Determination of landiolol, an ultra-short-acting β₁-receptor antagonist, in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A method for the determination of landiolol, an ultra-short-acting β₁-adrenoreceptor antagonist, in human plasma has been developed and validated. With the addition of pyridostigmine bromide to stabilize landiolol in the blood/plasma samples, and bisoprolol as internal standard, plasma samples were subjected to liquid–liquid extraction with diethyl ether:dicholoromethane (60:40, v/v) prior to assay by liquid chromatography–tandem mass spectrometry. Separation was performed on a TC-C_{18} column (150 mm × 4.6 mm, 5 μm) using a mobile phase of methanol:10 mM ammonium acetate containing 1% formic acid (65:35, v/v) in a run time of 3.5 min. Detection involved electrospray ionization in the positive ion mode followed by multiple reaction monitoring of the precursor-to-product ion transitions of landiolol at m/z 510.1 → 157.2 and bisoprolol at m/z 326.3 → 116.1. The method was linear over the concentration range 0.5–500 ng/ml with a lower limit of quantitation of 0.5 ng/ml. Intra- and inter-day precisions (as relative standard deviation, RSD) were <4.4% and <10.0%, respectively, with accuracy (as relative error, RE) <10.0%. The method was successfully applied to a clinical pharmacokinetic study involving a continuous infusion of landiolol hydrochloride to healthy Chinese volunteers.

1. Introduction

Tachycardia and associated myocardial ischemia is a major complication during general anesthesia and an important factor in the anesthetic management of patients. Numerous anesthetic drugs and techniques have been developed to minimize and treat acute hemodynamic responses during surgery [1,2] with short-acting adrenergic β₁-receptor antagonists being one of the most effective. Administration of this type of drug allows an adequate depth of anesthesia to be achieved with less anesthetic and less demand for postoperative analgesia [3,4].

Landiolol, (−)-[(S)-2,2-dimethyl-1,3-dioxolan-4-yl]methyl 3-[4-[(S)-2-hydroxy-3-(2-morpholinocarbonylamino)ethylamino] propoxy]phenylpropionate, is a novel ultra-short-acting β₁-adrenoreceptor antagonist with a structure similar to that of esmolol [5]. With a β₁/β₂ ratio of 255, the drug is some 5 times more β₁-selective than esmolol providing a more potent negative chronotropic effect and a less potent hypotensive effect [6,7].

Due to the presence of an ester group, landiolol is hydrolyzed very quickly by both pseudocholinesterase in plasma and carboxylesterase in the liver such that the elimination half-life ($t_{1/2}$ of 4 min) is significantly shorter than that of conventional β₁-receptor antagonists [8–11]. These characteristics make landiolol a promising alternative to esmolol.

To date, several methods for the determination of landiolol in human blood have been reported [12,13]. However, based on high performance liquid chromatography (HPLC) with UV or fluorescence detection, these methods have inadequate sensitivity and selectivity. Therefore they would require not only a large sample volume and complicated sample preparation but also a long run time to provide adequate separation from interfering substances in blood. In particular, with lower limits of quantitation of 10 ng/ml (fluorescence) and 50 ng/ml (UV), the methods are not suitable for application to clinical pharmacokinetic studies of landiolol.

Landiolol is a basic organic molecule containing electronegative nitrogen atoms that is readily protonated during electrospray ionization (ESI). Thus, the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) provides significantly superior sensitivity and selectivity compared to other detectors. MS/MS detection also simplifies sample preparation and shortens the chromatographic run time.

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Landiolol in blood/plasma cannot be quantified accurately because it will be rapidly hydrolyzed by pseudocholinesterase during storage and sample preparation [13]. In previous assays [12,13], blood samples were immediately mixed with an ethanolic solution of neostigmine to stabilize landiolol prior to sample preparation.

In preliminary experiments, we found that the concentration of landiolol in human plasma is almost equal to that in whole blood (unpublished results). Based on the preliminary results, concentrations of landiolol *in vitro* are not affected by any post-sampling redistribution of drug between plasma and red blood cells and therefore can reflect plasma concentrations *in vivo*.

