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Short communication

High-performance liquid chromatography for the determination of 3-*n*-butylphthalide in rat plasma by tandem quadrupole mass spectrometry: Application to a pharmacokinetic study

Zhanqi Niu, Fengju Chen, Jin Sun, Xiaohong Liu, Yongjun Wang, Dawei Chen, Zhonggui He*

Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, No. 103, Wenhua Road, Shenyang 110016, China

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ABSTRACT

A rapid, sensitive and specific high performance liquid chromatography–electrospray ionization tandem quadrupole mass spectrometry (HPLC–MS/MS) method was developed and validated for the determination of 3-*n*-butylphthalide in rat plasma. Following protein precipitation with acetonitrile, 3-*n*-butylphthalide and glipizide (internal standard, I.S.) were separated using a gradient elution program on a C_{18} column and detected by mass spectrometry in positive ion mode with the multiple reaction monitoring (MRM) mode using the respective precursor to product ion combinations of *m/z* 191/145 for 3-*n*-butylphthalide and *m/z* 446/321 for glipizide, respectively. The total chromatographic running time was 2.5 min. The method was linear over the concentration range of 11.14–3480.00 ng/mL, using as little as 100 μL plasma. The lower limit of quantification (LLOQ) was 5.57 ng/mL. Finally, the method was successfully used to support a preclinical pharmacokinetic study of 3-*n*-butylphthalide in rats following intravenous administration.

1. Introduction

3-*n*-Butylphthalide was volatile drug isolated from several plants including *Apium graveolens*, *Ligusticum sinensis* and *Ligusticum wallichii* [1–3]. It is a chiral compound, and the activities of the two enantiomers are not equivalent [4,5]. In preclinical and clinical experiments, *s*-(−)-NBP and (±)-NBP [(±)-3-butyl-1(3H)-iso-benzofuranone, Fig. 1] were found to attenuate cerebral ischemic damage mainly due to their ability to increase regional cerebral blood flow (rCBF) in the ischemic zone and their inhibitory effects on the release of glutamate and 5-hydroxytryptamine [6,7]. Consequently, 3-*n*-butylphthalide [(±)-NBP] may be a promising new drug for the treatment of ischemic cerebral diseases such as stroke. The State Food and Drug Administration of China had recently approved 3-*n*-butylphthalide for Phase III trials in the treatment of stroke.

Radiometric analysis with ^{3}H labeled 3-*n*-butylphthalide had been used to study its *in vivo* elimination in rat urine and feces [8], but this method cannot differentiate between 3-*n*-butylphthalide from its metabolites. A gas chromatography–mass spectrometry (GC–MS) method was developed to identify the metabolic pathway in rats after oral administration [9]. In our previous study, we set up a HPLC method with fluorescence detection to determine 3-*n*-butylphthalide in rabbit plasma [10]. Unfortunately, this method was rather time-consuming and insensitive, with a total run time over 10 min and a lower limit of quantification (LLOQ) of 21.2 ng/mL. Since 3-*n*-butylphthalide is highly volatile and is present in low concentrations in body fluid, speed of preparation and sensitivity are of great importance for its determination in biological samples.

Combined the above methods, we attempted to develop a novel, rapid, selective and highly sensitive method to determine 3-*n*-butylphthalide in rat plasma using HPLC–MS/MS. The validation results showed a higher sensitivity (with an LLOQ as low as 5.57 ng/mL) and need for a lower plasma volume (100 μL) compared with the previous methods. This method was then successfully applied to a preclinical pharmacokinetic study in rats after intravenous administration.

2. Experimental

2.1. Chemicals and reagents

3-*n*-Butylphthalide was provided by Shijiazhuang Pharmaceutical Co. (Shijiazhuang, China). The I.S., glipizide, was purchased from the National Institute for Control of Pharmaceutical and Biological

* Corresponding author at: Mailbox 59#, Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, No. 103 Wenhua Road, Shenyang 110016, China. Tel.: +86 24 23986321; fax: +86 24 23986321.
E-mail address: hezhonggui@gmail.com (Z. He).

