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Quantitation of catalpol in rat plasma by liquid chromatography/electrospray ionization tandem mass spectrometry and its pharmacokinetic study

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ABSTRACT

A sensitive, rapid and specific liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay has been established for the quantitation of catalpol in rat plasma. Plasma samples were treated by precipitating protein with methanol and were chromatographed by a Diamonsil C_{18} column (150 mm × 4.6 mm I.D., 5 μm) with the mobile phase consisting of methanol and 10 mM ammonium formate (30:70, v/v). The selected reaction monitoring (SRM) transitions were performed at m/z 380.1 → 183.0 for catalpol and m/z 364.3 → 167.0 for aucubin (IS) in the positive ion mode with electrospray ionization (ESI) source. Calibration curve was linear over the concentration range of 10–20,000 ng/mL. The mean recovery was 76.5 ± 5.2% and the matrix effect ranged from −5.1 to 13.0%. The intra- and inter-day precisions were less than 6.3 and 14.6%, respectively, and the accuracy was within ±5.6%. Catalpol was stable in possible conditions of storing and handling. The validated method has been successfully applied to determine the plasma concentration of catalpol for a pharmacokinetic study of catalpol after oral administration of 50 mg/kg to rats.

1. Introduction

Rehmannia glutinosa Libosch, an herbaceous plant of Scrophulariaceae, has a very high medicinal value and is thought as a "top grade" herb for various treatments as traditional Chinese medicine in China and Southeast Asia [1]. Catalpol, an iridoid glycoside found in the radix of *R. glutinosa* Libosch, is the most abundant and bioactive principle, which is proved to have important and extensive pharmacological action including hypoglycemic, diuretic, anticancer, antispasmodic and antiinflammatory effects based on *in vitro* and *in vivo* pharmacodynamic experiments [1–4]. Catalpol has also been proved to possess neuroprotective activity on astrocytes by suppressing the production of free radicals and elevating antioxidant capacity in recent years [5]. Besides *R. glutinosa*, catalpol is also found in plants of the genera *Veronica*, *Plantago*, *Melampyrum*, *Succisa*, *Valeriana*, *Thessalia leanira* and *Euphydryas anicia* in which catalpol is one of the major active principles [6,7]. Therefore, it is important to describe the pharmacokinetic properties of catalpol. Nevertheless, up till now, the analytical research mainly focused on the determination of catalpol in plants and crude drugs, and the pharmacokinetic study of catalpol has hardly been reported.

Catalpol is a small molecular and highly polar substance with poor ultraviolet absorption. Therefore, high performance liquid chromatography with ultraviolet detection (HPLC/UV) methods [8,9] is not sensitive enough for the pharmacokinetic study of catalpol. Mass spectrometric detection is often considered specific and sensitive, compared with other detection techniques such as ultraviolet, fluorometric and electrochemical detection, which are much better selective for most compounds. There were several reports about the determination of catalpol in plants or formulation by micellar electrokinetic capillary chromatography–mass spectrometry (MECC/MS) [6,7,10–13] and gas chromatography–mass spectrometry (GC/MS) [14,15] methods. However, these MECC and GC methods are not suitable for pharmacokinetic study, because of poor specificity, derivatization step, long runtime and complex sample preparation procedure before injection into the MS system. Generally, for routine analysis, the multiple-step extractions and derivatization step increases sample preparation time and the cost of the method.

In the present study, we have developed and validated a rapid, simple and sensitive LC/MS/MS method for the quantitation of catalpol in rat plasma for the first time. The method has the advantages of small volume plasma sample (50 μL), simple sample preparation (protein precipitation), good chromatographic resolution, specific and sensitive mass spectrometric conditions and short runtime (6.5 min), with the concentration range of 10–20,000 ng/mL. Then, we successfully applied the newly

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established method to the pharmacokinetic study of catalpol after oral administration of 50 mg/kg to rats.

2. Experimental

2.1. Chemicals and reagents

Catalpol (purity 99.5%) and aucubin (purity 99.0%) (IS) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade of methanol was from Tianjin Concord Tech Reagent Co. Ltd. (Tianjin, China). All other reagents were of analytical grade. Distilled water, prepared from demineralized water using a SYZ550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., Tianjin, China), was used for all aqueous solutions throughout the experiment.

