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A simple and sensitive HPLC–ESI-MS/MS method for the determination of andrographolide in human plasma

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ABSTRACT

A liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) method for the determination of andrographolide in human plasma was established. Dehydroandrographolide was used as the internal standard (I.S.). The plasma samples were deproteinized with methanol and separated on a Hanbon C_{18} column with a mobile phase of methanol–water (70:30, v/v). HPLC–ESI-MS/MS was performed in the selected ion monitoring (SIM) mode using target ions at $[M-H_2O-H]^-$, m/z 331.1 for andrographolide and $[M-H]^-$, m/z 331.1 for the I.S. Calibration curve was linear over the range of 1.0–150.0 ng/mL. The chromatographic separation was achieved in less than 6.5 min. The lower limits of quantification (LLOQ) was 1.0 ng/mL. The intra and inter-run precisions were less than 6.95 and 7.22%, respectively. The method was successfully applied to determine the plasma concentrations of andrographolide in Chinese volunteers.

1. Introduction

Andrographolide is one of the main active constituents of *Andrographis paniculate* (Burm) Nees, a famous traditional Chinese medicine that belongs to the Acanthaceae family, and is traditionally used as a medicinal herb to treat different diseases in India, China and Southeast Asia [1]. Andrographolide has many types of bioactivity, such as anti-inflammatory, anti-microbial [2,3], anti-platelet aggregation [4,5], hepatoprotective [6,7], and anti-HIV [8] activities. Andrographolide is widely used clinically with good results. Several HPLC [9–15], CE [15], GC–MS [15] and LC–MS [16] methods have been reported for the determination of andrographolide in plasma, in which the most sensitive assay is the LC–MS by liquid–liquid extraction (LLE) with an LLOQ of 9.9 ng/mL [16]. The method of liquid–liquid extraction (LLE) was time-consuming in sample pretreatment. The pilot pharmacokinetic study results in our laboratory showed that some of the human plasma levels of andrographolide on the terminal elimination phase were below 9.9 ng/mL after 6 h with the oral administration of 200 mg andrographolide. So, to evaluate the pharmacokinetics of andrographolide in humans, we needed to develop a more sensitive method for the determination of andrographolide in human plasma. This paper describes the development and validation of a sensitive LC–ESI-MS/MS method with an LLOQ as low as 1.0 ng/mL for the quantification of andrographolide in human plasma with one-step precipitation. The method was successfully applied to study the pharmacokinetics of andrographolide in healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and reagents

The standards of andrographolide (Fig. 1A, 99.8%, purity) and dehydroandrographolide (Fig. 1B, 99.6%, purity) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The test formulation was andrographolide dispersible tablets Containing 50 mg of andrographolide per tablet, which was provided by Jiangsu HeXin Pharmaceutical Co. Ltd. (Nanjing, China). Methanol was of HPLC grade (Merck, Darmstadt, Germany). Distilled water, prepared from demineralized water was used throughout the study.

2.2. Instrumentation and conditions

The HPLC–ESI-MS/MS equipment consisted of a Surveyor LC pump, a Surveyor auto-sampler, a Finigan TSQ Discovery Max

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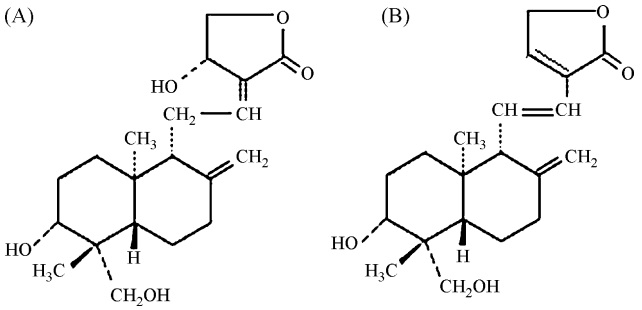

Fig. 1. Chemical structures andrographolide (A) and dehydroandrographolide (B).

