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Enantioselective determination of alprenolol in human plasma by liquid chromatography with tandem mass spectrometry using cellobiohydrolase chiral stationary phases

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ABSTRACT

A fast, sensitive, and enantioselective LC–MS/MS bioanalytical method was developed and validated for the direct determination of individual alprenolol enantiomers in human plasma using cellobiohydrolase (CBH) chiral stationary phases (CSP) along with supported liquid extraction (SLE) procedures. Complete baseline separation of enantiomeric alprenolol was achieved within 2 min in reversed phase chromatography at 0.9 ml/min. SLE in a 96-well plate format was used for sample extraction. The method validation was conducted over the curve range of 0.500–500 ng/ml for each alprenolol enantiomer using 0.0500 ml of plasma sample. The intra- and inter-day precision and accuracy of the quality control samples at low, medium, and high concentration levels showed ≤7.3% relative standard deviation (RSD) and −6.2 to 8.0% relative error (RE) for both alprenolol enantiomers.

1. Introduction

Alprenolol (Fig. 1) has been used as a nonselective β-blocking agent with intrinsic sympathomimetic activity and membrane stabilizing properties for the treatment of hypertension, angina pectoris, and arrhythmia [1]. Similar to most β-blockers, alprenolol is marketed as racemate. Generally, the major β-blocking activity can be attributed to the (S)-enantiomer of the aryloxy propanol amine type of β-blockers [2]. Same as many other β-blockers, alprenolol demonstrated significantly different pharmacokinetics for individual enantiomers [3]. Therefore, enantioselective determination of individual alprenolol enantiomers is needed for the analysis of biological samples from clinical studies.

Although various reports [4–7] as to enantioselective separation of alprenolol enantiomers have been published, only a few methods have been applied to the analysis of biological samples [1,3]. The high-performance liquid chromatographic (HPLC) method regarding the separation and quantitation of (*R*)- and (*S*)-alprenolol in human plasma, developed by Bahr [3], required derivatization with a chiral agent and a run time of over 12 min. Another study as to enantioselective quantitation of (*R*)- and (*S*)-alprenolol in human plasma by gas chromatography–mass spectrometry also required chemical derivatization and was only applicable in a very narrow concentration range of 2.50–20.0 ng/ml for each enantiomer [1]. Therefore, currently available bioanalytical methods for the analysis of individual alprenolol enantiomers have limited application owing to the inconvenience such as derivatization, long run time, low sensitivity, and narrow curve range. Nowadays, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has become the method of choice for bioanalysis due to its high sensitivity, specificity, and resolution [8–12]. To the best our knowledge, however, a validated LC–MS/MS based enantioselective bioanalytical method for the direct analysis of individual alprenolol enantiomers is not yet available.

The present study is to develop and validate a fast, sensitive and convenient bioanalytical assay for the direct analysis of individual alprenolol enantiomers in human plasma by LC–MS/MS. Chiral stationary phases-vancomycin [13], α1-acid glycoprotein (AGP) [14,15], and CBH [4–7] have been evaluated for the separation of alprenolol racemate and other β-blockers. High enantioselectivity was generally observed from CBH regarding the chiral separation of β-blockers [5]. Thus, CBH column (CHIRAL-CBH) was chosen to achieve the goals of the present study.

Systematic optimization of chromatographic conditions including mass spectrometer-friendly buffer concentrations, content of organic solvent, and pH in mobile phases was conducted to achieve fast separation of racemic alprenolol on CBH column. As a result, a bioanalytical method was developed for the high through-put

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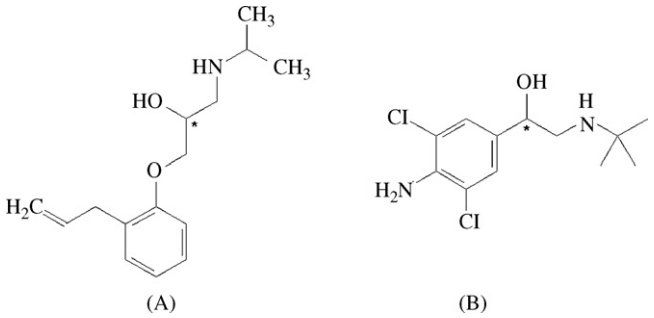

Fig. 1. Chemical structures of alprenolol (A) and clenbuterol (B). *Note*: (*) Indicates chiral center.

analysis of individual alprenolol enantiomers in human plasma. Complete baseline separation of individual alprenolol enantiomers was achieved on CBH column under reversed phase chromatographic condition with a run time of 2 min. Preparation of 96 samples using SLE plate in a 96-well format takes about 30 min, which further supports that this assay can be used for the high through-put sample analysis. Then, the optimized bioanalytical method was validated through the analysis of three separate batches of human plasma samples.

