2.4
		48 h in autosampler at 4 °C	99.8 ± 2.3
	200	4 h at room temperature	98.2 ± 1.3
		3 freeze–thaw cycles	99.1 ± 2.1
		30 days storage at −80 °C	103.8 ± 2.9
		48 h in autosampler at 4 °C	100.2 ± 1.7
	400	4 h at room temperature	97.6 ± 2.3
		3 freeze–thaw cycles	98.9 ± 2.6
		30 days storage at −80 °C	104.9 ± 3.3
		48 h in autosampler at 4 °C	99.2 ± 2.1

Table 6
Effect of experimental parameters on the percent recoveries of bufuralol enantiomers

Parameters	Modification	Recovery (%)	
		S-(+)-	*R*-(−)-
Flow rate (ml/min)	0.3	99.9	100.2
	0.5	99.8	100.2
	0.7	100.3	100.4
Wavelength (nm)	250	98.9	99.1
	254	100.3	100.2
	258	98.8	99.0
Temperature[a] (°C)	30	99.8	100.1
	50	99.5	99.6
	70	99.1	98.9
Day[b]	1	100.1	100.0
	2	100.2	100.4
	3	99.8	100.1
CSP[c]	New	100.1	100.1

[a] 7-Day stored solutions at 30, 50 and 70 °C.
[b] Solutions were stored at room temperature.
[c] Product of Advanced Separation Technologies (Whippany, NJ, USA).

mental parameters. Varying in the experimental parameters as well as carrying the experiment at room temperature provided an indication of its reliability during normal use and concluded that the method conditions were robust (Table 6).

4. Conclusion

An enantioselective HPLC method that enables sensitive determination of *S*- and *R*-bufuralol enantiomers in plasma and in pharmaceutical formulations was developed. The method used an efficient liquid–liquid extraction procedure for sample clean-up of plasma. The method is selective where coformulated drug excipients do not interference. With the present broad range of available CSPs and advances in column technology, the present enantioselective HPLC can be considered as the method of choice.

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