BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **21**: 508–513 (2007)
Published online 12 February 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/bmc.785

ORIGINAL RESEARCH

Liquid chromatographic–electrospray tandem mass spectrometric determination of bisoprolol in human plasma

Mingyuan Liu,[1,2] Dan Zhang,[1] Yantong Sun,[1] Yingwu Wang,[1] Zeyuan Liu[3] and Jingkai Gu[1]*

[1]Research Center for Drug Metabolism, College of Life Science, Jilin University, Changchun 130023, People's Republic of China
[2]Department of Pharmacology, School of Basic Medical Sciences, Jilin University, Changchun 130023, People's Republic of China
[3]Department of Clinical Pharmacology, Affiliated Hospital, Academic of Military Medical Sciences, Beijing 100039, People's Republic of China

Received 31 October 2006; revised 19 November 2006; accepted 20 November 2006

ABSTRACT: An analytical method for the determination of bisoprolol in human plasma has been developed based on liquid chromatography–tandem mass spectrometry (LC–MS/MS). The analyte and internal standard (IS) diphenhydramine were cleaned up by protein precipitation with acetonitrile, reconstituted in mobile phase and separated by reversed-phase high-performance liquid chromatography (HPLC) using methanol:10 mM ammonium acetate:formic acid (70:30:0.1 v/v/v) as mobile phase. Detection was carried out by multiple reaction monitoring (MRM) on an LC–MS/MS system and was completed within 2.5 min. The assay was linear over the range 0.5–100 ng/mL with a limit of quantitation (LOQ) of 0.5 ng/mL. The intra- and inter-day precision levels were within 5.54 and 9.95%, respectively, while the accuracy was in the range 89.4–113%. This method has been utilized in a pharmacokinetic study, where healthy volunteers were treated with an oral dose of 5 mg bisoprolol. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: bisoprolol; diphenhydramine; pharmacokinetics; LC–MS/MS

INTRODUCTION

Bisoprolol hemifumarate, (±)-1-{*p*-[(2-isopropoxyethoxy) methyl] phenoxy}-3-isopropyl-amino-2-propanol-hemifumarate, which is indicated for the treatment of hypertension and angina pectoris, is a highly β_1-selective adrenoceptor blocking agent without membrane-stabilizing activity or intrinsic sympathomimetic activity (Manalan *et al.*, 1981; Schliep and Harting, 1984).

Prior to the prevalent use of LC-MS techniques, various analytical methods, such as capillary electrophoresis (Awadallah *et al.*, 2003), gas chromatography (Kim *et al*., 2001) and HPLC with an ultraviolet (Zhang *et al.*, 2003; Ruiz-Angel *et al.*, 2002; Modamio *et al.*, 1996), fluorescence (Buhring and Garbe, 1986; Eastwood *et al.*, 1990; Suzuki *et al.*, 1993) or diode array detector (Caudron *et al.*, 2004), have been developed to determine bisoprolol in formulation and biological samples. These methods suffer from a number of limitations including low sensitivity, the need for a large volume of plasma, long run time and/or complicated sample preparation.

Currently, LC–MS and LC–MS/MS methods have been widely employed for the analysis of drug compounds in biological fluids because of their excellent specificity, speed and sensitivity. LC–MS systems equipped with atmospheric pressure chemical ionization (APCI) interface were used to identify 32 beta-blockers (including bisoprolol) in human urine and 22 beta-blockers (including bisoprolol) in human plasma, respectively (Thevis *et al.*, 2001; Maurer *et al.*, 2004). These LC–MS methods, however, are not appropriate for pharmacokinetic studies of bisoprolol because of their insufficient sensitivity and long run time. Furthermore, the use of selected ion monitoring (SIM) is associated with a loss of specificity.

To facilitate the pharmacokinetic studies of bisoprolol, a novel LC–MS/MS method was developed and validated. In addition to an improvement in sensitivity, the volume of plasma required and the analytical time were dramatically reduced. The method has been successfully used in a pharmacokinetic study of bisoprolol, where 20 healthy volunteers were each given a 5 mg bisoprolol tablet.

*Correspondence to: Jingkai Gu, Research Center for Drug Metabolism, College of Life Science, Jilin University, Changchun 130023, Jilin Province, People's Republic of China.
E-mail: gujk@mail.jlu.edu.cn

Abbreviations used: IS, internal standard; MRM, multiple reaction monitoring.

Copyright © 2007 John Wiley & Sons, Ltd.