2. Experimental

2.1. Chemical, materials, reagents, and apparatus

Racemic alprenolol hydrochloride (≥98%) and its internal standard (I.S.) clenbuterol hydrochloride (≥95) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Individual alprenolol enantiomers with purity of ≥99% for (*R*)-enantiomer and of ≥95% for (*S*)-enantiomer were prepared in the laboratory using racemic alprenolol under the chromatographic condition used for method validation as described in Section 2.2. The assignment of absolute configuration of individual alprenolol was referred to the work conducted by Pettersson's group [6], which demonstrated that (*S*)-alprenolol showed stronger inhibitory effects and also longer retention time on CBH than (*R*)-alprenolol. Ammonium acetate (HPLC-grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (≥96%), acetic acid (≥99.7%), as well as HPLC-grade acetonitrile and ethyl acetate (≥99.9%) were obtained from Sigma–Aldrich. PURELAB Ultra system from ELGA (Marlow, UK) was used in the laboratory to produce deionized water. Supported liquid extraction (SLE) 96-well plates containing 200 mg support media per well, setting limit of the maximum aqueous load of 200 μl, were obtained from Biotage (Uppsala, Sweden). Human plasma with K2-EDTA as the anticoagulant was obtained from Biochemed (Winchester, VA, USA).

A positive pressure processor (Speedisk®96) used for speeding sample absorption to SLE plate was purchased from J.T. Baker (Phillippsburg, NJ, USA). An automated SPE system (Quadra 96 model 96-320) for adding organic solvents and transferring samples during sample preparation was obtained from Tomtec (Hamden, CT, USA). A 96-well sample concentrator (SPE DRY-96) with temperature control was obtained from Jones Chromatography (Lakewood, CO, USA) for drying samples. A pH meter (model 520A) was purchased from Orion Research (Boston, MA, USA).

2.2. Chromatographic conditions

The HPLC system consisting of solvent delivery system LC-20AD, autosampler SIL-20AC, column oven CTO-20AC, degasser DGU-20A$_3$, UV detector SPD-10Avp, and controller CBM-20A was purchased from Shimadzu (Kyoto, Japan). Chromatographic separation of alprenolol enantiomers was evaluated on CHIRAL-CBH column (100 mm × 3.0 mm, 5 μm) from ChromTech (Cheshire, UK) in reversed phase mode chromatography. The separation of alprenolol enantiomers was optimized by tuning of the LC mobile phase composition. Mobile phase A (MA) consisted of aqueous buffer with various concentration of ammonium acetate and pH, while pure acetonitrile was constantly used as mobile phase B (MB) at all chromatographic conditions for the present study. Detailed description of various chromatographic conditions and their corresponding impacts on the enantioselectivity and retention of alprenolol enantiomers was included in Section 3.2. For method validation, the chromatographic condition was composed of 65% of MA of 10 mM ammonium acetate and 0.02% acetic acid (pH 5.0) in water and 35% of MB of acetonitrile. Complete baseline separation was achieved for alprenolol enantiomers with a run time of 2 min when running at 0.9 ml/min. However, no separation was observed for individual clenbuterol enantiomers under various chromatographic conditions evaluated in present study. The sample injection volume was 8 μl. The column temperature was set as 25 °C.

2.3. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer (MDS-Sciex, Concord, Canada) with turboionspray (TIS) interface was used for LC–MS/MS analyses. Positive ionization mode was used for multiple reactions monitoring (MRM). The mass spectrometric parameters were optimized to maximize the MRM sensitivity by infusing an approximately 10 ng/ml neat solution of alprenolol and clenbuterol enantiomers, respectively, in acetonitrile/water (50/50, v/v) using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA). The optimized instrument parameters for monitoring alprenolol and were as follows: TIS temperature, 650 °C; TIS voltage, 1500 V; curtain gas (CUR), 40; nebulizing gas (GS1), 70; TIS gas (GS2), 60; collision gas, 6; declustering potential (DP), 50 V; entrance potential (EP), 10 V; collision energy (CE), 25 eV; collision cell exit potential (CXP), 18 V. Most optimized instrument parameters for clenbuterol were same as those parameters for alprenolol except for some parameter including DP, 40 V; CE, 42 eV; CXP, 10 V. The following precursor to product ion transitions were used for the MRM of alprenolol and clenbuterol, respectively, at *m*/*z* 250.4 → 98.1 and at *m*/*z* 277.2 → 168.0 with dwell time of 200 ms. The mass spectrometer was operated at unit mass resolution for both Q1 and Q3 quadrupoles.

