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Stereoselective analysis of carvedilol in human plasma using HPLC/MS/MS after chiral derivatization

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Abstract

A relatively high-throughput high-performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) method using a chiral derivatization reagent was developed for the quantitative determination of carvedilol enantiomers in human plasma. *S*-carvedilol and *R*-carvedilol are extracted from human plasma by protein precipitation using acetonitrile containing racemic [$^2H_5$]-carvedilol as an internal standard. Extracts are then derivatized with 2,3,4,6-tetra-*O*-acetyl-beta-glucopyranosyl isothiocyanate (GITC) and analysed using HPLC–MS/MS with a TurboIonspray (TIS) interface and selected reaction monitoring. Using 150 μL of plasma, the method was validated over a concentration range of 0.2–200 ng/mL. The maximum within-run precision observed in a three run quality control was 8.2% for *S*-carvedilol and 6.7% for *R*-carvedilol, respectively. The maximum percentage bias observed at all quality control sample concentrations was 9.4% for *S*-carvedilol and 11.6% for *R*-carvedilol, respectively. The HPLC–MS/MS method was also compared with a previously developed high-performance LC/fluorescence method by analysing 25 samples containing racemic carvedilol. Based on results obtained, these two methods were found to be equivalent. However, compared with LC/fluorescence method, HPLC–MS/MS method is more sensitive, uses less plasma, and also employs a less time-consuming sample preparation process.

Keywords: Carvedilol enantiomers; HPLC–MS/MS; GITC derivatization; Human plasma; Quantitative

1. Introduction

Carvedilol is an anti-hypertensive agent with non-selective β- and α1-adrenergic receptor blocking activities [1–3]. Carvedilol has been used in treatment of congestive heart failure [4–6]. Recently published results from Metroprolol European Trial (COMET) study, the longest and largest beta blocker heart failure trial ever conducted and involving 3029 patients from 15 European countries and 317 centers enrolled in double-blinded and randomized parallel group trial, has shown carvedilol significantly reduced cardiovascular mortality by 20% and prolonged median survival [7]. Like other β-blockers, carvedilol contains one asymmetric carbon and exists in two enantiomeric forms (*S* and *R*). Pharmacological studies indicate that the β-blocking activity is essentially limited to the *S*-enantiomer [8]. In order to determine the stereospecificity exhibited in the metabolism and disposition of carvedilol, an analytical method for the quantitative analysis of each enantiomer is required.

The currently established analytical chiral methods for carvedilol involve solid-phase extraction (SPE) or liquid–liquid extraction (LLE) of the plasma samples, followed by derivatization of the extract with the chiral reagent, 2,3,4,6-tetra-*O*-acetyl-beta-D-glucopyranosyl isothiocyanate (GITC), an optically pure chiral reagent, and injection of the resultant diastereoisomers onto a reversed-phase HPLC column coupled to a fluorescence detector [9–11]. The LC run time is at least 10 min in order to separate the diastereoisomers and also to avoid endogenous interference. The lower limit of quantification (LLQ) is around 0.5–1.0 ng/mL in ∼1 mL of plasma. The chiral method for carvedilol enantiomers can also be performed without derivatization. The resolution of *R*- and *S*-carvedilol was achieved with HPLC using

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a chiral stationary phase column [12] or with capillary electrophoresis (CE) using cyclodextrins as the chiral selectors [13]. Long run times (>20 min) are typically needed to resolve two carvedilol enantiomeric forms, which makes it unfit for the high-throughput bioanalysis.

This paper describes a relatively high-throughput and high-sensitivity stereoselective method using protein precipitation and high-performance liquid chromatography coupled with tandem mass spectrometry detection (HPLC/MS/MS) for the simultaneous determination of *S*-carvedilol and *R*-carvedilol in human plasma. The method was modified from the previously reported carvedilol enantiomer method using GITC as the chiral derivatization reagent and liquid chromatography with fluorescence detection [9–11]. The tremendous sensitivity and selectivity gain offered by mass spectrometry has allowed us to use less plasma, employ a simpler sample preparation technique, and also reduce HPLC run time.

