Stereoselective HPLC Assay of Acebutolol Enantiomers with Fluorescence Detection and its Application to a Pharmacokinetic Study

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Abstract: Drug enantiomers can have biologically distinct interactions within the biological system and consequently different pharmacological or toxicological effects. Development of a better and safer drug product may be considered if one of the enantiomers has a significantly better effect/side effect ratio than the other. In this context, a stereoselective liquid chromatographic (LC) method was developed and validated to determine S-(-)- and R-(+)-acebutolol in mice plasma. Baseline resolution was achieved by using teicoplanin macrocyclic antibiotic chiral stationary phase (CSP) known as Chirobiotic T with a polar ionic mobile phase (PIM) consisting of methanol - glacial acetic acid - triethylamine, (100:0.025:0.050), (v/v/v) at a flow rate of 0.8 mL min\(^{-1}\) and fluorescence detection set at excitation/emission wavelengths 333/470 nm. The calibration curves in plasma were linear over the range of 10-500 ng mL\(^{-1}\) (r = 0.999) for each enantiomer with detection limit 2 ng mL\(^{-1}\). The proposed method was validated in compliance with ICH guidelines; in terms of linearity, accuracy, precision, limits of detection and quantitation and other aspects of analytical validation.

Key words: Acebutolol · Teicoplanin column liquid chromatography · Fluorescence detection

INTRODUCTION

Chirality remains an important consideration for many compounds including pharmaceuticals, biological molecules and agrochemicals [1, 2]. It has been established that frequently only one of the two enantiomers of a drug is pharmacologically active whereas the other can be inactive or toxic [3, 4]. Differences between the biological activities 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 [5-7]. In the last two decades extensive research has been performed on the resolution of the enantiomers by liquid chromatography (LC) and capillary electrophoresis (CE). A search of the literature indicates that the most interesting research in this area involves the development of new chiral selectors and different types of chiral selectors have been used in chromatography for direct enantiomer resolution [4].

Macrocyclic glycopeptides, such as teicoplanin, represent a recent class of powerful chiral selectors [8]. Their success can be attributed to the diversity of their structures that have multiple stereogenic centers and a variety of functional groups which are known to provide multiple interactions necessary for enantioselectivity [9, 10]. The glycopeptide antibiotic chiral stationary phases (CSPs) have great potential for the resolution of a variety of racemates [11, 12]. Due to the strong polar groups present in the macrocyclic peptides, it was possible to convert the mobile phase to 100% methanol with an acid/base added to effect selectivity. The key factor in obtaining complete resolution is still the ratio of acid to base [12].

Acebutolol \(N\{\text{3-acetyl-4-[2-hydroxy-3-}
\text{(isopropylamino) propoxy]} \text{phenyl] butanamide}\) is a cardioselective \(\alpha\)-blocker with a potent antihypertensive and antiarrhythmic effect. It works by relaxing blood vessels and slowing heart rate to improve blood flow and decrease blood pressure [13]. Acebutolol is a chiral compounds and is clinically administered as a racemic mixture, although it is reported that the S-(-)-acebutolol was about 50-fold more potent than R-(+)-acebutolol as antagonist [14]. The analytical methods reported for chiral separation of acebutolol include capillary electrophoresis techniques by adding various chiral selectors to the back
ground electrolyte [15-21] and LC methods utilizing direct and indirect chiral methods [22-27]. An indirect methods involve derivatization with either S-(+)-naphthylethylisocyanate to form the urea derivative [24], or with (-)-menthol chloroformate [25] and separated by RP-LC. Direct methods used LC chiral stationary phases consisting of amylas tria (3, 5-dimethylethylamide) [26] or cellulose tria (4-methylbenzoxe) CSP [27]. Recently, Jiang et al. [28], reported enatiomeration of acebutolol in spiked human plasma using chellobiobiodextrin CSP-based LC-MS. These methods were suffered from some major drawbacks such as expensive instrument [28] and just enatiomeration of the drug [26, 27]. Furthermore, laborious multiple derivatization steps and the procedure was time-consuming [24, 25]. For these reasons, our laboratory undertook the search for a new, simple, sensitive and direct chiral LC method that can overcome the drawbacks of the existing methods.