WILEY InterScience® DISCOVER SOMETHING GREAT

EXPERIMENTAL

Instrumentation. Chromatography was performed using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA), which was connected to an Applied Biosystems Sciex Q-trap™ mass spectrometer (Applied Biosystems Sciex, Ontario, Canada). Applied Biosystems/MDS SCIEX Analyst software (Version 1.3) was used for data acquisition and processing.

Reagents and chemicals. Bisoprolol hemifumarate was provided by Beijing Xinlicheng Pharmaceutical Co. Ltd (>99%, Beijing, China). Diphenhydramine hydrochloride (>98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Chemicals (Fair Lawn, NJ, USA), while all other chemicals (analytical grade) were purchased from commercial sources and used without further purification. Distilled water, prepared from demineralized water, was used throughout the study.

Preparation of solutions. All concentrations of bisoprolol and diphenhydramine refer to the free bases. Stock solutions (1 mg/mL) of bisoprolol and diphenhydramine were prepared in methanol. A series of bisoprolol standard solutions with concentrations of 0.5, 1, 3, 10, 30 and 100 ng/mL were prepared by dilution of individual aliquots of the stock solution with methanol and water (1:1, v/v). A working internal standard solution, diphenhydramine (30 ng/mL) was also prepared in methanol and water (1:1, v/v). Quality control (QC) solutions with low, medium and high concentrations (1, 10 and 80 ng/mL, respectively) were prepared in methanol and water (1:1, v/v). The calibrators were weighted according to $1/x$ [x = (concentration)^2] least-squared regression, and standard curves were constructed using linear regression of the peak area ratios of bisoprolol against internal standard, which was obtained from LC–MS/MS analysis of standard solutions against actual standard concentrations.

Sample preparation. Thawed samples were vortexed thoroughly at room temperature and employed as follows: to 100 μL plasma sample (or blank plasma) in a 1.5 mL Eppendorf tube, 100 μL methanol and water (1:1, v/v) (or bisoprolol standard solution or QC solution) and 50 μL internal standard solution were added. After vortexing, 0.5 mL acetonitrile was added and the contents of the tube were vortexed again for 30 s and then centrifuged at 6500***g*** for 10 min. The supernatant (150 μL) was then transferred to a fresh 10 mL glass tube and dried under nitrogen gas. Finally, the residue was reconstituted in 150 μL of the mobile phase and 20 μL was injected for analysis.

LC–MS/MS. HPLC was carried out on a Venusil MP C_{18} analytical column (5 μm, 100 × 4.6 mm i.d. from Agela Technologies Inc., USA) operated at 35°C. The mobile phase was methanol:10 mM ammonium acetate:formic acid (70:30:0.1 v/v/v). Chromatography was performed at a flow rate of 1.0 mL/min. An approximate 1:1 split of the column eluant was incorporated so that only 0.5 mL/min entered the mass spectrometer. Detection was performed on a Q TRAP™ LC–MS/MS system equipped with an electrospray ion source. A divert valve was programmed to send LC effluent to waste except during the 1.0–2.5 min period after injection.

The LC–MS/MS detector was operated at unit resolution in the MRM mode using the transitions of the protonated molecule of bisoprolol at m/z 326 → m/z 116 and diphenhydramine at m/z 256 → m/z 167. In order to optimize MS parameters, a standard solution of analyte and IS was infused into the mass spectrometer using a syringe pump. The determined parameters were as follows: curtain gas, gas 1 and gas 2 (nitrogen), 20, 50 and 60 psi, respectively, dwell time 200 ms, source temperature 500°C, IonSpray voltage 1500 V. Declustering potential (DP) and collision energy (CE) were, respectively, 50 V and 23 eV for bisoprolol and 50 V and 25 eV for diphenhydramine.

Assay validation. The specificity of the assay for the analyte and IS vs endogenous substances in the matrix was assessed by comparing the lowest concentration in the calibration curves with reconstitutions prepared with blank plasma from six different volunteers. Three different calibration curves using six plasma concentrations (0.5–100 ng/mL) of bisoprolol were prepared to validate the linearity of the method. Precision and accuracy were evaluated through determining replicate QC samples (1, 10 and 80 ng/mL; n = 6) on three different days. Recovery was determined by comparing peak area response of cleaned-up replicate QC samples (1, 10 and 80 ng/mL; n = 3) with that of cleaned-up drug-free plasma spiked with appropriate amounts of standards. The stability of bisoprolol at three QC plasma sample concentrations (1, 10 and 80 ng/mL) after three successive freeze–thaw cycles, stored at −20°C for 1 month and processed samples stored in room temperature for 8 h, in triplicate, was determined by comparing the concentrations calculated by the standard curve and the nominal concentrations of 1, 10, and 80 ng/mL.