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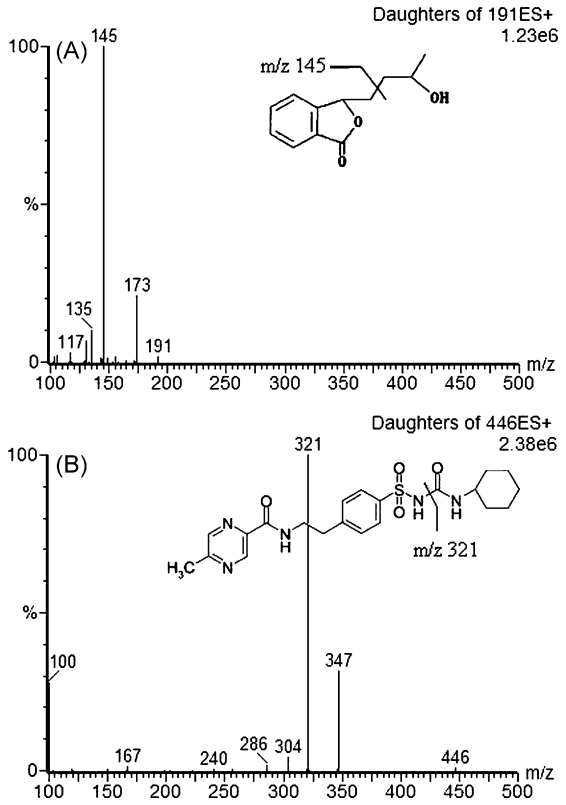

Fig. 1. Daughter scan product ion mass spectra of 3-*n*-butylphthalide (A) and glipizide (B).

Products (Beijing, PR China). Acetonitrile and methanol of HPLC-grade were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water (>18 MΩ) was obtained using an EASYPURE® II RF/UV ultrapure water system (Barnstead International Corp., USA). Formic acid (HPLC) was purchased from Dikma (Richmond Hill, NY, USA) and high-purity nitrogen (99.999%) was used.

2.2. Equipment and operating conditions

2.2.1. Liquid chromatography

Analyses were acquired on an ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA) with a cooling auto-sampler and column oven allowing accurate temperature control of the analytical column. An ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was used. The chromatographic separations were accomplished using gradient elution with a mobile phase composed of acetonitrile and water containing 0.1% formic acid. The gradient elution program in the initial stage was maintained at 50% water for 1 min, then changed linearly to 90% water (1–3 min) followed by a return to the initial conditions. The column temperature was maintained at 35 °C with the flow rate set at 0.2 mL/min. The auto-sampler temperature was conditioned at 7 °C. The injection volume was 5 μL using the partial loop mode for sample injection. The chromatographic run time per sample was 3.0 min. From 0.8 min to 2.5 min, the eluent was injected into detector, and the remainder was diverted to waste.

2.2.2. Mass spectrometry

The separated compounds were detected by a Waters Tandem Quadrupole (TQ) Detector (Waters). The mass spectrometer was operated with an electrospray ionization (ESI) interface in positive ionization mode. The ionization source conditions were: capillary voltage of 3.0 kV, cone voltage of 30 V, the optimized collision energy was 15 V and 28 V for 3-*n*-butylphthalide and glipizide, respectively; source temperature 120 °C and desolvation temperature 250 °C. The cone and desolvation gas flow rates were 50 L/h and 600 L/h, respectively, and were obtained from an in-house nitrogen source, Argon was used as collision gas at a pressure of approximately 3.30×10^{-3} mbar and the multipliers were set to 650 V. Under these HPLC–MS/MS conditions, the compounds were analyzed by multiple reaction monitoring (MRM) of the transitions of m/z 191 → 145 for 3-*n*-butylphthalide and m/z 446 → 321 for glipizide (I.S.), respectively. The scan time was set at 0.02 s per transition. Data were acquired using Masslynx 4.1 software.

2.3. Preparation of standards and quality control samples

Calibration standards were prepared by spiking 100 μL drug-free rat plasma with 100 μL of the appropriate standard solutions. The effective concentrations in plasma samples were 11.14, 27.84, 55.68, 139.20, 696.00, 1044.00 and 3480.00 ng/mL. The quality control (QC) samples were prepared at concentrations of 27.84, 139.20 and 2320.00 ng/mL in a similar way to the calibration standards. These calibration standard samples and QC samples were stored at −20 °C. Before processing each analytical batch, the appropriate standards and QCs were brought to room temperature and processed together with the biological samples.