2.4. Preparation of standard solutions

Primary stock solutions of alprenolol racemate at 0.100 mg/ml in acetonitrile/water (1/1, v/v) were prepared from separate weighing for calibration standards and quality control (QC) samples. The stock solutions were stored in glass vials and kept refrigerated (2–8 °C). Intermediate standard solutions at the desired concentration for the calibration curve and QC samples were made by serial dilution with acetonitrile/water (1/1, v/v) starting with their respective concentrated stock solutions. In addition, standard solutions of individual alprenolol enantiomers at 10.0 ng/ml were obtained by dissolving and diluting the respective single enantiomers. The intermediate internal standard solution at 80.0 ng/ml was diluted from the stock internal standard solution of clenbuterol racemate at 0.100 mg/ml with acetonitrile/water (1/1, v/v). These standard solutions of individual alprenolol enantiomers and internal standard solutions were also stored in glass vials and kept refrigerated (2–8 °C).

2.5. Preparation of calibration standards and quality control samples

Fresh calibration standards were prepared daily by spiking appropriate amount of the intermediate standard solutions into pooled human plasma. Eight calibration standards of individual alprenolol enantiomers were prepared at 0.500, 1.00, 2.50, 10.0, 50.0, 250, 450, and 500 ng/ml. Quality control samples were prepared by spiking an appropriate amount of intermediate standard solutions into human plasma to reach the desired concentration with non-matrix composition less than 5% of the final volume. Lower limit of quantitation QC (LLOQ), low QC (LQC), medium QC (MQC), high QC (HQC), and dilution QC (DQC) were prepared at 0.500, 1.50, 20.0, 400, and 2500 ng/ml for each alprenolol enantiomer. In addition, QC samples of individual alprenolol enantiomers were prepared at 0.500 ng/ml (LLOQ) for the investigation of possible conversion between these two individual alprenolol enantiomers under sample preparation and storage conditions. All QC samples were aliquoted into 1.4 ml polypropylene vials and stored at −20 °C.

2.6. Sample preparation

A volume of 50.0 μl of each calibration standard, QC sample, zero sample, and blank matrix control sample were aliquoted into individual wells in a 96-well plate. Next, 25.0 μl of the intermediate internal standard solution of clenbuterol racemate at 100 ng/ml were added to each sample with the exception of the blank control samples, to which 25.0 μl of acetonitrile/water (1/1, v/v) were added. Then, 150 μl of deionized water were then added to each sample. Thereafter, 200 μl of each sample (225 μl) in the above 96-well plate were transferred to individual wells of a 2 ml 96-well SLE plate using an automated SPE system. Minimum positive pressure was applied to facilitate the sample absorption into the cartridge within 10 s using a positive pressure processor. Then, 800 μl of ethyl acetate were added to each well of the SLE plate to elute the compounds. A minimum positive pressure can be used to speed the elution. The complete elution of 800 μl of ethyl acetate takes approximately 5 min. The eluate was evaporated to dryness using a 96-well sample concentrator (SPE DRY-96) set at 50 °C for about 5 min. Then the samples were reconstituted in 200 μl of acetonitrile/water (1/1, v/v) for LC–MS/MS analysis.

2.7. Data analysis

The chromatographic peaks of alprenolol and clenbuterol enantiomers were integrated using Sciex Analyst software (version 1.4.1). The calibration curves (analyte peak area/IS peak area for *Y*-axis and analyte concentration/IS concentration for *X*-axis) of individual alprenolol enantiomers were obtained based upon the least square linear regression fit ($y = mx + b$) and a weighting factor of $1/x^2$. The single chromatographic peak for clenbuterol racemate (no separation as described in Section 2.2) was used as internal standard for the quantitation of individual alprenolol enantiomers. The coefficient of determination (r^2) was set as >0.98 for acceptance criteria of calibration curves. The accuracy and precision were required to be within 100 ± 15% of the nominal concentration and ≤15% RSD, respectively, for LQC, MQC, HQC, and DQC samples while the accuracy and precision were required to be within 100 ± 20% of the nominal concentration and ≤20% RSD, respectively, for LLOQ samples in the intra- and inter-batch assay.

2.8. Method validation

The current LC–MS/MS assay was conducted in the range of 0.500–500 ng/ml for each alprenolol enantiomer. The assay was validated for matrix effect, specificity, carryover, sensitivity, linearity, precision, accuracy, dilution integrity, and stability. The matrix effect was determined by comparing the absolute peak areas of analytes and internal standards in six lots of blank plasma extract fortified with analytes at MQC level with those in blank water extract fortified with analytes at the same concentration level. The specificity was assessed by screening six lots of blank plasma for the presence or absence of interference as well as the lot-to-lot variation. Carryover was determined by the blank plasma extract run immediately after an upper limit of quantification (ULOQ) sample. The signal to noise ratio of LLOQ samples was used to assess the sensitivity. The linearity of the calibration curve was evaluated as described in Section 2.7.