Application of the assay. A single oral dose of 5 mg of bisoprolol was administrated to each of the 20 healthy volunteers after overnight fasting. Blood samples were collected before administration and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h after administration. The plasma samples were labeled and kept frozen at −20°C until analysis.

RESULTS AND DISCUSSION

LC–MS/MS

Both analyte and internal standard responded best to positive ionization, and protonated molecules $[M + H]^+$ were present as major peaks in the mass spectra of both compounds. The chemical structures of bisoprolol and IS as well as the full-scan product ion spectra of $[M + H]^+$ are shown in Fig. 1. The transitions m/z 326 → 116 and m/z 256 → 167 were used for quantitation of bisoprolol and IS, respectively.

Various combinations of acetonitrile, methanol and formic acid were investigated with a view to optimizing the mobile phase for sensitivity, speed and peak shape. Ammonium acetate buffer and formic acid were used,

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/bmc

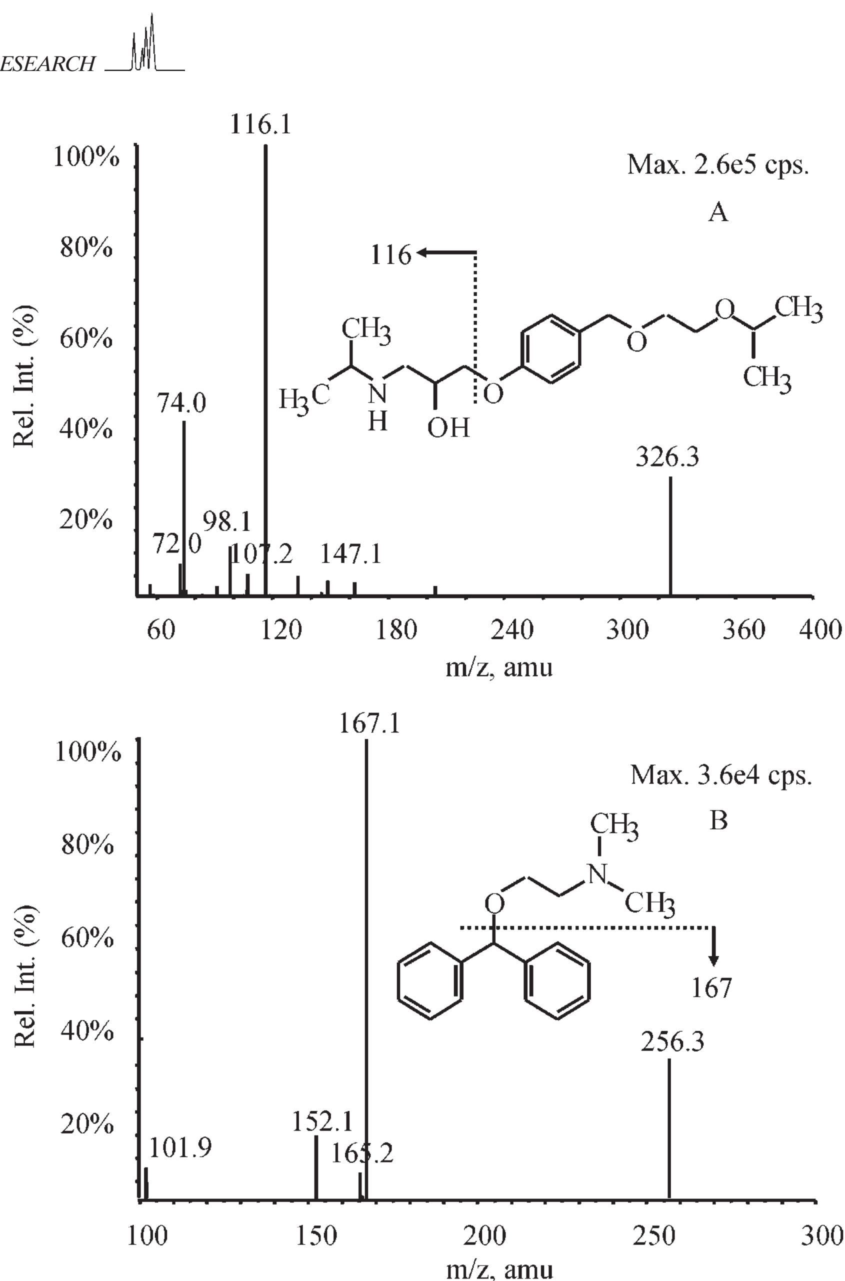

Figure 1. Full-scan product ion spectra of $[M + H]^+$ and the structures for (A) bisoprolol and (B) diphenhydramine.