2. Experimental

2.1. Chemicals and reagents

Racemate of carvedilol (SKF-105517, COREG® marketed by GlaxoSmithKline Pharm, $C_{24}H_{26}N_2O_4$, MW = 406) and racemic [$^2H_5$]-carvedilol (MW = 411) were obtained from in-house synthesis. Analytical reagent grade of GITC, triethylamine (TEA) and ammonium formate were purchased from Sigma (St. Louis, MO, USA), and HPLC grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ, USA). Human control plasma containing heparin as anticoagulant was purchased from Bioreclamation, Inc. (Hicksville, NY). Acetonitrile was the solvent used to make 3 mg/mL GITC solution (derivatization reagent) and 0.5% TEA solution (catalyst for derivatization). The GITC solution was prepared fresh daily. The buffer of 2 mM ammonium formate was adjusted to pH 3.0 with formic acid.

2.2. Equipment

A Tomtec Quadra 96 model 320 automated liquid handling system (Hamden, CT, USA), an Eppendorf 5810R centrifuge with a four 96-well plate rotor (Brinkmann Instrument, Westbury, NY, USA), a Mettler AE240 balance (Hightstown, NJ, USA), 1.2 mL polypropylene 96-well tubes (Micronic Systems, Lelystad, Holland) and silicone sealing mats with PTFE coating (ArcticWhite LLC, Bethlehem, PA, USA), 96-well plate sample concentrator (Jones Chromatographs, Lakewood, CO, USA) and a Harvard Apparatus Model 22 infusion pump (South Natick, MA, USA) were used.

2.3. Sample preparation and extraction

Carvedilol was dissolved in dimethyl formamide (DMF) to give a 1 mg/mL of racemic stock solution. The concentration of each enantiomer in the stock solution is 500 μg/mL. The stock solution was further diluted with acetonitrile/water (50/50) to make working solutions at concentrations of 4000, 2000, 1000, 400, 200, 100, 40, 20, 10 and 4 ng/mL. These working solutions were used to spike heparinized plasma to make duplicate calibration standards at 200, 100, 50, 20, 10, 5, 2, 1, 0.5 and 0.2 ng/mL, and quality control samples at 200, 160, 10, 0.8 and 0.2 ng/mL in replicates of six. The internal standard of [$^2H_5$]-carvedilol was supplied by the in-house synthesis. The chemical purity for [$^2H_5$]-carvedilol was determined to be >96.5% with the non-deuterium content at <0.1%. Internal standard solution was prepared by diluting the 1 mg/mL stock of [$^2H_5$]-carvedilol into acetonitrile to give a 10 ng/mL solution.

One hundred fifty microliters of plasma sample (blank, standard, quality control (QC) or in vivo sample) and 450 μL internal standard solution containing 10 ng/mL [$^2H_5$]-carvedilol were transferred in sequence into a single well of a 96-well tube plate. After vortex-mixing for 2 min the 96-well tube plate was centrifuged for 20 min at ~3200 g. Approximately 500 μL of supernatant was transferred into a new 96-well tube plate and then was evaporated to dryness under a stream of N_2 at 40 °C. Two hundred microliters of 0.5% TEA and twenty microliters of 3 mg/mL GITC solution were added to each tube. Following a brief vortex-mixing, samples were held at room temperature for 20 min for GITC reaction to take place. The solvent from the derivatized samples was then evaporated into complete dryness under N_2 at 40 °C (TEA, also an ion-paring reagent, would cause interference to LC separation). The residue was reconstituted in 100 μL of 2 mM ammonium formate (pH 3)/acetonitrile (50/50, v/v). Samples were briefly mixed and centrifuged for 5 min at approximately 3220 g before injection onto HPLC–MS/MS system for analysis.

2.4. Chromatographic conditions

HPLC separation was performed on a quaternary Rheous Flux liquid chromatography model 2000 system. The makeup flow was delivered by a quaternary Rheous Flux liquid chromatography model 4000 system. Separation was achieved on an Ace 3 C18 column (3 μm, 50 mm × 2 mm) using an isocratic flow at 550 μL/min. Column temperature was maintained at 45 °C. The mobile phase consisted of a 50:50 mixing of 2 mM ammonium formate buffer (pH 3.0) and acetonitrile. The solvent front (up to 1.4 min) was diverted to waste while another LC pump delivered a makeup flow of acetonitrile/water (50/50, v/v) at 250 μL/min into MS. The LC cycle time for each sample run was 2.8 min. A sample volume of 2 μL was injected into LC/MS system by loop filling using a CTC HTS PAL autosampler (Leap Technology, Greensboro, NC).