Three separate batches of human plasma samples were analyzed to assess the precision and accuracy of the method. Each batch consisted of one set of calibration standards of eight levels and six replicates of QC samples at each of LLOQ, LQC, MQC, HQC, and DQC levels. Dilution integrity was assessed by 10-fold dilution of the DQC sample with blank plasma prior to extraction. The short-term stability was evaluated in one of the three validation batches, in which the LQC, MQC, and HQC samples were subjected to three freeze–thaw cycles (freeze–thaw stability) or sitting on lab-bench at room temperature (~22 °C) for approximately 24 h (room temperature stability) prior to extraction. To determine the storage and re-injection reproducibility of the processed samples, one of the three batches of extracted samples was stored in the autosampler for approximately 48 h before re-injection for LC–MS/MS analysis. The long-term stability was assessed in an additional batch, in which LQC, MQC, and HQC samples were stored at approximately −20 °C for 2-month while all calibration standards were freshly prepared. The investigation of the possible mutual conversion between individual alprenolol enantiomers in human plasma was conducted at the LLOQ level under the same storage conditions including freeze–thaw, room temperature, and re-injection stability as those of regular QCs.

3. Results and discussion

3.1. Sample preparation

Supported liquid extraction (SLE) serves as an alternative to traditional liquid–liquid extraction (LLE) for providing cleaner samples, while it is easy to automate and reduces other liquid handling issues [16,17]. An automated SLE extraction procedure (as described in Section 2.6) was developed for samples clean-up. The total sample preparation time takes approximately 30 min. Especially, the sample preparation was conducted in a 96-well plate format with automation; thus, it can be adapted to high through-put analysis of biological samples.

The recovery of extraction for alprenolol was obtained by comparing the absolute peak areas of the analytes extracted from QC samples at the low, medium, and high level prepared in plasma with those of blank plasma post-extraction fortified with neat analytes solution at the same concentration as LQC, MQC, and HQC. The average recovery of six replicates of QCs at three levels was 84.3% for individual alprenolol enantiomers. Similarly, the average recovery of 90.8% for clenbuterol as an internal standard was obtained at the intermediate standard solution level (80.0 ng/ml). Although different recovery was obtained for alprenolol and clenbuterol, recoveries for alprenolol at different concentration levels including LQC, MQC and HQC are very consistent (≤4.1% RSD). The recovery for clenbuterol is also consistent (≤3.8% RSD). Therefore, minimal/no impact on the quantitation of alprenolol could be resulted from the different recovery of alprenolol and clenbuterol, which are supported by the data described in the following sections.

3.2. Chromatographic separation

CBH, widely used as a chiral selector, especially for the enantiomeric separation of β-blockers, is the quantitatively dominating cellulose-degrading enzymes (cellulases) produced by fungus *Trichoderma reesei* [4,18]. The major chiral recognition mechanism of CBH column has been attributed to the electrostatic and hydrophobic interactions between CBH and the chiral analytes [6,19]. Major factors such as pH, buffer concentrations, content of organic solvent in the mobile phases, controlling the retention and enantioselectivity of analyte enantiomers on the CBH column, have been studied and reviewed [5–7,20].

The chiral recognition mechanisms of alprenolol on CBH have been studied [5,6]. However, systematic optimization of chromatographic conditions, especially using mass spectrometer-friendly buffers or solvents in mobile phases, for fast enantioselective analysis of individual alprenolol enantiomers has not been investigated. The present study is used as an example on developing chiral assays on CBH column with sufficient resolution (complete baseline separation) and acceptable retention time (short run time) by adjustment of factors including buffer concentrations, content of organic solvent, and pH in mobile phases. Capacity factor ($k' = (t_R - t_m)/t_m$), selectivity factor ($\alpha = k'_2/k'_1$), and resolution ($Rs = 2(t_R, 2 - t_R, 1)/(w_2 + w_1)$) were used to evaluate the retention, enantioselectivity, and separation power, respectively, of individual alprenolol enantiomers under various chromatographic conditions. The inflection point from UV detection was used to determine t_m. Parameters including k'_2, $t_{R,2}$, and w_2 were used to describe the more retained enantiomer (*S*)-alprenolol.

To assess the chromatographic behaviors of individual alprenolol enantiomers under various buffer concentrations in mobile phases, 5, 10, and 20 mM ammonium acetate in water was used as MA, respectively, and 35% acetonitrile was used as MB. With the increase of concentration of ammonium acetate, the retention and resolution of (*R*)- and (S)-alprenolol were reduced while enantioselectivity did not change as shown in Table 1. This result is different from previous study on separation of β-blocker propranolol, which indicated that higher concentrations of buffer ions (phosphate buffer) improved the enantioselectivity and decreased the analysis time [5].