EXPERIMENTAL

Instrumentation: The LC system consisted of a Waters binary pump, Model 1525 (Milford, MA, USA), equipped with a fluorescence detector model 2487, an autosampler model 717 plus and an optical rotation detector (Chiralyzer, JM Science Inc., Grand Island, NY, USA) operating at room temperature. The CSP used in this study was the macrolide-type antibiotic teicoplanin, known as Chirobiotic T (250 x 4.6 mm i.d) purchased from Advanced Separation Technologies (Whippany, NJ, USA). The mobile phase was methanol-glacial acetic acid-triethylamine (100: 0.025: 0.050, v/v/v). The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon, Millipore (Yonezawa, Japan) and degassed before used. The flow rate was 0.8 mL min⁻¹ and the detection wavelengths were set at 333 nm for excitation and 470 nm for emission. Collection of data was performed using Empower® Software from Waters.

Chemical and Reagents: (±) Acebutolol hydrochloride, S-(−)-acebutolol and R-(+)-acebutolol were obtained from RBI (Natisk, MA, USA). Timolol (internal standard) was obtained from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade methanol and ethanol and analytical grade triethylamine and glacial acetic acid were purchased from BDH Chemicals (Poole, UK). Deionized water was purified using a cartridge system (PicoTech water system, RTP, NC, USA). Oasis HLB and Sep-Pak C18, C8, C2 and CN cartridges were obtained from Water Corp (Milford, MA, USA). Plasma mice was obtained from Research Center, College of Pharmacy, King Saud University (Riyadh, KSA) and was kept frozen until use.

Preparation of Stock and Standard Solutions: Stock solutions of individual S-(−) - and R-(+)-acebutolol and timolol were prepared in methanol to give a concentration of 1.0 mg mL⁻¹. Appropriate dilutions of the individual acebutolol stock solutions were made to provide 5 μg mL⁻¹ standard solutions which were used for spiking plasma. A five-point non-zero calibration standard curve, ranging from 10-500 ng mL⁻¹ (10, 50, 100, 250 and 500 ng mL⁻¹), was prepared by spiking the drug-free blood with appropriate volume of S-(−) - and R-(+)-acebutolol standard solutions. The quality control (QC) samples, at three concentration levels, i.e. 50, 200, 450 ng mL⁻¹ were prepared in similar manner from the stock solutions. Before the spiking, the drug-free plasma was tested to make sure that there was no endogenous interference at retention times of acebutolol enantiomers and internal standard. The quality control samples were extracted with the calibration standards to verify the integrity of the method.

Animals and Preparation of Actual Blood Sampling: All animal experimentation was conducted in accord with accepted standards of human animal care in accordance with the NIH guidelines and the legal requirements in Kingdom of Saudi Arabia. 30 male Wister mice weighing (25±5g) were used in this study. Mice were randomly divided into six groups (n = 5) for different sampling times. Each group was marked and housed in one large cage. The animals were maintained under standard conditions of humidity, temperature (25±2°C) and light (12h light/12h dark) for 1-2 weeks prior to treatment. The animals were fed with a standard mice pellet diet and had free access to water. Groups of mice were i.p. injected with 40 mg kg⁻¹ of (±)-acebutolol in normal saline.

The experiment including a control group of mice administered only normal saline to provide the blank mice plasma. The injected volume was 0.01 mL g⁻¹ body weight. Blood sampling from treated and control groups were collected from the orbital plexus in eppendorf tubes at 60, 120, 180, 240 and 300 min after injection. Before blood collection animals were light anaesthetized by halothane. The plasma samples were separation from blood by centrifugation and then stored at −20°C until analysis. The samples were extracted by methanol and 20μL volume of each sample was injected into LC system. Each data point is the mean of five replicates and all results were expressed as the mean ±SD of five replicates.
Preparation of Spiked Plasma and Assay Method:

Accurately measured aliquots of the individual standard S(-) and R(+)-acebutolol solutions were pipetted into individual 1.5 mL Eppendorf tube containing 0.5 mL plasma mice. Then 70 μL of the internal standard solution was added to each tube and diluted with acetonitrile to 1 mL and vortex for 5 min. to give final concentrations of 50, 200 and 450 ng mL⁻¹ of each acebutolol enantiomers. Blank plasma mice samples were processed in the same manner using deionized water instead of acebutolol enantiomers. Oasis HLB and Sep-Pak C18, C8, C2 and CN cartridges and different elution solvents consisting of methanol 100%, ethanol 100%, methanol containing 1% triethylamine, methanol containing 1% acetic acid and mobile phase were studied. Cartridges were conditioned with 2 X 1 mL methanol and 2 X 1 mL deionized water before applying the plasma samples. Care was taken that the cartridges did not run dry. Blank and spiked plasma samples were transferred into the cartridges and vacuum was applied to obtain a flow of 0.5 mL min⁻¹. After the entire plasma samples had been aspirated through the cartridges, the cartridges were washed with 2 X 500 μL deionized water. The cartridges were then dried under vacuum for 3 min. All cartridges were eluted with 2 X 500 μL of absolute methanol and 20 μL was injected into the LC system. The absolute recoveries of each enantiomer from plasma were calculated by comparing drug peak area of the spiked analyte samples to unextracted analyte of stock solution that has been injected directly into the LC system. Calibration curves were constructed by diluting stock solutions with pooled mice plasma to yield five concentrations over the range of 10-500 ng mL⁻¹ for each acebutolol enantiomers. Linear regression analysis of normalized drug / internal standard (D/IS) peak area ratio versus concentration gave slope and intercept data for each analyte, which were used to calculate the concentration of each analyte in the mice plasma sample. The within-run and between-run precision (reported as % RSD) and accuracy (reported as % error) of the assay in plasma were determined by assaying three quality control samples in triplicate over a period of 3 days. The concentrations represented the entire range of the calibration curve. The regression equations were used to determine the concentrations in the quality control samples.

Limit of Detection and Limit of Quantitation: The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as 3 and 10 times the baseline noise, respectively. 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 (Sₓ) and intercept (Sᵧ) on the ordinate and the standard deviation of the residuals (Sₑₑ) were shown. 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.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions:

Macro cyclic antibiotic chiral stationary phases have been widely used for enantiomer resolution because they very effectively recognize the enantiomer of ionic compounds. The selectivity towards these compounds is because of the presence of amine groups in the chiral selector [2]. The polar ionic mobile phase (PIM) has been described as a developed method to obtain difficult enantioselective separation with macrocyclic antibiotic-based CSPs [29]. This approach uses a non-aqueous polar component (methanol) with both glacial acetic acid and triethylamine, which are necessary to achieve enantioseparation.

The LC method carried out in this study, aimed at developing a chromatographic system, capable of eluting and resolving acebutolol enantiomers from mice plasma. The preliminary investigations were directed toward the effect of various factors on the system. The factors assessed include the detection wavelength, the type of column and the composition of mobile phase. Acebutolol enantiomers showed two excitation wavelength maxima at 333 and 470 nm. The 470-nm wavelength showed a better sensitivity. The separation of acebutolol enantiomers was first attempted using Chirobiotic V and Chirobiotic TAG columns. However, despite the use of a range of ratios of acetic acid and triethylamine in the mobile phase, complete separation was not achieved on both columns.