2.5. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Concord, ON, Canada) with a TurboIonspray

(TIS) interface operated in the positive ion mode was used for the selected reaction monitoring (SRM) LC/MS analyses. The mass spectrometric conditions were optimized for GITC derivatized carvedilol by continuously infusing to the mass spectrometer the standard solution at 10 μL/min using a Harvard infusion pump via a "T" connector into the post-column mobile phase flow (0.5 mL/min). The TurboIonspray source temperature was maintained at 750 °C to ensure high sensitivity. The ionspray voltage was set at 5000 V. The curtain gas was set at 20, the declustering potential (DP) at 61 V, and the nebulizer (GS1) and TIS (GS2) gases at 60 and 70 psi, respectively. The CID gas was set at 4, and the collision energy was set at 53 eV. The following precursor to product ion transitions were used for the SRM analyses: derivatized carvedilol (*S* and *R*) *m*/*z* 796–222 with the dwell time set at 150 ms and derivatized [$^2H_5$]-carvedilol (*S* and *R*) *m*/*z* 801–227 with the dwell time set at 100 ms. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.6–0.8 Da) for both Q1 and Q3 quadrupoles. Data were acquired using the Analyst 1.1 Software (AB/MDS-Sciex).

2.6. Data acquisition and processing

HPLC–MS/MS data were acquired and processed (integrated) using Analyst™ Software (Version 1.1, MDS Sciex, Canada). A calibration line of analyte/internal standard peak area ratio versus *S*-carvedilol and *R*-carvedilol concentration was constructed and a weighted $1/x^2$ linear regression applied to the data. Concentrations of *S*-carvedilol and *R*-carvedilol in quality control samples were determined from the calibration line and used to calculate the bias and precision of the method with an in-house LIMS (Study Management System, SMS2000, version 1.4, GlaxoSmithKline).

3. Results and discussion

3.1. Derivatization of carvedilol with GITC

Structures of carvedilol, [$^2H_5$]-carvedilol, GITC derivatized *S*-carvedilol and *R*-carvedilol are shown in Fig. 1. GITC is an optically pure chiral reagent, and GITC derivatized carvedilol racemate thus becomes diastereoisomers because of the presence of an additional chiral center in the molecule. Optimum conditions for the chiral derivatization of the secondary amine have been investigated before and were only slightly modified for our experiment [9–11]. It was found that at least 126 nmole GITC was needed as the reaction reagent for the derivatization to proceed completely. Triethylamine was used as the reaction catalyst. The optimum amount of triethylamine to use is 0.4%. The derivatization