Different from previous observation that enantioselectivity and resolution were improved with higher organic solvent in the mobile phase [5,20], the capacity factors, enantioselectivity, and resolution of individual alprenolol enantiomers were decreased with increasing the acetonitrile as organic modifier from 10 to 35% in mobile phases (Table 1), which might be resulted from competition for hydrogen binding or hydrophobic interaction between CBH and analytes. It is very obvious that the capacity factor of the (*S*)-alprenolol decreases more rapidly than that of (*R*)-alprenolol. In addition, CBH has the possibility to be used under high concentrations of organic modifiers in the mobile phases without adverse effects on the chiral discrimination properties of the cellulase phase [20]. Based on our experiences with using CBH column, however, its discrimination power can be permanently affected or damaged when exposed to ≥50% acetonitrile in mobile phase for a long period of time due to the possible denaturation of CBH (data not shown). Therefore, the increase of organic modifier in mobile phases with caution can be used to shorten the analysis.

The retention of alprenolol enantiomers was decreased with reducing the pH of MA from 6.5 to 5.0 as shown in Table 1. In contrast, the enantioselectivity and resolution did not follow the same trend with the decrease of the pH, supporting that electrostatic interaction is not the only key point controlling the enantioselectivity.

As indicated above, increasing the buffer concentration or organic content in mobile phases or lowering the pH of mobile phase can be applied to shorten the analysis time for the determination of individual alprenolol enantiomers if complete baseline separation is achievable. The selection of chromatographic conditions (shown in Section 2.2) for method validation was a result of compromising the short run time and good resolution (complete baseline separation). Representative chromatograms for the analysis of individual alprenolol enantiomers at ULOQ and LLOQ levels under chromatographic conditions for validation were shown in Figs. 2A1 and 3, respectively. However, clenbuterol enantiomers, also a β-blocker with a chemical structure different from alprenolol at both side chain and aromatic ring (Fig. 1), have not been separated at chromatographic conditions evaluated for the separation of alprenolol enantiomers. It was still used as internal standard due to its similar retention with alprenolol enantiomers at chromatographic conditions for method validation.

Table 1
Chromatographic separation of individual alprenolol enantiomers under various mobile phase conditions

	k'_1	k'_2	α	Rs
Condition A1	0.74	1.20	1.62	2.25
Condition A2	0.64	1.04	1.62	2.12
Condition A3	0.52	0.84	1.62	1.79
Condition B1	1.16	4.58	3.95	6.94
Condition B2	0.80	1.94	2.43	3.73
Condition B3	0.64	1.04	1.62	2.12
Condition C1	2.32	5.72	2.47	6.99
Condition C2	1.80	3.68	2.04	5.19
Condition C3	0.94	2.52	2.68	5.47
Condition C4	0.64	1.04	1.62	2.12

Note: Mobile phase conditions of A1–A3 consist of acetonitrile as MB (35%) and 5, 10, and 20 mM NH_4OAC in water with pH 5 as MA (65%), respectively; B1–B3 consist of acetonitrile as MB (10%, 20%, and 35%) and 10 mM NH_4OAC in water with pH 5 as MA (90%, 80%, and 65%), respectively; C1–C4 consist of acetonitrile as MB (35%) and 10 mM NH_4OAC in water with pH 6.5, 5.9, 5.6, and 5.0 as MA (65%), respectively. In addition, condition A2 = B3 = C4.

3.3. Matrix effect, specificity, and carryover

Six lots of human plasma were used to evaluate matrix effects. The average absolute peak areas of alprenolol enantiomers and clenbuterol (Table 2), in six lots of blank plasma extracts spiked post-extraction with these enantiomers at MQC level, and in three blank water extracts spiked post-extraction with these enantiomers at the same concentration level, were very close (≤5.1% difference). Matrix enhancement was observed at similar extent for both analytes and internal standards. Therefore, the matrix effect on the quantitation of alprenolol was minimal. In addition, lot-to-lot variation in matrix effects from six lots of blank plasma extracts fortified with alprenolol at MQC level was also negligible (RSD% ≤ 3.9).

The assay selectivity was also evaluated using six lots of blank rat plasma. Under the current LC–MS/MS conditions, no interference peaks were observed for both alprenolol enantiomers and their internal standards from any of these six lots of blank plasma sample. In addition, contribution between alprenolol enantiomers and their internal standards to each other was not observed as shown in Fig. 2.

To evaluate carryover, blank plasma extract was analyzed immediately after the ULOQ sample. No peaks were observed in the chromatogram of blank matrix sample analyzed immediately after ULOQ (data not shown). As a result, carryover from previous concentrated samples of alprenolol enantiomers should be negligible.