Here we report a novel method for quantitatively determination of landiolol in human plasma by using LC–MS/MS with bisoprolol as internal standard (I.S.). Landiolol in the blood/plasma samples is stabilized with the pseudocholinesterase inhibitor pyridostigmine bromide. The method has been fully validated and successfully applied to a clinical pharmacokinetic study involving a continuous infusion of landiolol hydrochloride to healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and reagents

Landiolol hydrochloride, bisoprolol hemifumarate and pyridostigmine bromide (purity in all cases >99.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was purchased from Fisher Chemicals (Fair Lawn, NJ, USA), all other chemicals were analytical grade from commercial sources and used as received. Distilled water, prepared from demineralized water, was used throughout the experiments. Heparinized blank (drug-free) human blood was obtained from the Changchun Blood Donor Service (Changchun, China) and stored at −20 °C until use.

2.2. Instrumentation

The LC–MS/MS system consists of an Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) connected to an QTRAP 2000™ mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), equipped with an ESI source. Applied Biosystems/MDS SCIEX Analyst software (Version 1.5.1) was used for data acquisition and processing.

2.3. LC–MS/MS conditions

HPLC separation was performed on an Agilent TC-C$_{18}$ column (150 mm × 4.6 mm, 5 μm, Agilent Technologies) maintained at 30 °C using a mobile phase of methanol:10 mM ammonium acetate containing 1% formic acid (65:35, v/v). The flow rate was set at 0.8 ml/min and an approximately 1:1 split of the column eluant was included before entry into the mass spectrometer. The electrospray voltage was set at 5500 V and the heater gas temperature was 500 °C. Nitrogen was used as nebulizing gas (55 p.s.i), auxiliary gas (50 p.s.i) and curtain gas (15 p.s.i). Multiple reaction monitoring (MRM) detection mode was carried out with the precursor to product ion transitions for landiolol of *m/z* 510.1 (Q1) → 157.2 (Q3). Declustering potential (DP) was set at 80 V and collision energy (CE) was set at 45 eV. For bisoprolol transition of *m/z* 326.3 (Q1) → 116.2 (Q3) was used with DP of 55 V and CE of 15 eV. The pause time and dwell time were set at 10 ms and 200 ms, respectively.

2.4. Preparation of calibration standards and quality control (QC) samples

All solutions were prepared in methanol:water (50:50, v/v). A stock solution of landiolol hydrochloride (1 mg/ml) was diluted to produce standard solutions with the concentrations of 0.5, 1.5, 5, 15, 50, 150, and 500 ng/ml. QC solutions were prepared independently at the concentrations of 1.5, 15 and 150 ng/ml in the same way. Aliquots (50 μl) of each standard solution were mixed with 50 μl blank human plasma to produce landiolol calibration standards with the concentrations of 0.5, 1.5, 5, 15, 50, 150, and 500 ng/ml in plasma. QC samples (1.5, 15, 150 ng/ml) were generated from QC solutions in the same way. A stock solution of bisoprolol hemifumarate (1 mg/ml) was diluted to obtain an I.S. working solution with the concentration of 100 ng/ml. All solutions were stored at 4 °C before use.

2.5. Sample collection

Immediately after collection, human blood samples (1 ml) were accurately aliquotted into prechilled heparinized Eppendorf tubes containing 200 μg pyridostigmine bromide and centrifuged at 12,000 × g for 10 min at 4 °C. Plasma was then transferred into prechilled Eppendorf tubes and stored at −80 °C pending analysis.

2.6. Sample preparation

50 μl I.S. working solution and 50 μl methanol:water (50:50, v/v) were added into 50 μl human plasma in a glass tube and the mixture subjected to liquid–liquid extraction (LLE) using 3 ml diethyl ether:dichloromethane (60:40, v/v). After centrifugation for 5 min at 2000 × g, the organic layer was transferred to another glass tube and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 500 μl methanol:water (50:50, v/v) and 20 μl injected into the LC–MS system.

2.7. Assay validation

The method was fully validated according to Guidance for Industry, Bioanalytical Method Validation of the FDA [14].

2.7.1. Specificity

Specificity of the method was based on demonstrating the ability of the assay to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, blank samples of analyses were obtained from six different healthy subjects.