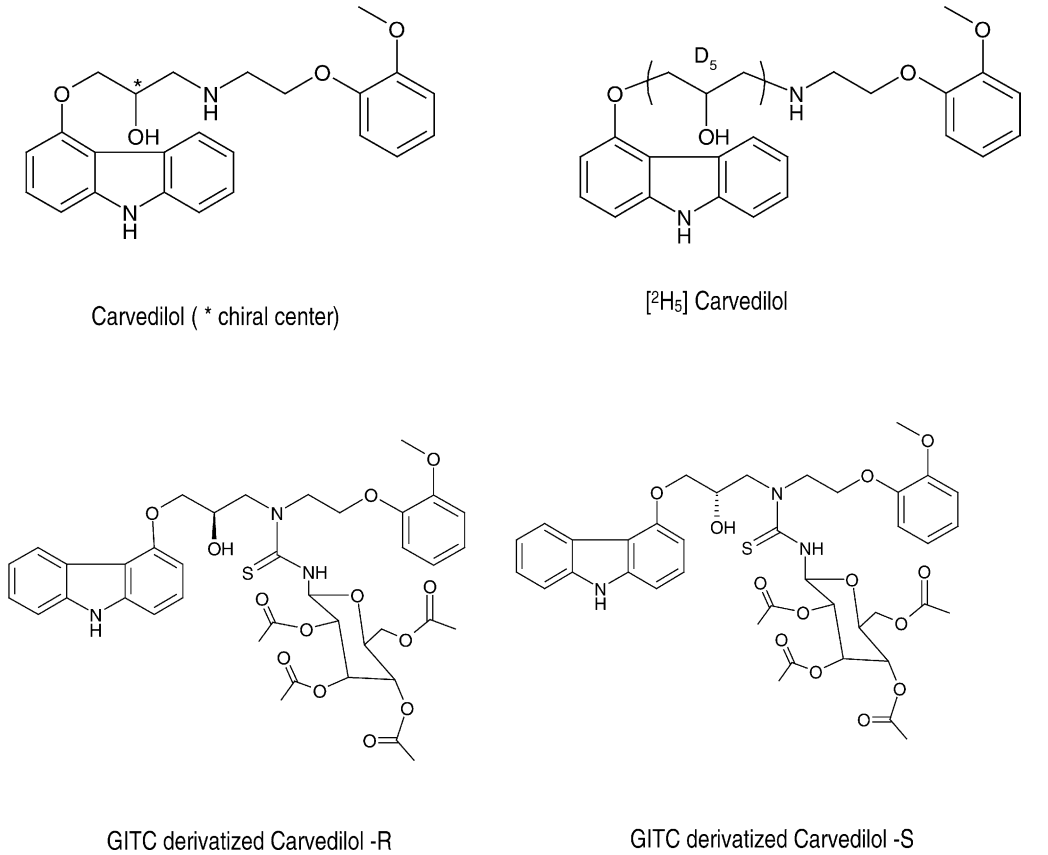

Fig. 1. Structures of carvedilol, [$^2H_5$]-carvedilol, GITC derivatized *R*-carvedilol and GITC derivatized *S*-carvedilol.

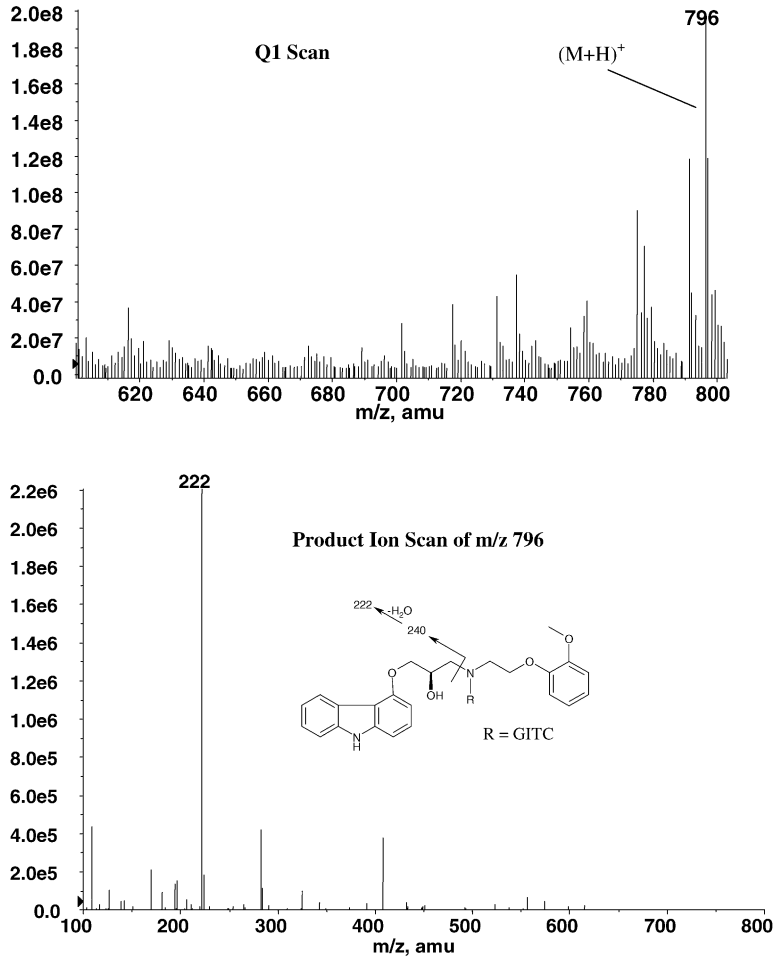

Fig. 2. Full scan Q1 and product ion scan spectrum of GITC derivatized carvedilol.

reaction proceeded quickly and the response from both enantiomers plateau 10 min after the start of the reaction. Chiral inversion did not occur in derivatization reactions described above.