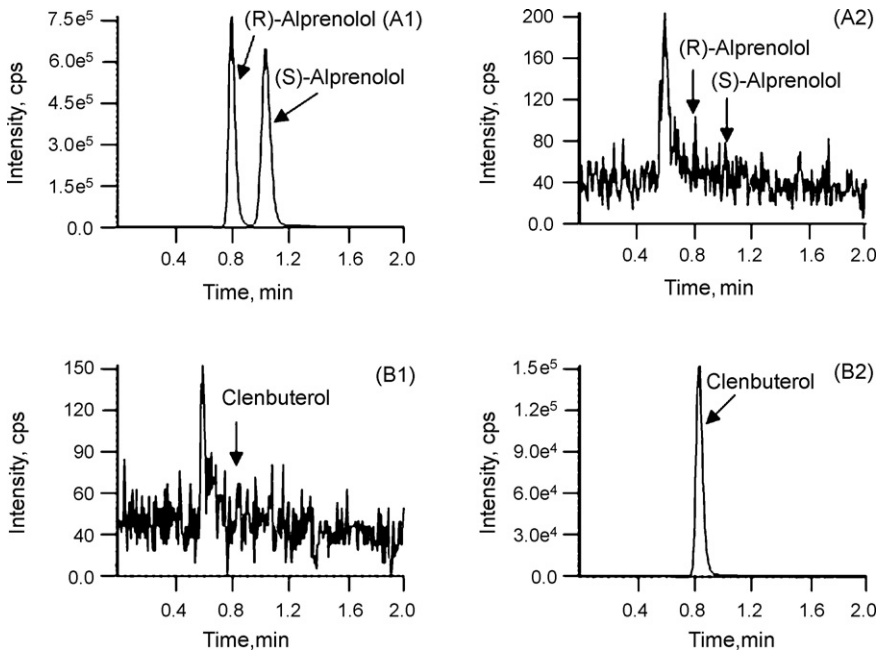

Fig. 2. Representative chromatograms of extracted human plasma samples spiked with alprenolol enantiomers at 500 ng/ml (ULOQ) and no internal standards (clenbuterol) by monitoring alprenolol enantiomers at *m/z* 250.4 → 98.1 (A1) and clenbuterol at *m/z* 277.2 → 168.0 (B1). Representative chromatograms of extracted human plasma sample spiked with clenbuterol enantiomers at 80.0 ng/ml (drug free) by monitoring alprenolol enantiomers at *m/z* 250.4 → 98.1 (A2) and clenbuterol at *m/z* 277.2 → 168.0 (B2). Arrows indicate approximate retention times of these individual enantiomers.

3.4. Sensitivity and linearity

To evaluate the assay sensitivity, LLOQ samples ($n = 6$) were analyzed in each of three validation batches. A signal-to-noise ratio (S/N) of approximately 20 and 16 for (*R*)- and (*S*)-alprenolol, respectively, was obtained at the LLOQ of 0.500 ng/ml level (shown in Fig. 3). Acceptable precision (≤6.5% RSD) and accuracy (−4.0 to 2.0% RE) for (*R*)-alprenolol were obtained (Table 3). Similarly, acceptable precision (≤7.3% RSD) and accuracy (−5.8 to 2.0% RE%) were obtained for (*S*)-alprenolol.

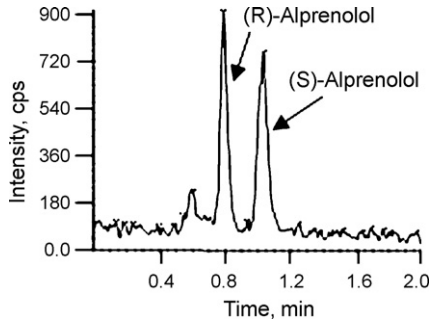

Fig. 3. The chromatogram of extracted human plasma sample spiked with alprenolol enantiomers at 0.500 ng/ml (LLOQ).

The linearity was evaluated based on the average of eight calibrators analyzed in three separate batches. Acceptable linearity was achieved in the range of 0.500–500 ng/ml for each alprenolol enantiomer. For (*R*)-alprenolol, the slope was 0.0141 with an intercept of 0.000418. A slope of 0.0160 with an intercept of −0.000105 was determined for (*S*)-alprenolol. The coefficients of determination (r^2) for both enantiomers were greater than 0.998 in all validation batches.

3.5. Precision and accuracy

As summarized in Table 4, the back-calculation data of all calibration standards exhibited ≤6.1% RSD and −6.7 to 4.0% RE for (*R*)-alprenolol and showed ≤8.8% RSD and −3.2 to 4.0% RE for (*S*)-alprenolol in all three validation batches. The precision and accuracy of the method were determined by analyzing six replicates of QC samples at low (1.50 ng/ml, LQC), medium (20.0 ng/ml, MQC), and high levels (400 ng/ml, HQC) in three separate batches for each alprenolol enantiomer. For (*R*)-alprenolol, the precision

Table 2
Results of the matrix effect

	(*R*)-Alprenolol		(*S*)-Alprenolol		Clenbutolol (I.S.)	
	Samples[a]	Samples[b]	Samples[a]	Samples[b]	Samples[a]	Samples[b]
N	6	3	6	3	6	3
Mean (peak area)	110103.6	104745.2	132395.9	131593.9	264660.4	255802.4
SD	4316.3	979.2	1829.0	1261.5	7980.6	4584.4
RSD (%)	3.9	0.9	1.4	1.0	3.0	1.8
Matrix effect (%)	5.1		0.6		3.5	

[a] Samples prepared by post-extraction spiking with pure standard solutions of alprenolol racemate and clenbuterol. Samples were spiked at the MQC (20.0 ng/ml) level in extracted blank human plasma.