tandem mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA), equipped with an ESI ion source. Xcalibur software v.1.3 was used for data acquisition and analysis. LC separation was performed on a Hanbon C_{18} column (4.6 mm × 150 mm, 5 μm, Jiangsu Hanbon Science & Technology Co. Ltd., China) with a mobile phase of methanol–water (70:30, v/v) at a flow rate of 0.7 mL/min. The column temperature was maintained at 25 °C. HPLC–ESI-MS/MS was carried out using nitrogen to assist nebulization. Tuning the ion spray, operated in the negative ionization mode, for andrographolide resulted in a 4500 V spray voltage and a 350 °C capillary temperature with the nitrogen sheath, ion sweep and auxiliary gasses set at 40, 5 and 10 psi, respectively. The source collision-induced dissociation (sid) was set at −2 eV and the tube lens offsets were both selected at 140 V. The selected ion monitoring (SIM) mode was used and the collision energy was selected at 10 V with argon as the collision gas at 1.4 mTorr (1 Torr = 133.3 Pa). The target ions were set at $[M-H_2O-H]^-$ m/z 331.1 for andrographolide and $[M-H]^-$ m/z 331.1 for I.S.

2.3. Preparation of standard solutions

The stock solutions of andrographolide (1.0 mg/mL) and internal standard (1.0 mg/mL) were prepared in methanol and stored at −20 °C. Standard solutions of andrographolide with concentrations of 10, 1, 0.1, and 0.01 μg/mL, were prepared by serial dilution of andrographolide stock solution with methanol in separate 10 mL volumetric flasks. A solution containing 0.05 μg/mL internal standard was also obtained by further dilution of I.S. stock solution with methanol.

2.4. Sample preparation

All frozen standards and samples were allowed to thaw at room temperature and homogenized by vortexing. A 0.2-mL aliquot plasma sample was transferred to a 1.5 mL centrifuge tube together with 50 μL of I.S. (0.05 μg/mL). The sample mixture was mixed with 0.60 mL of methanol and vortex mixed for approximate 3 min, then allowed to stand for 5 min to deproteinize and the precipitate was removed by centrifugation at 16,000 rpm for 3 min. The supernatant was pipetted into an injected vial and a 10 μL aliquot was injected into the HPLC–ESI-MS/MS for analysis.

2.5. Calibration curves

Calibration standards of andrographolide were prepared by spiking appropriate amounts of the standard solutions in 0.20 mL blank plasma obtained from healthy volunteers. Standard curves were prepared in the range of 1.0–150.0 ng/mL for andrographolide at concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 150.0 ng/mL. The calibration curve was prepared and assayed along with quality control (QC) samples and each run of unknown plasma samples.

2.6. Preparation of QC samples

QC samples were prepared at concentration levels of 1.6, 16.0, and 64.0 ng/mL for andrographolide using the same method of preparing the calibration standards and stored at −20 °C. QC samples were analyzed with processed test samples at intervals in each run. The results of the QC samples provided the basis of accepting or rejecting the run.

3. Assay validation

3.1. Selectivity

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the protein precipitation procedure and HPLC–ESI-MS/MS conditions to ensure no interference of andrographolide and I.S. from plasma.

3.2. Linearity of calibration curve and LLOQ

Calibration standards of seven andrographolide concentration levels at 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 150.0 ng/mL were extracted and assayed. To evaluate the linearity, calibration curves were prepared and assayed on 5 days. The calibration curve was constructed by plotting the peak-area ratios of andrographolide to the I.S. versus the concentrations of andrographolide, using weighted least squares linear regression. The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [17], and it was established using five samples independent of standards. The QC samples were assayed along with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

3.3. Precision and accuracy

The validation samples were prepared and analyzed on three different days (one run per day) to evaluate the accuracy, intra-run and inter-run precision of the analytical method. The accuracy, intra-run and inter-run precisions of the method were determined by analyzing five replicates at 1.6, 16.0 and 64.0 ng/mL of andrographolide treated following the sample preparation procedures, including the addition of 50 μL I.S., along with one calibration curve on each of three runs. Assay precision was calculated using the relative standard deviation (RSD%). The accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions [17]. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated by using the formula $RE\% = (E - T)/T \times 100$.