In order to improve the resolution of acebutolol enantiomers, Chirobiotic T column was used and several mobile phase compositions were tested. The best results in terms of resolution, analysis time and separation factor were obtained with a mobile phase consisted of methanol - glacial acetic acid - triethylamine (100: 0.025: 0.050, v/v/v) (Table 1). No enantioseparation were observed in the absence of triethylamine. 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 of the triethylamine concentration in the mobile phase (to about 0.1%)
Table 1: Chromatographic parameters for acebutolol enantiomers and the internal standard timolol

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$R^a$</th>
<th>$x^b$</th>
<th>$R^a$</th>
<th>$T_0$ (min)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S(-)-Acebutolol</td>
<td>1.83</td>
<td>1.12</td>
<td>6.62 ± 0.02</td>
<td>35.59 ± 0.03</td>
</tr>
<tr>
<td>R(+)-Acebutolol</td>
<td>*</td>
<td>*</td>
<td>7.46 ± 0.04</td>
<td>37.79 ± 0.05</td>
</tr>
<tr>
<td>Timolol</td>
<td>*</td>
<td>*</td>
<td>12.61 ± 0.01</td>
<td>39.29 ± 0.02</td>
</tr>
</tbody>
</table>

$\text{a} R = 2 (t_2-t_1)/(w_2+w_1)$, where $t_2$ and $t_1$ are the retention of the S(-)-acebutolol and R(+)-acebutolol peaks and $w_2$ and $w_1$ are the half peak width of the S-O-acebutolol and R(+)-acebutolol peaks; $\text{b} \text{Separation factor, calculated as } k/\alpha; \text{c} \text{Retention factor, calculated as } T_0/T_\alpha; \text{d} \text{Retention time}; \text{e} \text{Not calculated}

Table 2: Validation parameters for the determination of acebutolol enantiomers in standard solution using the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S(-)-Acebutolol</th>
<th>R(+)-acebutolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration range (ng mL$^{-1}$)</td>
<td>10-500</td>
<td>10-500</td>
</tr>
<tr>
<td>Intercept ($a$)</td>
<td>-0.007</td>
<td>-0.006</td>
</tr>
<tr>
<td>Slope ($b$)</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Correlation coefficient ($r$)</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>$S_\nu$</td>
<td>0.015</td>
<td>0.014</td>
</tr>
<tr>
<td>$S_\alpha$</td>
<td>0.014</td>
<td>0.013</td>
</tr>
<tr>
<td>$S_b$</td>
<td>0.0002</td>
<td>0.0002</td>
</tr>
<tr>
<td>LOD (ng mL$^{-1}$)$^a$</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>LOQ (ng mL$^{-1}$)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

$\text{a} S/N = 3$

decreased the retention factors of the studied analytes. Increasing the concentration of acetic acid in the mobile phase (to about 0.1%) also decreased the capacity factors of the studied analytes. This demonstrates that it is the concentration of acetic acid and triethylamine in mobile phase that has a substantial influence on the capacity factors and not the ionic strength of the mobile phase that was constant.

The studied enantiomers of acebutolol contain nitrogen and oxygen atoms, along with a benzene ring, which interact with the complimentary groups on the chiral selector. The inclusion baskets and the other functional moieties provide the chiral sites in which the enantiomers fit stereogenically in a different fashion, which results in chiral discrimination between the acebutolol enantiomers. Besides, the steric effect is also playing an important role for the chiral resolution of the studied drug on this CSP.

**Application to Spiked Mice Plasma**: In current method, solid phase extraction (SPE) is increasingly used for sample preparation instead of traditional methods such as liquid-liquid extraction for the advantages of being less time-consuming and more compatible with automation. Five solid phase extraction cartridges (Water oasis HLB, C18, C8, C2 and CN) were investigated for plasma cleanup prior to HPLC assay. C8 cartridge gave the best recoveries for both acebutolol enantiomers. The eluting abilities of various elution solvents towards acebutolol enantiomers were also investigated. Of these solvents, only absolute methanol is able to break all the types of interactions in the case of acebutolol enantiomers and thus to elute them from C8 sorbent. The ability of other solvents to break these interactions is lower, resulting in poor elution of acebutolol enantiomers from the C8 sorbent. The extraction procedure used in this study afforded percentage recovery ranges from 95.15% to 101.25% in the range of 10-500 ng mL$^{-1}$ for both acebutolol enantiomers (Table 2).