since they improved peak symmetry and ionization. A number of commercially available columns, including Nucleosil, Hypersil and Venusil, were evaluated, and the Venusil MP C_{18} column showed suitable retention time and peak shape for bisoprolol and offered relatively short analytical runtimes (2.5 min). A stable-isotope labeled analyte would be preferable as the internal standard for the quantitation of the analyte in complex matrices by LC–MS/MS analysis. However, there are also many problems associated with the use of stable isotope-labeled internal standards. The major problems involve inadequate isotopic purity and stability, which often impose an unfavorable impact on highly sensitive quantitative analyses. Diphenhydramine was chosen as the internal standard from a number of evaluated compounds (tramadol, diazepam and diphenhydramine), because of its satisfactory peak shape, relatively high recovery and similar retention time with that of bisoprolol.

As shown in Fig. 2, analyte and IS were free of interference from endogenous substances, and gave retention times of 1.4 and 1.5 min, respectively. With these optimal conditions, no interference peak was detected for bisoprolol and IS throughout the study.

Sample preparation

In this study, one-step protein precipitation, which is economical and convenient, was adopted to simplify sample preparations. Several solvents were investigated,

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/bmc

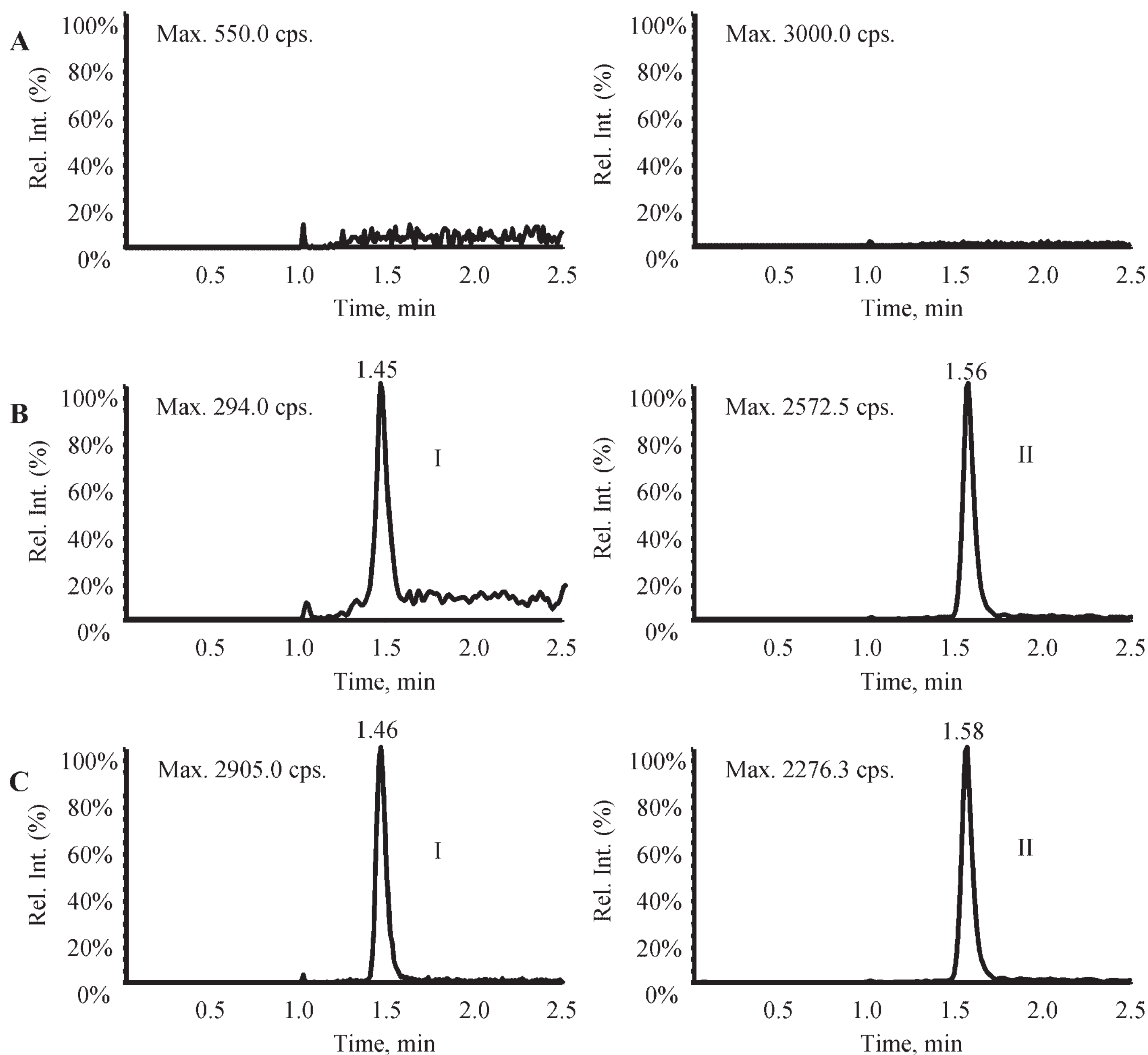

Figure 2. Representative single reaction monitoring chromatograms of (A) blank plasma, (B) plasma spiked with bisoprolol and diphenhydramine at the limit of quantitation (5 ng/mL), and (C) a plasma sample 24 h after an oral administration of bisoprolol (5 mg/day) to healthy volunteers. Peak I, bisoprolol; peak II, diphenhydramine.

but only acetonitrile provided an efficient protein precipitation. This sample preparation procedure was simple and rapid, and provided a clean chromatogram and a satisfactory recovery of the analyte from the plasma.

Assay validation