2.4. Sample preparation

A 100 μL aliquot of the I.S. solution (glipizide, 82.9 ng/mL) was added to 100 μL plasma samples, followed by 100 μL acetonitrile and 50 μL 1% formic acid. The samples were vortexed for 1 min and deproteinized with 500 μL acetonitrile, and the precipitate was removed by centrifugation at 13,000 × *g* for 10 min. Then, the supernatant acetonitrile layer was transferred to glass vials, and an aliquot of 5 μL was injected into the HPLC–MS/MS system for analysis. In addition, the samples at the first time point was double diluted with blank plasma because the 3-*n*-butylphthalide concentrations were above the stated upper limit of the linear range.

2.5. Method validation

The method was validated for linearity, selectivity, LLOQ, accuracy, precision, matrix effect, extraction recovery and stability. The different validation parameters and the values for accepting the range of validation parameters were in accordance with international guidelines [11].

Standard calibration graphs were constructed by linear least-squares regression analysis on the analyte/I.S. area ratio plotted against the sample concentration with $1/x^2$ (x is the concentration of 3-*n*-butylphthalide) as the weighting factor and assayed in duplicate on three consecutive days. During routine analysis, each analytical run included a set of calibration standards, a set of QC plasma samples in duplicate at intervals per batch and plasma samples to be determined.

Selectivity was assessed by comparing chromatograms of six different batches of blank plasma obtained from six rats with those of corresponding standard plasma samples spiked with 3-*n*-butylphthalide and glipizide (82.9 ng/mL) and a plasma sample after intravenous administration.

Calibration curves were prepared using seven standard plasma samples over the range 11.14–3480.00 ng/mL.

LLOQ was defined as a signal/noise ≥10, and the precision and accuracy were evaluated by analyzing six samples which were prepared in six replicates, and the results were less than 20%.

Precision and accuracy of the method were assessed using QC samples at three concentration levels (27.84, 139.20 and 2320.00 ng/mL) performed on three separate days. On each day, six replicates of QC samples at each concentration level were analyzed.

The extraction recovery of 3-*n*-butylphthalide following protein precipitation was assessed by comparing the mean peak area of the regularly prepared samples at three concentrations (27.84, 139.20 and 2320.00 ng/mL) with the mean peak area of spike-after-extraction plasma samples. To prepare the spike-after-extraction samples, blank rat plasma was processed according to the sample preparation procedure described above. The supernatant was mixed with the appropriate standard solutions of 3-*n*-butylphthalide at concentrations corresponding to the final concentration of the pretreated plasma samples. The recovery of I.S. was also evaluated.

Six different lots of blank plasma were extracted and then spiked with the analyte at 27.84, 139.20 and 2320.00 ng/mL. The corresponding peak areas of the analyte in the spiked plasma post-extraction (*A*) were then compared with those of the solution standards in mobile phase (*B*) at equivalent concentrations. The absolute matrix effect (ME) was calculated as follows:

$$\mathrm{ME} = \frac{A}{B} \times 100$$

The value of 100% indicates that the response for 3-*n*-butylphthalide in the plasma extracts and in the mobile phase was the same and no absolute ME was observed. The value >100% indicates ionization enhancement, and <100% is ionization suppression. Evaluation of the relative ME was made by a direct comparison of the analyte peak areas between different lots of plasma. The variability in the values, expressed as RSD (%), is a measure of the relative ME of the target analyte.

The stabilities of 3-*n*-butylphthalide in rat plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at concentrations of 27.84, 139.20 and 2320.00 ng/mL. The stability of the post-preparation samples was evaluated by keeping the prepared samples in an auto-sampler maintained at 7 °C for 6 h and 12 h, and then analyzing them. The freeze and thaw stability was determined by subjecting unextracted quality control samples to three freeze–thaw cycles. After completing three cycles, the samples were processed and analyzed. The long-term stability was examined by storing QC samples at −20 °C for 15 d. After that the samples were analyzed. Unextracted QC samples were kept at ambient temperature (25 °C) for 6 h and 12 h in order to establish the short-term stability of 3-*n*-butylphthalide in rat plasma. These analysis results were compared with those obtained from freshly prepared plasma samples. The analytes were considered stable in the biological matrix when 85–115% of the initial concentrations were found.