3.4. Extraction recovery

The extraction recovery of the method was determined by comparing the peak areas obtained from the plasma samples with those of direct injected standards. The extraction recovery of andrographolide was evaluated by analyzing five replicates at 1.6, 16.0, and 64.0 ng/mL andrographolide. Recovery was calculated by comparison of the peak areas of andrographolide extracted from plasma samples with those of injected standards.

3.5. Stability

The stability of andrographolide in plasma was studied under a variety of storage and handling conditions using the low (1.6 ng/mL) and high (64.0 ng/mL) QC samples. The short-term temperature stability was assessed by analyzing QC samples that were kept at ambient temperature for 10 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. The QC samples were stored at −20 °C for 24 h and thawed unassisted at room temperature (22 °C). When completely thawed, the samples were refrozen for 24 h under the same conditions and thawed unassisted at room temperature. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was performed at −20 °C in plasma for 3 weeks. The stability of stock solutions of andrographolide and the I.S. at −20 °C was also evaluated.

3.6. Application

The method described above was applied to the pharmacokinetic study in which plasma concentrations of andrographolide in 20 healthy Chinese male volunteers were determined up to 36 h after administration of 4 andrographolide dispersible tablets containing 200 mg andrographolide. Blood was sampled pre-dose and at 0.167, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 15, 24 and 36 h post-dose for determination of concentration of andrographolide. The clinical study protocol was approved by the ethics committee of Nanjing First Hospital of Nanjing Medical University. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Pharmacokinetic parameters were determined using the plasma concentration–time data. The maximum plasma concentrations (C_{max}) and the time to those (t_{max}) were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated from the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration (AUC_{0-t}) was calculated by the linear trapezoidal method. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$, where C_t was the last measurable plasma concentration and k_e was the elimination rate constant.

4. Results and discussion

4.1. Selection of I.S.

For a proper internal standard, it should be structurally or chemically similar to the analyte, and it should also have similar retention to the analyte, be well resolved from the analyte and other peaks, and mimic the analyte in any sample preparation steps [18]. Dehydroandrographolide was chosen as the internal standard for the assay because of its similarity of structure, retention and ionization to the analyte.

4.2. Conditions for MS/MS

Because andrographolide has several hydroxy groups in their structure, it was a medium-polarity compound, so the negative ion mode and ESI source were adopted for the assay of andrographolide. Fig. 2(a) shows a typical mass spectrum of the negative ions of andrographolide by the scan monitoring. The base peak in the mass spectrum is the dehydration ion $[M-H_2O-H]^-$ of andrographolide at m/z 331.1. Therefore, the $[M-H_2O-H]^-$ ion at m/z 331.1 was chosen as the parent ion for andrographolide monitoring in the ESI-MS assay. The optimization of the collision energy (CE) of the $[M-H_2O-H]^-$ ions of andrographolide and I.S. was performed for the purpose of selecting abundant daughter ions for MRM detection. Selection of abundant daughter ions and optimization for their yield against collision energy was automatically conducted by the instrument control software. At low collision energy (15 or 25 V), the $[M-H_2O-H]^-$ ions of andrographolide did not fragmented to daughter ions, and $[M-H_2O-H]^-$ ions were still dominant in the spectra (Fig. 2(b) and (c)). As the collision energy was increased, the $[M-H_2O-H]^-$ ions fragmented to multiple daughter ions. Unfortunately, as shown in Fig. 2(d) and (e), the fragment ions of $[M-H_2O-H]^-$ varied frequently even when the collision energies were both set at 30 V, so did the fragment ions at higher collision energy (35 V) in Fig. 2(f) and (g). The fragment ions of $[M-H_2O-H]^-$ of andrographolide were so unsteady that selected ion monitoring mode (SIM) was adopted instead of selected reaction monitoring mode (SRM) in the LC–MS/MS. In order to minimize the undesirable fragmentation of $[M-H_2O-H]^-$ of andrographolide, the collision energy was set at a lower value. At lower collision energy, ESI produced an abundant negative ion at $[M-H_2O-H]^-$ m/z 331.1 for andrographolide. In order to determine the optimal