3.2. Selectivity, sensitivity and linearity

The single MS Q1 and product ion scan spectrum of GITC derivatized carvedilol was shown in Fig. 2. The product ion at *m*/*z* 222 is generated by a two step process, a single bond cleavage to generate *m*/*z* 240 ion followed by loss of water. The characteristic precursor $[M + H]^+$ to product ions transitions, *m*/*z* 796–222 and 801–227, are consistent with the structures of GITC derivatized carvedilol and its stable isotope labelled internal standard, respectively, and are used as multiple reaction monitoring transitions to ensure high sensitivity and selectivity. The selectivity of the method was further established by the analysis of samples of drug-free human plasma from six individual subjects. Carvedilol in vivo metabolism was well-studied [14] and all the known metabolites, mostly through hydroxylation, are not expected to affect method selectivity. The selectivity of the method was also assessed by the inclusion of blank sample with internal standard and double blank samples (no analyte and no internal standard) prepared from drug-free human plasma in validation experiments. HPLC–MS/MS chromatograms of the blanks and quality control samples were visually examined and compared for chromatographic integrity and potential interference. Representative chromatograms of a blank sample with internal standard, a quality control sample at the LLQ of 0.2 ng/mL, a quality control sample at the higher limit of quantification (HLQ) of 200 ng/mL, a clinical sample with the concentration of *S*-carvedilol determined at 4.3 ng/mL, and the concentration of *R*-carvedilol determined at 9.5 ng/mL are shown in Fig. 3A–D, respectively. No unacceptable interference from endogenous material at the retention time of *S*-carvedilol and *R*-carvedilol were observed (Fig. 3A). The signal to noise ratio at LLQ was >50 for both enantiomers (Fig. 3B). *S*-carvedilol was baseline resolved from *R*-carvedilol at HLQ (Fig. 3C). Previous experiments have shown that *S*-carvedilol is metabolized faster than *R*-carvedilol [10,11]. As a result, *S*-carvedilol is normally of lower concentration than *R*-carvedilol in clinical samples (Fig. 3D).

Linear responses in the analyte/internal standard peak area ratios were observed over the range 0.2–200 ng/mL.