2.6. Pharmacokinetic study

The developed method was used to determine the plasma concentrations of 3-*n*-butylphthalide in a preclinical study in six healthy adult male Wistar rats (Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, Liaoning, China) weighing 200 ± 20 g (mean ± standard deviation). The rats were starved for 18 h prior to surgery but supplied with water *ad libitum*. The animal studies were approved by the Shenyang Pharmaceutical University Animal Care and Use Committee.

The injection was prepared with 0.9% NaCl:propylene glycol (3:2) to give a solution with a final concentration of 5 mg/mL.

The rats received a single injection of 5 mg/kg, then, 0.3 mL blood samples were collected into heparinized tubes by puncture of the retro-orbital sinus according to the following time schedule: 0, 2, 5, 10, 20, 40, 60, 120, 240 and 360 min post-dosing. The blood samples were centrifuged immediately at 3500 rpm for 10 min to obtain plasma. The plasma samples were labeled and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Mass spectrometry

The chemical structure (Fig. 1) of 3-*n*-butylphthalide indicates that it tends to capture a proton, so it gives a strong mass response in positive ion mode. Mass spectrometric detection was firstly investigated using APCI and ESI sources. The ESI source provided a better response than the APCI source. Further analysis development was therefore limited to the ESI source in this study. Then, 3-*n*-butylphthalide and glipizide were injected directly into the mass spectrometer along with the mobile phase to obtain the full scan spectra. The MS/MS parameters, cone voltage and collision energy of the compounds were evaluated for the best response of the parent ion and daughter ion respectively by the automatic optimization process and then transferred to the MRM method. This process was also verified by manual operation. The cone voltage was set at 30 V because 3-*n*-butylphthalide does not give highly protonated molecules $[M+H]^+$ at very low and high energy. At a different collision energy (CE), two major fragment ions of $[M+H]^+$ were obtained. Comparing the two transitions, m/z 191 → 145 and m/z 191 → 173, the former gave a considerably better response and a higher signal-to-noise ratio. As a result, the transition of m/z 191 → 145 at CE 15 V was chosen for use in MRM. For glipizide, its structure allows it also to give a high mass spectrometric response under positive ESI conditions. The collision energy 28 V was set to obtain the highest abundance. The full scan spectrum was dominated by protonated molecules $[M+H]^+$ m/z 191 and 446 for 3-*n*-butylphthalide and glipizide, and the major fragment ions observed in each product spectrum were m/z 145 and 321, respectively (Fig. 1).

3.2. Chromatography

Faster chromatographic separation for determination of multiple components in biological samples is a common goal in pharmaceutical analysis. This method used an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm, i.d. 1.7 μm particle size) to provide fast chromatographic separations and excellent stability while maintaining satisfactory chromatographic resolution. The maximum operating pressure was about 5800 psi under the conditions used, which is not a very high-pressure separation in this system. The retention time of 3-*n*-butylphthalide and glipizide were very short, 1.90 min and 1.12 min, respectively. The mobile phase was composed of acetonitrile–water (0.1% formic acid) and the presence of a small amount of formic acid was able to improve the detection response of the analytes in positive ion mode. Gradient elution was employed in order to extend the column life, elute the analyte rapidly and improve peak shape. Under these chromatographic conditions, the total run time was just 2.5 min per sample and this met the requirement for a high sample throughput.

3.3. Choice of internal standard

An appropriate choice of I.S. will reduce errors occurring during the sample preparation, injection and detection. Similar behavior is expected during the sample preparation stage as well as the chromatographic separation. The selection of glipizide as I.S. in the present study was based on its ready availability, low cost and its

unlikely presence in plasma. However, glipizide belongs to a different chemical class, and has a significantly different fragmentation pattern, but a strong mass response in positive ESI mode, and the pretreatment steps as well as the chromatographic retention time were similar to those of 3-*n*-butylphthalide.

3.4. Sample preparation

Since 3-*n*-butylphthalide is a volatile drug, the more complex pretreatment, the lower the extraction recovery. In the present study, a one-step protein precipitation procedure was adopted to obtain a high extraction recovery. The protein precipitant was acetonitrile because of its efficient protein precipitation and extraction. The chromatograms show that, even at low levels, the target drugs can be determined without any significant interference (Fig. 2).