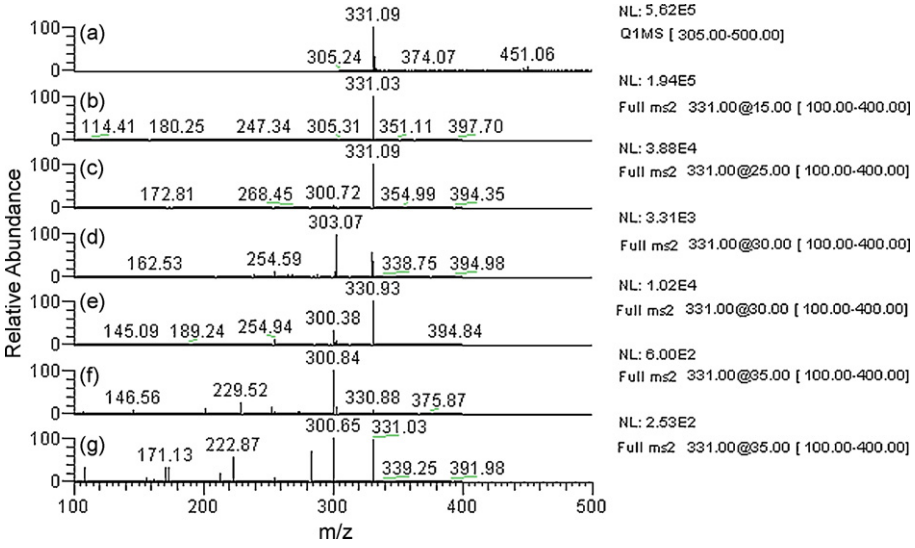

Fig. 2. ESI(−) mass spectra of andrographolide (a) and tandem mass spectra of andrographolide at collision energies (CE) of (b) 15 V, (c) 25 V, (d and e) 30 V and (f and g) 35 V.

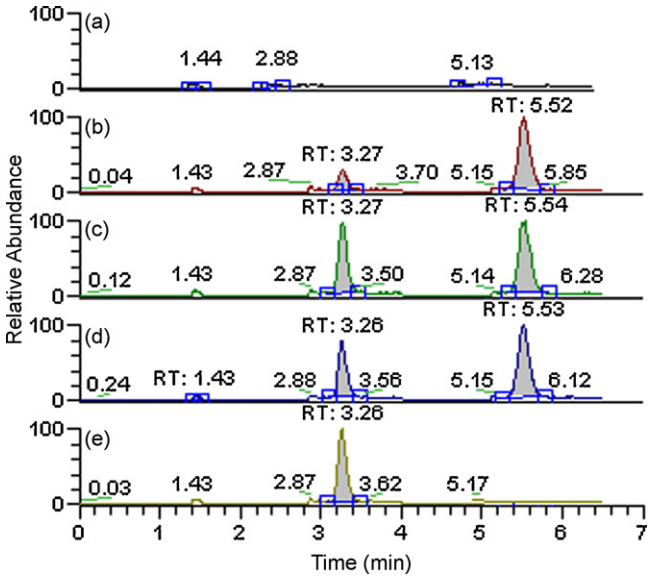

Fig. 3. Typical SIM chromatograms of blank plasma (a), LLOQ for andrographolide in plasma and I.S. (b), plasma spiked with 50.0 ng/mL for andrographolide and I.S. (c), plasma obtained from a volunteer at 1.5 h after administration of 200 mg andrographolide orally and I.S. (d), and plasma obtained from a volunteer (e).

collision energy, the intensities of the negative ion $[M-H_2O-H]^-$ of andrographolide at m/z 331.1 were compared at low collision energy of 5, 7, 10, 13, 15 and 18 V. The results showed that while selecting the negative ion $[M-H_2O-H]^-$ at m/z 331.1 as the target ion of andrographolide, the highest sensitivity of the assay could be achieved by using a 10 V collision energy. Therefore, a collision energy of 10 V was used to carry out the ESI-MS/MS in the assay.