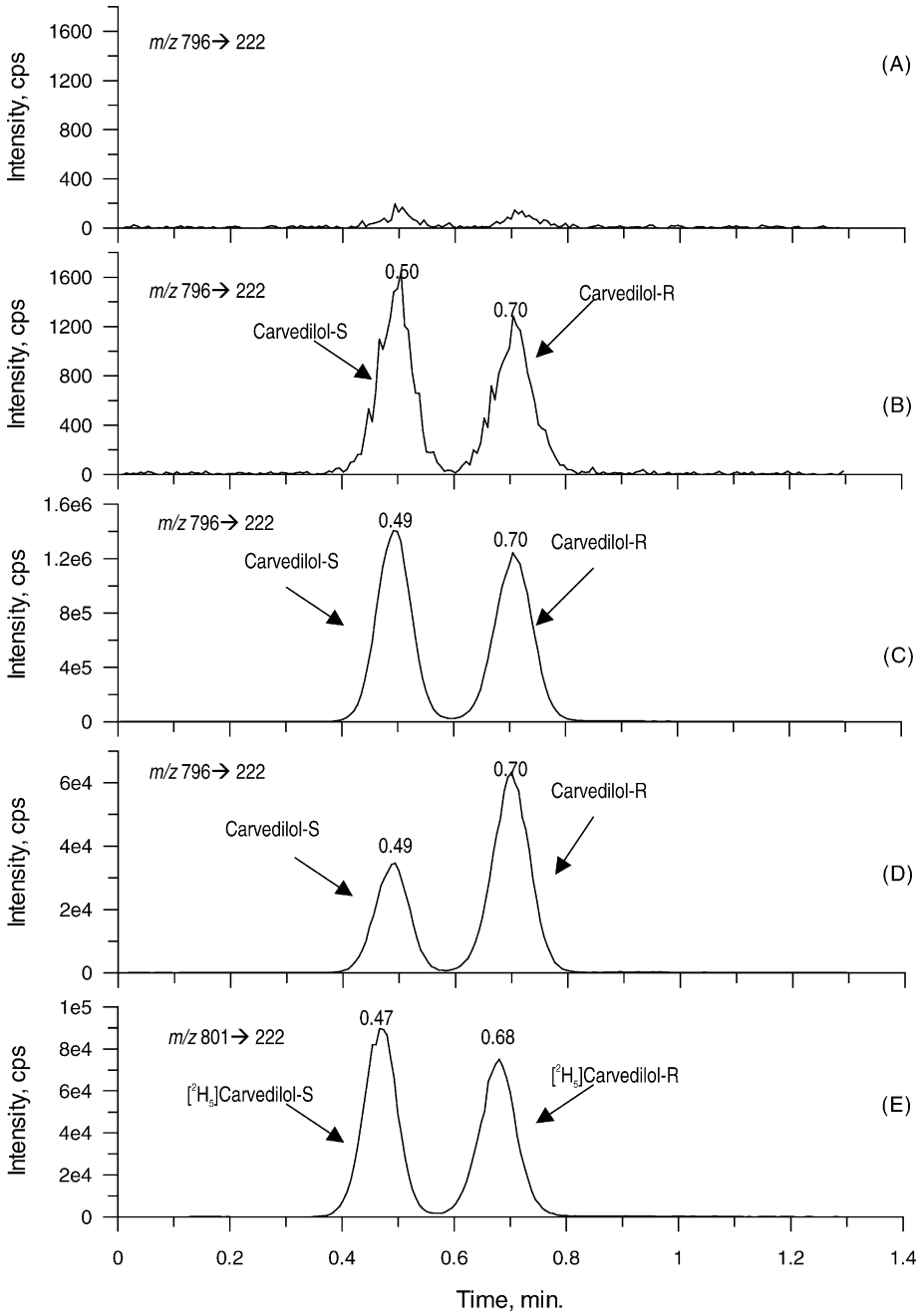

Fig. 3. Representative HPLC–MS/MS chromatograms of blank sample (A); LLQ at 0.2 ng/mL (B); HLQ at 200 ng/mL (C); a clinical sample at 4.3 ng/mL for *S*-carvedilol and 9.5 ng/mL for *R*-carvedilol (D); internal standard (E). MS data acquisition starts at 1.4 min.

Correlation coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9976 for *S*-carvedilol and 0.9962 for *R*-carvedilol.

3.3. Bias and precision

Concentrations of *S*-carvedilol and *R*-carvedilol in quality control samples were determined from the calibration line on each occasion. At all quality control sample concentrations examined, the within-run percent bias is within ±15%. The maximum percent bias observed was 9.4% for *S*-carvedilol and 11.6% for *R*-carvedilol, respectively. At all quality control sample concentrations examined, the within- and between-run precision are less than 15%. The maximum within-run precision observed was 8.2% for *S*-carvedilol and 6.7% for *R*-carvedilol, respectively. The maximum between-run precision observed was 4.8% for *S*-carvedilol and 5.5% for *R*-carvedilol, respectively. As defined by the lower and upper quality control sample concentrations possessing acceptable accuracy and precision, the validated range of this method for *S*-carvedilol and *R*-carvedilol based on 150 μL of human plasma is 0.2–200 ng/mL.