3.5. Method validation

3.5.1. Selectivity

Fig. 2 shows that there is no interference from endogenous substances observed at the retention time of the analytes.

3.5.2. Matrix effect

All the ratios of ME for 3-*n*-butylphthalide and glipizide were between 85% and 115%, and the relative matrix effect result was RSD <10%, which means that there is no significant matrix effect in this method.

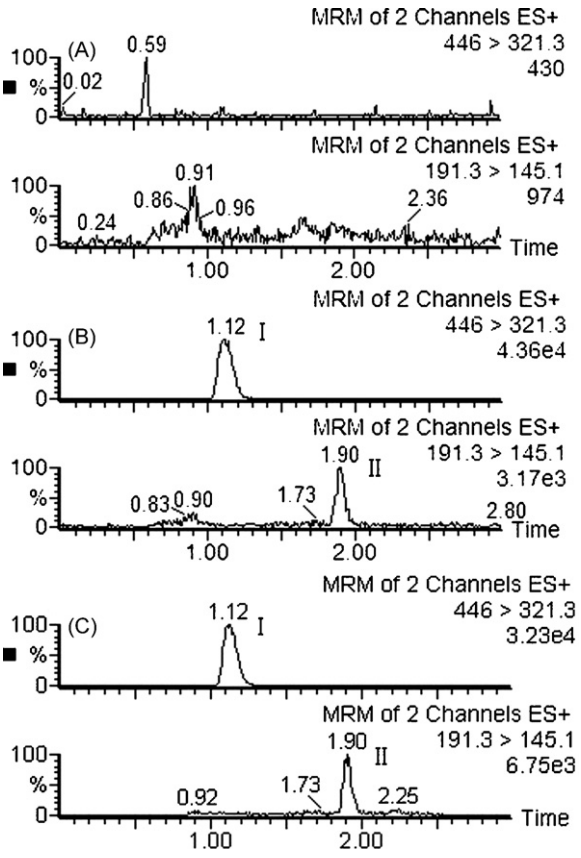

Fig. 2. Representative MRM chromatograms of 3-*n*-butylphthalide (peak II, channel 2) and glipizide (peak I, channel 1) in rat plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with 3-*n*-butylphthalide at an LLOQ of 5.57 ng/mL and I.S. (82.9 ng/mL); (C) plasma sample from a rat, 24 h after intravenous administration of 3-*n*-butylphthalide, the concentration of 3-*n*-butylphthalide is 15.20 ng/mL. The retention times for 3-*n*-butylphthalide and glipizide were 1.12 min and 1.90 min, respectively.

Table 1
Precision and accuracy for the determination of 3-*n*-butylphthalide in rat plasma ($n = 6$)

Concentration (ng/mL)		RSD (%)		RE (%)
Nominal	Found (mean)	Intra-day	Inter-day	
5.57 (LLOQ)	5.43	6.72	4.75	−2.42
27.84	28.78	8.19	10.51	3.37
139.20	138.99	3.84	5.27	−0.15
2320.00	2272.32	3.54	7.21	−2.06

3.5.3. Linearity and LLOQ

The linear regression of the peak area ratio versus concentration was fitted over the concentration range of 11.14–3480.00 ng/mL in rat plasma. A typical equation for the calibration curve was as follows: $y = 0.892109x + 4.22463$ ($r = 0.9990$), where y is the peak area ratio of 3-*n*-butylphthalide to glipizide, and x (ng/mL) is the plasma concentration of 3-*n*-butylphthalide. The standard errors of slope and intercept were 0.05 and 0.53, respectively. Good linearity ($r > 0.9967$) was seen over this concentration range in all analytical runs.

LLOQ was 5.57 ng/mL (Table 1). Under the present LLOQ, the 3-*n*-butylphthalide concentrations could be determined in plasma samples up to 36 h after a single intravenous dose of 5 mg/kg, which is sensitive enough to investigate the pharmacokinetic behavior of the drug.

3.5.4. Precision and accuracy

The intra-day precision (R.S.D.) for QC samples (27.84, 139.20 and 2320.00 ng/mL) was 10.51%, 5.27% and 7.21%, and that of inter-day analysis was 8.19%, 3.84% and 3.54%, respectively; The relative errors ranged from −2.06% to 3.37%. More information is given in Table 1. The errors were high, which may be due to the sample preparation procedure. Controlling the pretreatment steps will improve the method reproducibility.