2.7.2. Linearity and lower limit of quantitation

Linearity in the range 0.5–500 ng/ml was assessed by weighted (1/x^2) least squares linear regression of calibration curves based on analyte/I.S. peak area ratios prepared in triplicate. The precision and accuracy of lower limit of quantitation (LLOQ), which was the lowest standard on the calibration at 0.5 ng/ml, was also assessed by six replicates on three consecutive days.

2.7.3. Precision and accuracy

Precision and accuracy were determined by assay of six replicates of QC samples on three consecutive days. Intra- and inter-day precision (RSD) and accuracy (RE) were required to be <15% and ±15% respectively.

2.7.4. Recovery and matrix effects

Recovery was evaluated by comparison of peak areas of analyte and I.S. in six replicates of QC samples with those of analyte and I.S. added to extracted blank plasma at the same concentrations. Matrix

effects were determined by comparing peak areas of analyte and I.S. added to six replicate extracted blank plasma samples at the concentrations of 1.5, 15 and 150 ng/ml with the mean peak areas of standard solutions at the same concentrations.

2.7.5. Stability

Stability of landiolol in human plasma was evaluated by assay of three replicates of QC samples under the following conditions: freeze/thaw stability after three freeze/thaw cycles (−80 to 25 °C); long-term stability after storage at −80 °C for 60 days; and short-term stability after storage at ambient temperature (25 °C) for 4 h. Stability in processed samples was assessed after 3 h in the autosampler set at 18 °C.

2.8. Pharmacokinetic study

The study was performed according to the guidelines of the World Medical Assembly of Helsinki concerning the ethical consideration in human experiments. Protocol was approved by the Ethics Committee of the Second Clinical Hospital Affiliated to the Chinese Medical University, Shenyang (China).

A loading infusion at 50 μg/kg/min for 1 min was followed by a continuous infusion at 10 μg/kg/min for 60 min to 8 healthy volunteers with ethics approval. Blood samples were collected before infusion, at 6, 16, 31 and 46 min during the continuous infusion and at 1, 2, 5, 8, 11, 14, 17, 20, 23, 26, and 30 min after completion of the continuous infusion. Blood samples were immediately treated as described in Section 2.5 and stored at −80 °C for further analysis.

3. Results and discussion

3.1. LC–MS conditions

Mass spectrometric detection was carried out on a Q-trap instrument equipped with an ESI source operated in the positive ion mode. During optimization of the mass spectrometric parameters, the strong signals of landiolol and bisoprolol were observed in the form of their $[M+H]^+$ molecular ions with mass to charge ratios of m/z 510.1 and m/z 326.3, respectively. Different CE values for landiolol were tested, and the product ion at m/z 157.2 was more abundant and stable than other product ions with CE of 45 eV. Fragmentation of I.S. produced the most abundant product ion at m/z 116.1. Accordingly, the ion transitions m/z 510.1 → 157.2 and 326.3 → 116.1 were selected for MRM of analyte and I.S., respectively (Fig. 1).

Separation was evaluated using several C_{18} columns and mobile phases containing mixtures of methanol, acetonitrile, water and ammonium acetate to obtain suitable retention times, symmetrical peak shapes and low matrix effects. Methanol produced higher signals than acetonitrile with no solvent-clustered ions. The addition of 1% formic acid to the aqueous component improved peak shape significantly and the inclusion of 10 mM ammonium acetate produced a buffer system with stable pH. Finally the best combination of peak shape and retention time was achieved using a TC-C_{18} column (150 mm × 4.6 mm, 5 μm) with a mobile phase of methanol:10 mM ammonium acetate containing 1% formic acid (65:35, v/v).