[b] Samples prepared by post-extraction spiking with pure standards solutions of alprenolol racemate and clenbuterol. Samples were spiked at the MQC (20.0 ng/ml) level in extracted blank water.

Table 3
Precision and accuracy of quality control samples of enantiomeric alprenolol pairs

	LLOQ (0.500 ng/ml)		LQC (1.50 ng/ml)		MQC (20.0 ng/ml)		HQC (400 ng/ml)		DQC[a] (2500 ng/ml)	
	Alprenolol		Alprenolol		Alprenolol		Alprenolol		Alprenolol	
	(*R*)-isomer	(*S*)-isomer	(*R*)-isomer	(*S*)-isomer	(*R*)-isomer	(*S*)-isomer	(*R*)-isomer	(*S*)-isomer	(*R*)-isomer	(*S*)-isomer
Inter-Batch										
N	18	18	18	18	18	18	18	18	18	18
Mean	0.500	0.480	1.50	1.5	21.0	20.0	385	386	2442	2461
RSD (%)	5.4	5.2	4.0	4.7	2.9	2.5	2.6	2.0	3.2	3.6
RE (%)	0.0	−4.0	0.0	0.0	5.0	0.0	−3.7	−3.5	−2.3	−1.6

[a] A 10-fold dilution of the DQC with blank matrix prior to extraction was applied.

Table 4
Accuracy and precision of calibration standards for enantiomeric alprenolol pairs

	0.500 (ng/ml)	1.00 (ng/ml)	2.50 (ng/ml)	10.0 (ng/ml)	50.0 (ng/ml)	250 (ng/ml)	450 (ng/ml)	500 (ng/ml)
(*R*)-Alprenolol								
N	3	3	3	3	3	3	3	3
Mean	0.500	1.00	2.59	10.4	52.0	254	429	467
RSD (%)	1.6	3.2	0.9	1.1	6.1	0.5	6.0	2.1
RE (%)	0.0	0.0	3.6	4.0	4.0	1.6	−4.7	−6.7
(*S*)-Alprenolol								
N	3	3	3	3	3	3	3	3
Mean	0.497	1.01	2.42	10.4	50.0	257	443	490
RSD (%)	1.6	2.3	0.9	2.4	7.2	0.8	8.8	4.1
RE (%)	−0.6	1.0	−3.2	4.0	0.0	2.8	−1.6	−2.0

was 0.5–4.0% RSD and the accuracy was in the range of −6.2 to 8.0% RE over the three concentration levels evaluated in all three batches. Similarly, the precision and accuracy for (*S*)-alprenolol were also evaluated at the LQC, MQC, and HQC levels with 1.2–4.7% RSD and −4.0 to 4.0% RE, respectively, over these batches. The inter-batch precision and accuracy of QC samples at different levels were shown in Table 3. These results suggested that excellent precision and accuracy can be achieved for this newly developed assay in the concentration range of 0.500–500 ng/ml under current experimental conditions.

3.6. Dilution integrity

To assess sample dilution integrity of alprenolol enantiomers, the dilution QC samples (DQCs) were subjected a 10-fold dilution with blank matrix prior to extraction in all three batches. As shown in Table 3, the results demonstrated that samples with concentration greater than the upper limit of the standard curve could be quantified with reliable precision and accuracy after being appropriately diluted with blank matrix.

3.7. Stability

In order to evaluate the short-term stability of incurred samples pre- and post-processing, the experiments were designed and conducted under different conditions that incurred plasma samples may encounter during sample shipment such as several freeze–thaw cycles and short-term storage at room temperature. For extracted samples, conditions such as sitting in the autoinjector or refrigerator were assessed as described in Section 2.8. All stability data were summarized in Table 5. After three freeze–thaw cycles, the QC samples showed ≤5.7% RSD in precision and −3.0 to 1.0% RE in accuracy for both alprenolol enantiomers. For the QC samples stored at room temperature for an approximately 24 h, the precision (≤2.4% RSD) and accuracy (−3.0 to 2.0% RE) were obtained. One of validation batches was stored in the HPLC auto-

Table 5
Freeze–thaw stability, room temperature stability, re-injection reproducibility, and 2-month stability of enantiomeric alprenolol pairs