The calibration curves showed good linearity within the range 0.5–100 ng/mL, with all coefficients of correlation greater than 0.99. As shown in Table 1, the method gave good precision and accuracy. Intra- and inter-day precisions were better than 5.54 and 9.95%, respectively, while the accuracy was in the range 89.4–113%. The lower limit of detection of the method was 0.1 ng/mL (S/N > 3), and the LOQ was 0.5 ng/mL [Fig. 2(B)]. The recoveries of bisoprolol were 92.7 ± 8.1, 94.8 ± 4.9 and 95.9 ± 3.1% at concentrations of 1, 10 and 80 ng/mL, respectively. The recovery of the IS at working concentration of 30 ng/mL was 82.3 ± 5.0%. The lower recovery of the IS observed may be explained by the non-specific binding to certain components of the

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/bmc

Table 1. Precision and accuracy for the determination of bisoprolol in human plasma (data are based on assay of six replicate QC samples on three different days)

Added concentration (ng/mL)	Found concentration (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Relative error (%)
1.00	1.00	3.48	5.70	0.33
10.00	9.79	2.34	9.95	–2.14
80.00	80.57	5.54	4.19	0.71

precipitated proteins. In processed samples, the analytes were stable for a period of more than 8 h at room temperature. No instability of analytes in spiked samples was observed over three freeze–thaw cycles or during storage at –20°C for a one-month period.

The matrix effects were evaluated by comparing the peak areas of bisoprolol (1, 10 and 80 ng/mL) and internal standard (30 ng/mL) in three QC samples (prepared by three different drug-free plasmas) with those of the standard solutions, which were prepared in the same way as QC samples except water substituted for drug-free plasma. The average peak areas of the QC samples vs that obtained from the corresponding neat solution were 0.71, 0.72 and 0.77 for bisoprolol at concentrations of 1, 10 and 80 ng/mL, respectively, and 0.79 for the IS, which indicated that there was approximately a 21–29% matrix suppression for this method. Combined with the fact that there was no significant lot-to-lot variation in LOQ and specificity results, it was concluded that the matrix effect for this method did not compromise the performance of the method.

Pharmacokinetic study

The mean bisoprolol plasma concentration–time profile obtained after a single oral administration of bisoprolol of 5 mg is shown in Fig. 3. The C_{max} was 33.46 ± 11.02 ng/mL occurring at 3.3 ± 1.8 h. The mean plasma elimination half-life ($t_{1/2}$) was 8.67 ± 1.09 h and the mean area under the plasma concentration-time curve (AUC_{0-t}) was 421.27 ± 139.30 ng h/mL.