3.2. Selection of I.S.

Due to their similar structures, bisoprolol tracked landiolol during LLE with almost the same recovery. It also eluted close to landiolol from the column and gave satisfactory positive-ion electrospray ionization. Thus bisoprolol was selected as a suitable I.S. in order to guarantee high accuracy and precision of LC–MS/MS assay.
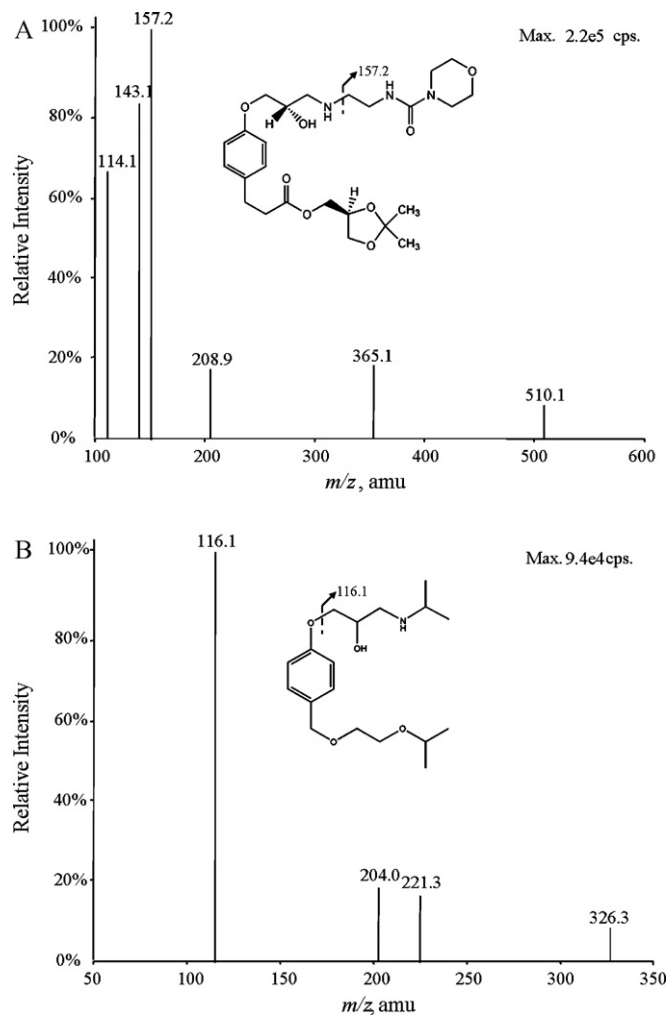

Fig. 1. Full-scan product ion mass spectra of $[M+H]^+$ ions of (A) landiolol and (B) bisoprolol.

3.3. Sample collection

Landiolol in the blood/plasma samples would not be quantitated exactly as landiolol was enzymatically hydrolyzed very quickly by pseudocholinesterase during storage and pretreatment without any process of samples. Therefore landiolol would be prevented from degradation by adding the pseudocholinesterase inhibitor of pyridostigmine bromide into blood [15]. Meanwhile the activity of pseudocholinesterase would be inhibited partially at the ice-cold temperature. Stability of landiolol in human blood was evaluated. Landiolol (15 ng or 150 ng) was spiked in the freshly blank blood samples (1 ml) with or without pyridostigmine bromide (200 μg). The above blood samples were immediately incubated on ice. The stability of landiolol in blood samples was determined at a time course of 0–60 min. The results showed landiolol in human blood was stable with pyridostigmine bromide on ice (Fig. 2).

3.4. Sample preparation

Sample preparation by protein precipitation, SPE and LLE was evaluated. Protein precipitation resulted in poor peak shape and significant ion suppression. SPE produced relatively clean extracts but recovery was low. One step LLE with diethyl ether:dichloromethane (60:40, v/v) at a relative volume of 3 ml to 50 μl plasma gave efficient extraction of both landiolol and I.S. in an extract suitable for determination.
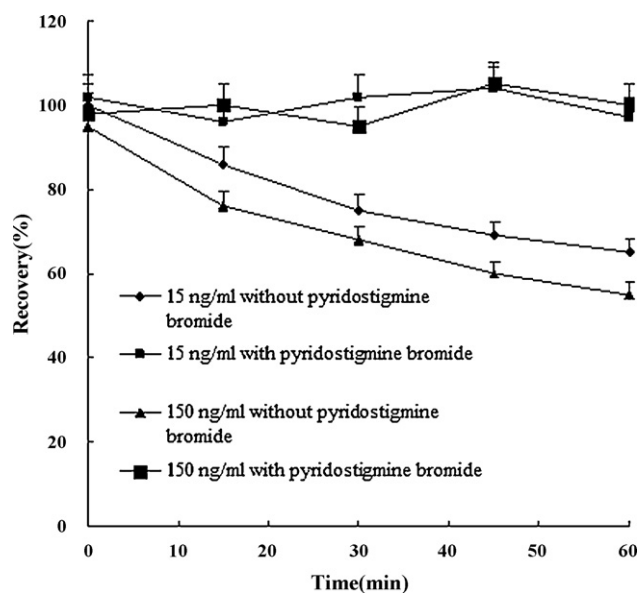
Fig. 2. Effects of pyridostigmine bromide on the stability of landiolol in human blood (data are means ± SD, $n = 3$).