	LQC (1.50 ng/ml)		MQC (20.0 ng/ml)		HQC (400 ng/ml)	
	(*R*)-Alprenolol	(*S*)-Alprenolol	(*R*)-Alprenolol	(*S*)-Alprenolol	(*R*)-Alprenolol	(*S*)-Alprenolol
Stability after three freeze–thaw cycles, *N* = 6						
Mean	1.52	1.50	19.6	19.6	396	388
RSD (%)	5.7	3.5	2.1	4.8	1.1	1.3
RE (%)	1.0	0.0	−2.0	−2.0	−1.0	−3.0
Room temperature stability ~24 h, *N* = 6						
Mean	1.50	1.48	20.3	19.4	391	396
RSD (%)	2.4	1.0	0.9	1.5	2.4	1.5
RE (%)	0.0	−1.3	2.0	−3.0	−2.2	−1.0
Re-injection reproducibility ~48 h, *N* = 6						
Mean	1.55	1.52	20.6	20.6	395	400
RSD (%)	1.5	1.5	1.0	1.1	1.4	1.7
RE (%)	3.3	1.3	3.0	3.0	−1.2	0.1
Two-month QC samples stored at −20 °C, *N* = 6						
Mean	1.66	1.59	22.2	22.6	432	448
RSD (%)	9.6	7.6	2.4	1.7	3.3	3.5
RE (%)	10.7	6.0	11.0	13.0	8.0	12.0

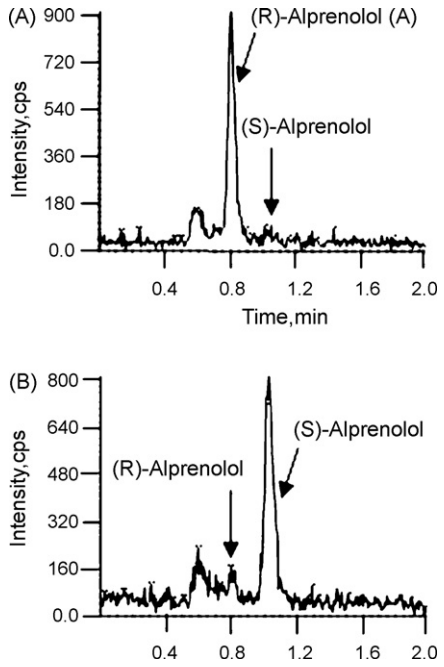

Fig. 4. The chromatograms of extracted human plasma sample spiked with individual alprenolol enantiomers at 0.500 ng/ml (LLOQ).

injector and refrigerator (2–8 °C) for approximately 48 h prior to its re-injection for quantitation. The precision was ≤1.7% RSD and the accuracy was in the range of −1.2 to 3.3% RE for all three levels of QC samples. Through the evaluation the short-term stability of QC samples, short-term storage of plasma samples or extracted samples was therefore acceptable to obtain reliable data.

The 2-month storage of QC samples has been tested to evaluate the long-term stability of incurred samples and the data were included in Table 5. The results showed the precision (≤9.6 RSD) and accuracy (6.0–13.0% RE) for all three levels of QC samples, indicating that plasma samples were stable for at least 2 months if stored at approximately −20 °C.

3.8. Conversion between two alprenolol enantiomers

Stability experiments same as in Section 3.7 were conducted to investigate the possible conversion between two enantiomers under sample storage conditions using samples of individual alprenolol enantiomers. After stored at room temperature for an approximately 24 h, no conversion between two enantiomers was observed from LLOQ samples of each alprenolol enantiomers (Fig. 4). The data for LLOQ was shown in Fig. 4A and B. Similar observation was obtained from freeze–thaw stability, storage stability of processed samples, and 2-month storage stability at −20 °C experiments (data not shown). Therefore, this assay can be reliably used to determine individual alprenolol enantiomers in biological samples.

4. Conclusion

A fast, sensitive, and reliable bioanalytical method on direct separation and quantitation of individual alprenolol enantiomers in human plasma using LC–MS/MS has been successfully developed and validated. The complete baseline separation of the enantiomers was achieved within 2 min on CHIRAL-CBH column under the reversed phase mode. Increasing the buffer concentrations and organic content in mobile phases or lowering the pH of mobile phase can be applied to reduce the run time for the separation of the enantiomers but often at the cost of sacrificing the resolution. Therefore, balancing the run time and resolution is very important for developing fast and reliable bioanalytical methods for the determination of individual alprenolol enantiomers. The present assay demonstrated highly reproducible chromatographic and statistical results in terms of precision and accuracy during method validation. Thanks to the speed and convenience for the sample preparation by using SLE as well as short LC run time, this bioanalytical assays can be used for high through-put analysis of individual alprenolol enantiomers in biological samples.

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