3.5.5. Extraction recovery and stability

Mean extraction recoveries of 3-*n*-butylphthalide at three concentrations (27.84, 139.20 and 2320.00 ng/mL) were 84.20 ± 4.13%, 88.84 ± 0.44% and 88.69 ± 0.85%, respectively ($n = 6$). The mean recovery of the I.S. (82.9 ng/mL) was 91.16% ($n = 6$).

The stability of 3-*n*-butylphthalide is shown in Table 2. The results indicate that the analyte was stable under the storage conditions during the chromatography, extraction and storage of the plasma samples. Glipizide was also stable at ambient temperature and in the auto-sampler, confirming the suitability of this method of determination.

Table 2
Summary of stability of 3-*n*-butylphthalide in rat plasma under various storage conditions ($n = 3$)

Storage conditions	Concentration (ng/mL)		RSD (%)	RE (%)
	Nominal	Found		
Ambient temperature (12 h)	27.84	29.70	0.89	6.68
	139.20	137.63	5.01	−1.12
	2320.00	2166.40	2.63	−6.62
Auto-sampler (7 °C, 12 h)	27.84	29.30	1.56	5.24
	139.20	131.33	3.62	−5.65
	2320.00	2174.30	1.70	−6.28
Three freeze–thaw cycles	27.84	30.37	4.12	10.03
	139.20	135.40	4.12	−3.60
	2320.00	2293.80	0.88	−1.72
Long-term (15 d at 20 °C)	27.84	30.07	9.01	8.00
	139.20	146.33	5.33	5.12
	2320.00	2413.67	4.63	4.04

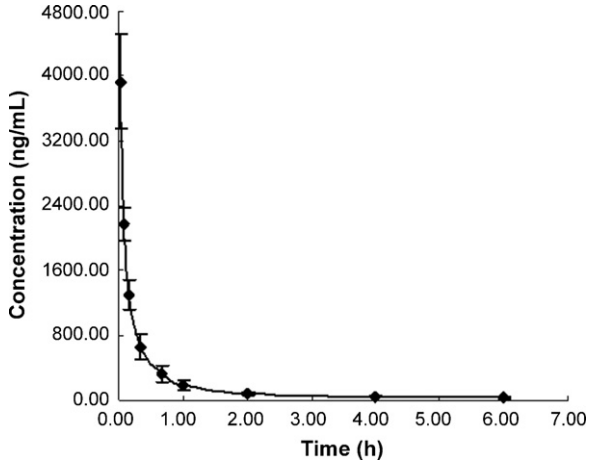

Fig. 3. The logarithm of mean plasma concentration–time curves of 3-*n*-butylphthalide after a single intravenous dose of 5 mg/kg to six rats. Each point represents the mean ± S.D.

3.6. Application of the method to a pharmacokinetic study

This validated analytical method has been successfully applied to determine the plasma concentrations of 3-*n*-butylphthalide to support preclinical pharmacokinetic studies in rats following intravenous administration. The profile of the mean plasma concentration of 3-*n*-butylphthalide versus time is shown in Fig. 3. The pharmacokinetic modeling was carried out using DAS 2.0. The plasma concentration–time data best conformed to a two-compartment intravenous model with a weight of $1/C \times C$, AIC = 2.007. The area under plasma concentration–time curve ($AUC_{0-6\,h}$) of 1140.16 ng·h/mL, an apparent volume of distribution (Vc) of 1.22 L/kg, a distribution half-life ($T_{1/2\alpha}$) of 0.098 h, an elimination half-life ($T_{1/2\beta}$) of 2.62 h, a clearance [CL(s)] of 3.67 L/h kg. The results were similar with those reported before.

4. Conclusion

This paper describes a rapid, sensitive and specific HPLC–MS/MS method for the determination of 3-*n*-butylphthalide in rat plasma as well as its validation. Compared with the previous methods, HPLC–MS/MS has a high column efficiency, a very short single run time, reduced ion suppression, superior sensitivity and satisfactory selectivity. The method has also been successfully applied to a preclinical pharmacokinetic study of 3-*n*-butylphthalide in rats.

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