3.5. Assay validation

3.5.1. Specificity

As shown in Fig. 3A, no significant interference was observed from endogenous substances in plasma at the retention times of landiolol and bisoprolol. Fig. 3B shows a representative ion chromatogram of a standard sample at a concentration close to the LLOQ (0.5 ng/ml) with a signal to noise ratio of about 20. Fig. 3C shows the chromatogram of a plasma sample from a healthy volunteer taken during infusion of landiolol hydrochloride.

3.5.2. Linearity and LLOQ

The method was linear over the concentration range 0.5–500 ng/ml with correlation coefficients in the range 0.9975–0.9996. A typical equation of a calibration curve was $y = 0.0255x + 0.000726$, $r = 0.9975$. The LLOQ for landiolol was found to be 0.5 ng/ml. The ratio of signal to noise was about 20 (Fig. 2B). The response of LLOQ was found to be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% (Table 1).

3.5.3. Precision and accuracy

Precision and accuracy data are presented in Table 1. Intra- and inter-day precisions (RSD) were <4.4% and <10.0%, respectively, with accuracy (RE) <10.0%.

3.5.4. Recovery and matrix effects

Recoveries of landiolol (mean ± SD) for low, medium and high QC samples were 97.2 ± 2.0%, 100.1 ± 2.9% and 102.6 ± 0.9%, respectively. Recovery of I.S. was 101.7 ± 2.0%. The results indicate that recoveries of analyte and I.S. are satisfactory and reliable. In terms of matrix effects, the ratios of peak responses for low, medium and high QC samples were 100.5 ± 3.9%, 98.2 ± 0.6%, and 96.4 ± 1.1%, respectively. The ratio for bisoprolol was 98.6 ± 2.4%. On this basis matrix effects do not compromise the performance of the method.
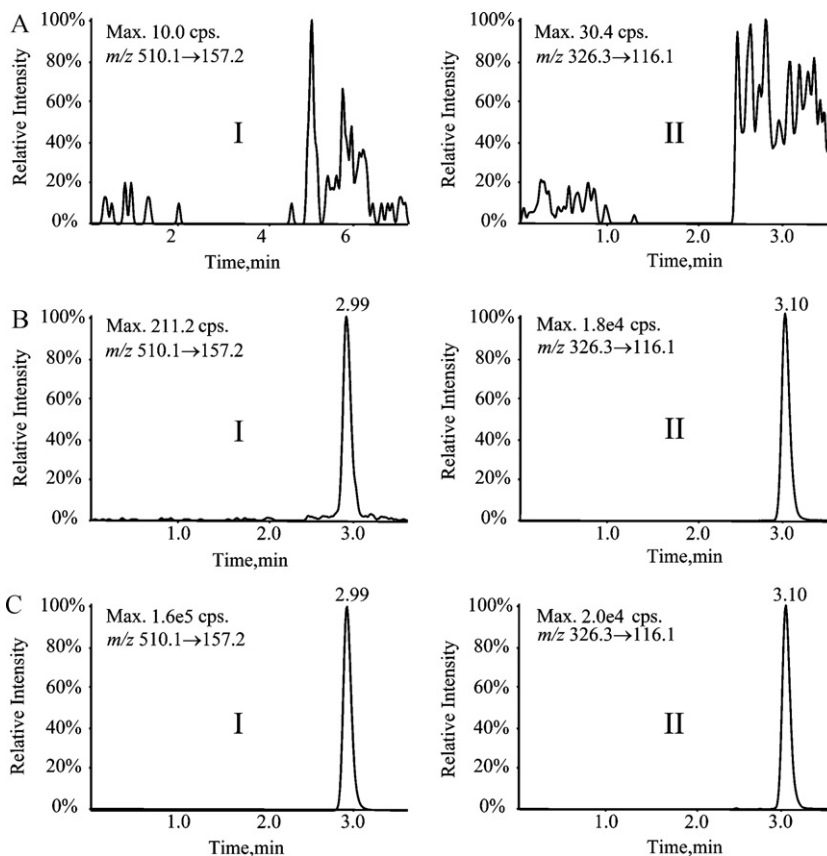

Fig. 3. Typical MRM chromatograms of (I) landiolol and (II) bisoprolol in human plasma: (A) blank plasma; (B) blank plasma spiked with 0.5 ng/ml landiolol and 100 ng/ml bisoprolol (LLOQ); and (C) a plasma sample from a human volunteer at 46 min during commencement of a continuous infusion of landiolol hydrochloride (280 ng/ml).