4.3. Conditions of HPLC

The selection of mobile phase components is a critical factor in achieving good chromatographic peak shapes and resolution. To suppress the tailing phenomena of chromatographic peaks of alkaline compound, ammonium acetate buffer solution is usually adopted in the mobile phase of the HPLC [19]. But because andrographolide was not an alkaline compound, the peak shapes would not be improved with the addition of ammonium acetate. Meanwhile, further experiment results showed that acidifying the mobile phase with formic acid decreased the MS sensitivity to andrographolide and I.S. because the negative ion mode was adopted in the LC–MS/MS period, So an elution system of methanol–water was adopted. In order to optimize the separation and get the maximal signal of peaks, different concentrations of methanol were used as the mobile phase. As the concentration of organic solvent increased, the signal of the two analytes, especially the dehydroandrographolide, was increased. When the organic solvent concentration was too high, the analytes were not separated fully. The acceptable retention and separation of andrographolide was obtained by using an elution system of methanol–water (70:30, v/v) as the mobile phase. Typical retention times were about 3.3 min for andrographolide and 5.54 min for I.S. Fig. 3 shows typical HPLC profiles of the andrographolide and dehydroandrographolide in one run. The peaks are completely separated and the shapes are symmetrical.

4.4. Method validation

4.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 3 showed the typical chromatograms of a blank (a), a LLOQ for andrographolide in plasma and I.S. (b), a spiked plasma sample with andrographolide (50.0 ng/mL) and I.S., and plasma samples from healthy volunteers (d and e). There was no significant interference from endogenous substances observed at the retention times of the analytes. Fig. 3(e) showed that dehydroandrographolide was not existing in the plasma samples of volunteers, so dehydroandrographolide could be used as I.S.

Table 1
Matrix effect evaluation of andrographolide and I.S. in human plasma ($n = 5$).

Samples	Nominal concentration (ng/mL)	Matrix effect (%)
Andrographolide	1.6	108.7
	16.0	101.3
	64.0	103.8
I.S.	50.0	105.6

Note: n, number of replicates.

4.4.2. Matrix effect

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [17]. It was examined by comparing the peak areas of the analytes and I.S. between two different sets of samples. In set 1, analytes was resolved in the blank plasma sample's reconstituted solution, and the obtained peak areas of analytes were defined as A. In set 2, analytes was resolved in mobile phase, and the obtained peak areas of analytes were defined as B. ME was calculated by using the formula: $ME(\%) = A/B \times 100$. The matrix effect of the method was evaluated at three andrographolide concentration levels of 1.6, 16.0 and 64.0 ng/mL and the I.S. concentration level of 50 ng/mL. Five samples at each level of the analytes were analyzed. The blank plasma samples used in this study were from five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an endogenous matrix effect is implied. As shown in Table 1, the results obtained were well within the acceptable limit, which indicated that there was no matrix effect of the analytes observed in this study.

4.4.3. Linearity of calibration curves and LLOQ

Five calibration analyses were performed on five consecutive days and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges of 1.0–150.0 ng/mL for the andrographolide. The typical calibration curve had a slope of 0.02291 ± 0.000776, an intercept of 0.00309 ± 0.00450 (weighting factor was $1/C^2$). The LLOQ for andrographolide in plasma was 1.0 ng/mL.