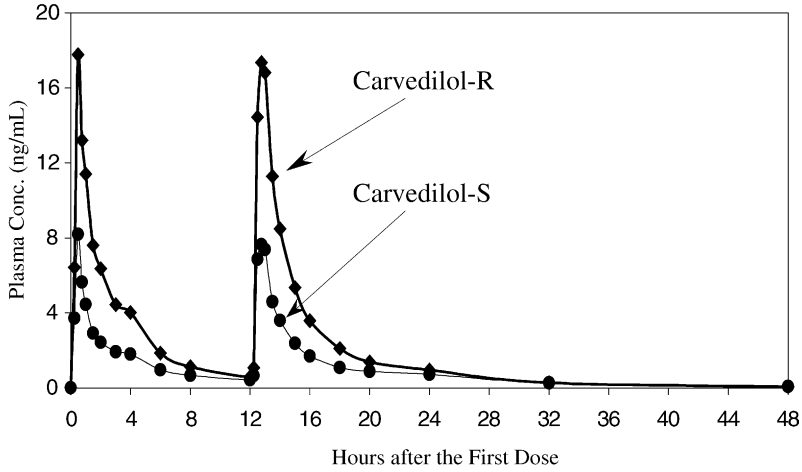

Fig. 4. The pharmacokinetic profile of *S*-carvedilol and *R*-carvedilol from a healthy volunteer after administration of a 12.5 mg tablet of carvedilol (COREG®) at 0 and 12 h.

3.4. Stability

The stability of derivatized carvedilol in processed extracts of human plasma samples was assessed by re-injecting a validation run batch after storage at room temperature for 24 h. The accuracy, precision and sensitivity of these samples were found to be acceptable on re-injection, indicating that the processed extracts were stable stored at room temperature for 24 h.

Storage stability of carvedilol in human plasma has been studied previously [15]. Carvedilol is stable in spiked human heparin plasma at −20 °C for at least 30 months. Ex vivo stability of carvedilol in authentic human plasma samples is acceptable upon storage at −20 °C for at least 40 months. Carvedilol is also stable in human plasma after at least three freeze–thaw cycles from −20 °C.

3.5. Comparison with HPLC/fluorescence method

The HPLC/MS/MS method was compared with an already established LC/fluorescence method to determine whether two carvedilol methods are equivalent. The LC/fluorescence method was performed following the experimental process described previously [11]. Twenty-five samples prepared by pooling in vivo samples from a previous study were split into two aliquots and were analysed using both methods. Concentrations of *S*-carvedilol and *R*-carvedilol ranged from 1 to 190 ng/mL and concentration of *S*-carvedilol is generally two times lower than that of *R*-carvedilol. Regression analysis has shown excellent correlation between two methods even without any weighting applied. The correlation coefficients for *S*-carvedilol and *R*-carvedilol were 0.9996 and 0.9986, respectively. The slope of the regression line was 1.0498 for *S*-carvedilol and 1.0393 for *R*-carvedilol, which was <5% from 1.0. Both regression lines show small negative intercept (−0.1489 for *S*-carvedilol and −0.4609 for *R*-carvedilol), meaning results obtained from LC/MS/MS were slightly higher than the ones from LC/fluorescence method. However, the difference was negligible and we concluded that the two methods generated equivalent results.

3.6. Application to pharmacokinetic study

The HPLC/MS/MS method developed has been used to investigate plasma profile of carvedilol enantiomers in a number of clinical pharmacokinetic studies. A pharmacokinetic profile of *S*-carvedilol and *R*-carvedilol from a healthy volunteer, after administration of the immediate release form of carvedilol (COREG®) 12.5 mg each at 0 and 12 h, was shown in Fig. 4. Blood samples were drawn pre-dose (0) and at intervals from 0.25 to 48 h post-dose. The C_{max} (maximum drug concentration in plasma) for *S*- and *R*-carvedilol from this healthy volunteer was found to be 5.65 and 13.2 ng/mL, respectively. Both *S*- and *R*- carvedilol reached their high concentration in plasma at ∼0.75 h (t_{max}). The small volume of plasma (150 μL per analysis) needed for the analysis allowed clinicians to draw less blood from healthy volunteers or heart patients at each time point and also to take blood samples at more time points to better define C_{max} and t_{max}. In addition, the sensitivity and speed the HPLC/MS/MS method provided has enabled us to provide results with relatively fast turnaround time. To this end, the HPLC/MS/MS method described in this paper has been employed in-house and was also transferred to a contract bioanalytical lab to successfully analyse more than 50,000 pharmacokinetic samples.

4. Conclusion

A relatively simple, selective and sensitive HPLC/MS/MS method, using protein precipitation and chiral derivatization, has been developed and validated for the determination

of carvedilol enantiomers in human plasma. The method was shown to be equivalent to a previously validated HPLC/fluorescence method, and it has been successfully employed to support a number of pharmacokinetic studies.

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