**Method Validation**: The linear regression analysis of S(-) and R(+)-acebutolol 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 10-500 ng mL$^{-1}$ for S(-) and R(+)-acebutolol, with correlation coefficient (r) of more than 0.998. A typical calibration curve has the regression equation of $y = 0.008x - 0.007$ for S(-)-acebutolol and $y = -0.006x - 0.006$ for R(+)-acebutolol. A summary of the accuracy and precision results is given in Table 3. The accuracy criteria (within-run and between-run % RSD of < 15% and accuracy between 85 and 115%) were met in all cases. The precision and accuracy of the method 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 percentage RSD and percentage error were 3.4-5.2% and 1.4-4.4%, respectively for S(-)-acebutolol and 3.5-5.4% and 1.8-3.5% for R(+)-acebutolol, respectively.
The between-run precision and accuracy (n=6) expressed by percentage RSD and percentage error were 3.8 - 6.0% and 1.3-3.6% for $S$(-)-acebutolol and 4.0-6.1% and 1.4-3.6% for $R$(-)-acebutolol, respectively. The LOD as defined in the experimental section were 2 ng mL$^{-1}$ for $S$(-)- and $R$(-)-acebutolol. The LOQ of each calibration graph was 10 ng mL$^{-1}$ for each enantiomer. The LOD was calculated using the equations $y - \alpha = 3.3 \times S\alpha$ and $y - \alpha = b \times$ LOD, while the limit of quantitation, LOQ, was attained using the equations $y - \alpha = 10 \times S\alpha$ and $y - \alpha = b \times$ LOQ (where $b$ is the slope and $S\alpha$ is the standard deviation of the intercept of the regression line). In particular, LOD and LOQ were calculated taking under consideration data obtained from the calibration equations [30]. 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. The analytical figures of merit for this method are shown in Table 1. $S$(-) - and $R$(-)-acebutolol enantiomers were well separated under the LC conditions applied. Retention times were 35.59 and 37.79 for $S$(-)- and $R$(-)-acebutolol enantiomers, respectively. No interference was observed in drug free human plasma samples (Figures 1 and 2).

**Pharmacokinetic Studies:** The described method was further applied to a pharmacokinetic study of both acebutolol enantiomers in mice. The concentration of acebutolol in mice serum at different times (1, 2, 3, 4 and 5h) after dosing was determined individually.
Fig. 2: Chromatogram of blank mice plasma spiked with S(-)-acebutolol (35.59 ± 0.03 min), R-(+)-acebutolol (37.79±0.05 min) and timolol (30.29 ± 0.02 min)

Fig. 3: Mean concentration-time profile of S(-)-acebutolol in mice plasma after intraperitoneal (i.p.) administration of 40mg kg$^{-1}$ of acebutolol. Each point represents the mean ± S.D. of the five mice

Fig. 4: Mean concentration – time profile of R-(+)-acebutolol in mice plasma after intraperitoneal (i.p.) administration of 40 mg kg$^{-1}$ of acebutolol. Each point represents the mean±S.D. of the five mice
After the determination, a plasma concentration–time curve (AUC) of acetobutol enantiomers could be drawn, as shown in (Fig. 3 = S(-)-acetobutol) and (Fig. 4 = R(+) - acetobutol). The main pharmacokinetic parameters of acetobutol enantiomers were calculated from the curve. After i.p. administration of 40 mg kg⁻¹ acetobutol, the main value of Tmax and Cmax were 1h and 755.56 ± 37.94 ng ml⁻¹, respectively for S(-)-acetobutol. And the main value of Tmax and Cmax were 1h and 362.44 ± 25.86 ng ml⁻¹, respectively for R(+) - acetobutol. There were no similar peaks observed from samples collected from treated control animals. In this study unknown metabolite has been detected only in the first h of treatment and disappear in the next timing programmed (2, 3, 4 and 5h).

CONCLUSIONS

A sensitive and selective HPLC method has been developed and validated for the analysis of acetobutol enantiomers in mice plasma. The enantiomers were separated with teicoplanin chiral stationary phase. The method used an efficient solid phase extraction procedure for sample clean-up. The developed method allowed quick trace analysis of acetobutol enantiomers in plasma. Finally, the pharmacokinetic study could be performed by using the developed method.

REFERENCES