Table 1
Precision and accuracy for the determination of landiolol in human plasma (data were based on assay of six replicates on three consecutive days).

Spiked concentration (ng/ml)	Calculated concentration (ng/ml) (mean ± SD)	Intra-day, RSD (%)	Inter-day, RSD (%)	Accuracy, RE (%)
0.5 (LLOQ)	0.48 ± 0.03	1.31	2.17	1.52
1.5	1.52 ± 0.06	4.36	4.59	0.34
15	15.5 ± 0.6	1.93	9.96	2.57
150	152 ± 1	2.27	8.98	1.36

Table 2
Stability of landiolol in human plasma under different storage conditions (n = 3).

Storage conditions	Initial concentration (ng/ml)	Final concentration (ng/ml) (mean ± SD)	RE (%)
In human plasma at	1.5	1.66 ± 0.04	10.7
25 °C for 4 h	15	15.3 ± 0.1	2.00
	150	147 ± 0	−2.00
In human plasma after	1.5	1.57 ± 0.13	4.67
three freeze/thaw	15	16.3 ± 0.5	8.67
cycles	150	163 ± 1	8.67
In human plasma for	1.5	1.52 ± 0.07	1.33
60 days at −80 °C	15	14.8 ± 0.3	−1.33
	150	148 ± 1	−1.33
In processed samples at	1.5	1.54 ± 0.07	2.67
18 °C for 3 h	15	15.2 ± 0.3	1.33
	150	149 ± 3	−0.67
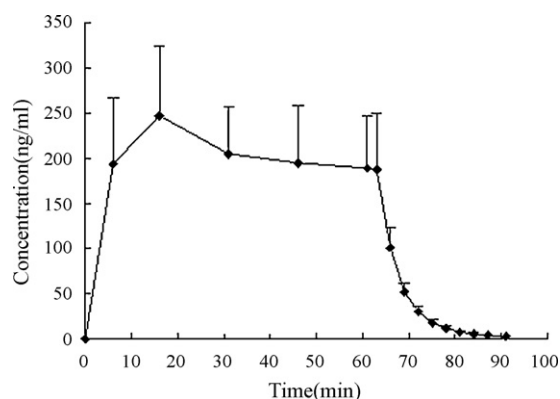
Fig. 4. Mean plasma concentration–time curve for landiolol in healthy volunteers involving a continuous infusion of landiolol hydrochloride after a loading infusion (data are means ± SD, n = 8).

3.5.5. Stability

The results of stability tests are shown in Table 2. With the addition of pyridostigmine bromide, landiolol was stable in human plasma under all the conditions evaluated.

3.6. Pharmacokinetic study

The method was successfully applied to the determination of landiolol in human plasma in a pharmacokinetic study involving continuous infusion of landiolol hydrochloride to healthy volunteers. A concentration–time profile for landiolol in these volunteers is shown in Fig. 4. Landiolol could be determined in all plasma samples for 30 min after cessation of the continuous infusion. The results confirm that the method is sufficiently sensitive to allow investigation of the pharmacokinetics of landiolol involving continuous infusion under the dosing condition mentioned above for healthy Chinese volunteers.

4. Conclusion

A rapid and sensitive LC–MS/MS method for the determination of landiolol in human plasma has been developed and validated. Addition of an esterase inhibitor (pyridostigmine bromide) ensures stability of landiolol in the blood/plasma samples under normal storage conditions. The main advantage of the method is that it only needs small volume of plasma with simple and rapid sample preparation of high recovery, short analytical run time and excellent sensitivity. The method was shown to be suitable for the application to clinical pharmacokinetic studies.

Acknowledgments

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