4.4.4. Precision and accuracy

Andrographolide plasma samples at three concentration levels of 1.6, 16.0, and 64.0 ng/mL were analyzed for accuracy and precision. The data obtained for andrographolide is shown in Table 2. The precision was calculated by using one-way ANOVA. For the three concentration levels of andrographolide, the intra-run precision was less than 6.95%, the inter-run precision was less than 7.22% and the accuracy was within ±9.36%. The data obtained for andrographolide was within the acceptable limits to meet the guidelines for bioanalytical methods [17].

4.4.5. Extraction recovery

The mean extraction recoveries were measured at three different concentration levels for andrographolide (1.6, 16.0, and 64.0 ng/mL) by comparing the peak areas of andrographolide prepared in plasma with those obtained from direct injection of

Table 2
Precision and accuracy of the assay for determination of andrographolide in plasma ($n = 3$ runs, five replicates per run).

Spiked concentration (ng/mL)	Mean found concentration (ng/mL)	RE (%)	Intra-run RSD (%)	Inter-run RSD (%)
1.6	1.73 ± 0.12	7.94	6.95	7.22
16.0	17.5 ± 0.96	9.36	5.23	5.71
64.0	68.1 ± 4.09	6.40	5.94	5.87

Note: RE: relative error; RSD: relative standard deviation.

Table 3
The mean extraction recoveries data of andrographolide in plasma ($n = 5$).

Spiked concentration (ng/mL)	Extraction recovery (%, mean ± SD)	RSD (%)
1.6	93.6 ± 6.3	6.7
16.0	91.2 ± 5.7	6.3
64.0	91.3 ± 3.9	4.3

Note: *n*, number of replicate; SD, standard deviation; RSD: relative standard deviation.

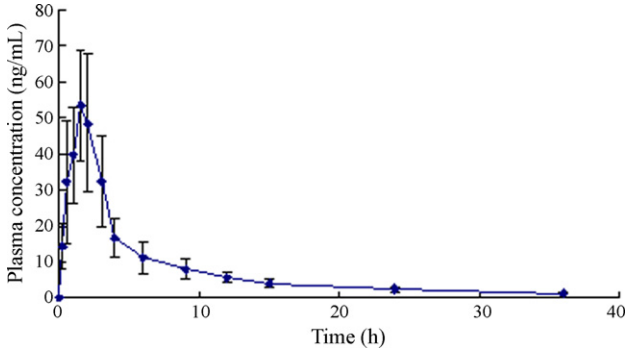

Fig. 4. The mean plasma concentration–time curve of andrographolide after administration of 200 mg andrographolide orally in 20 Chinese volunteers.

standards dissolved in the supernatant of the processed blank plasma. The data of recovery obtained are shown in Table 3.

4.4.6. Stability

The results of stability experiments showed that no significant degradation occurred at room temperature for 10 h and during the three freeze–thaw cycles for andrographolide plasma samples. The accuracy values of low (1.6 ng/mL) and high (64.0 ng/mL) concentration of andrographolide in plasma were 102.0 and 105.0% after 10 h at room temperature, 112.4 and 106.3% after three freeze–thaw cycles, and 96.4 and 106.0% at −20 °C for 3 weeks, respectively.

4.5. Pharmacokinetic studies

The described method was applied to a pharmacokinetic study in human. The mean plasma concentration–time curve of andrographolide is shown in Fig. 4. The main pharmacokinetic parameters of andrographolide were calculated. After oral administration of 200 mg andrographolide, the mean values of T_{max} and C_{max} were 1.6 h (range 1.5–2.0 h) and 58.62 ng/mL (range 29.34–81.21 ng/mL), respectively. The elimination half-life of andrographolide was 10.50 ± 2.07 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 262.50 ± 61.78 and 285.53 ± 65.40 ng h/mL, respectively.

5. Conclusion

The assay achieved good sensitivity and specificity for the determination of andrographolide in human plasma. No significant interferences caused by endogenous compounds were observed. This simple and sensitive assay is suitable for pharmacokinetic studies of andrographolide in human subjects.

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