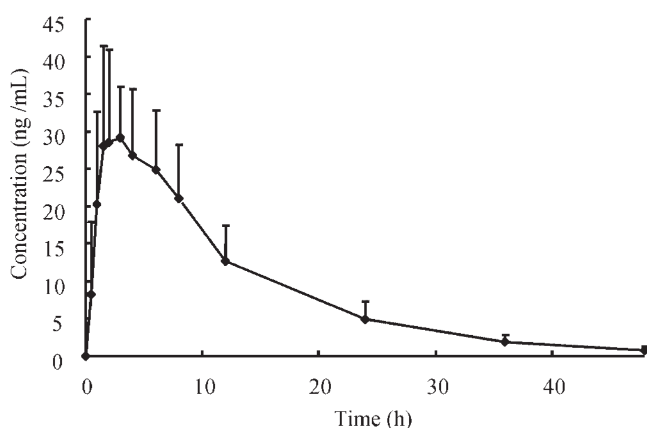

Figure 3. Mean plasma concentration–time profile of bisoprolol after administration of 5 mg tablet of bisoprolol to healthy volunteers (n = 20). Data are mean ± SD.

CONCLUSION

A rapid LC–MS/MS method requiring a small volume sample for the determination of bisoprolol in human plasma was reported. The precision, accuracy, sensitivity and selectivity of the method make it suitable for human pharmacokinetic studies. The method allows high sample throughput due to its short run time and relatively simple sample preparation procedure.

REFERENCES

Awadallah B, Schmidt PC, Holzgrabe U and Wahl MA. Quantitation of talinolol and other beta-blockers by capillary electrophoresis for in vitro drug absorption studies. *Electrophoresis* 2003; **24**: 2627.

Buhring KU and Garbe A. Determination of the new beta-blocker bisoprolol and of metoprolol, atenolol and propranolol in plasma and urine by high-performance liquid chromatography. *Journal of Chromatography* 1986; **382**: 215.

Caudron E, Laurent S, Billaud EM and Prognon P. Simultaneous determination of the acid/base antihypertensive drugs celiprolol, bisoprolol and irbesartan in human plasma by liquid chromatography. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences* 2004; **801**: 339.

Eastwood RJ, Jerman JC, Bhamra RK and Holt DW. High performance liquid chromatographic measurement of bisoprolol in plasma. *Biomedical Chromatography* 1990; **4**: 178.

Kim KH, Lee JH, Ko MY, Hong SP and Youm JR. Chiral separation of beta-blockers after derivatization with (–)-alphamethoxy-alpha-(trifluoromethyl)phenylacetyl chloride by gas chromatography. *Archives ofPpharmaceutical Research* 2001; **24**: 402.

Manalan AS, Besch HR Jr and Watanabe AM. Characterization of [^{3}H](+/–)carazolol binding to beta-adrenergic receptors. Application to study of beta-adrenergic receptor subtypes in canine ventricular myocardium and lung. *Circulation Research* 1981; **49**: 326.

Maurer HH, Tenberken O, Kratzsch C, Weber AA and Peters FT. Screening for library-assisted identification and fully validated quantification of 22 beta-blockers in blood plasma by liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization. *Journal of Chromatography A* 2004; **1058**: 169.

Modamio P, Lastra CF and Marino EL. Development and validation of liquid chromatography methods for the quantitation of propranolol, metoprolol, atenolol and bisoprolol: application in solution stability studies. *International Journal of Pharmaceutics* 1996; **130**: 137.

Copyright © 2007 John Wiley & Sons, Ltd.

Ruiz-Angel MJ, Carda-Broch S, Torres-Lapasio JR, Simo-Alfonso EF and Garcia-Alvarez-Coque MC. Micellar-organic versus aqueous-organic mobile phases for the screening of β-blockers. *Analytica Chimica Acta* 2002; **454**: 109.

Schliep HJ and Harting J. Beta 1-selectivity of bisoprolol, a new beta-adrenoceptor antagonist, in anesthetized dogs and guinea pigs. *Journal of Cardiovascular Pharmacology* 1984; **6**: 1156.

Suzuki T, Horikiri Y, Mizobe M and Noda K. Sensitive determination of bisoprolol enantiomers in plasma and urine by high-performance liquid chromatography using fluorescence detection, and application to preliminary study in humans. *Journal of Chromatography* 1993; **619**: 267.

Thevis M, Opfermann G and Schanzer W. High speed determination of beta-receptor blocking agents in human urine by liquid chromatography/tandem mass spectrometry. *Biomedical Chromatography* 2001; **15**: 393.

Zhang X, Ouyang J, Baeyens WR, Zhai S, Yang Y and Huang G. Enantiomeric separation of beta-blockers by HPLC using (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid as chiral stationary phase. *Journal of Pharmaceutical and Biomedical Analysis* 2003; **31**: 1047.

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/bmc