2.2. Instrumentation and analytical conditions

The LC/MS/MS system consists of a Surveyor auto-sampler, a Surveyor LC pump, a TSQ Quantum Discovery Max^{TM} triple-quadrupole mass spectrometer with an electrospray ionization (ESI) source and the Xcalibur 1.4 software for data acquisition and analysis (Thermo Finigan, USA). The analyte and IS were chromatographed at 25 °C by injection of 5 μL sample onto a Diamonsil C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Dikma Technologies, China). The mobile phase consisting of methanol and 10 mM ammonium formate (30:70, v/v) was performed to elute isocratically at a flow rate of 0.4 mL/min.

Mass spectrometer equipped with an ESI source run in positive ion mode. The ESI source was operated with a spray voltage of 4500 V. The sheath gas and auxiliary gas were nitrogen delivered at 40 psi and at 5 L/min, respectively. The capillary temperature was 320 °C. The collision gas (argon) pressure was 0.8 mTorr. The MS recordings were carried out in SRM mode with specific ion transitions of ammonia adducted precursor ion to product ion at m/z 380.1 → 183.0 with collision energy (CE) of 15 eV for catalpol, and at m/z 364.3 → 167.0 with CE of 15 eV for IS. The total analytical runtime was 6.5 min.

2.3. Preparation of standards and quality control (QC) samples

The stock solutions of catalpol and IS were dissolved in methanol at 1.0 mg/mL, respectively. The stock solution of catalpol was then serially diluted with methanol to obtain the working solutions at the concentrations of 10, 20, 100, 500, 2000, 10,000 and 20,000 ng/mL. The IS working solution was also prepared by diluting the IS stock solution with methanol to get the concentration of 2000 ng/mL. All the solutions were kept at 4 °C and were brought to room temperature before use.

The 50 μL of working solution was evaporated to dryness at 40 °C under a gentle stream of nitrogen, and then added 50 μL of blank rat plasma, which was vigorously vortex-mixed for 30 s. The concentration levels of catalpol in plasma ranged from 10 to 20,000 ng/mL. Quality control (QC) samples were prepared in a similar manner at low, medium and high levels (20, 500, 16,000 ng/mL). All the spiked plasma samples were then treated according to sample preparation procedure. Both the calibration standard samples and the QC samples were applied in the method validation and the pharmacokinetic study.

2.4. Sample preparation

Rat plasma sample was performed by protein precipitation. An aliquot of 50 μL plasma samples were added with 50 μL methanol and 100 μL IS working solution (2000 ng/mL) in polypropylene tubes. Subsequently, the tubes were vigorously vortex-mixed for 30 s to precipitate plasma proteins and centrifuged for 5 min at 6000 × g. An aliquot of 150 μL of the upper organic layers were transferred to polypropylene tubes and diluted with 150 μL water. The tubes were briefly vortex-mixed, and then 5 μL aliquots of the supernatant were injected into the LC/MS/MS system for analysis. Samples that were found to exceed the upper limit of quantitation (20,000 ng/mL) were diluted five times with blank plasma. Aliquot of 50 μL diluted plasma sample was treated as the same sample preparation manner, and re-analyzed.

2.5. Method validation

The method was validated in terms of selectivity, linearity, sensitivity, accuracy, precision, recovery, stability and matrix effect [16].

2.5.1. Selectivity

To investigate whether endogenous constituents interfered with the assay, six different blank rat plasma samples were analyzed to detect the potential interferences co-eluting with analyte and IS. Chromatographic peaks of analyte and IS were identified on the basis of their retention times and SRM responses.

2.5.2. Matrix effect

The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes, which was evaluated by comparing the corresponding peak areas of the post-extraction spiked samples to those of the standard solutions evaporated directly and reconstituted in mobile phase. Experiments were performed at the three QC levels, in triplicate.

2.5.3. Linearity

To evaluate linearity of this method, calibration curves, over a linear range from 10 to 20,000 ng/mL in rat plasma, were prepared and assayed in triplicate on three consecutive days. Calibration curves were constructed by plotting the peak area ratio (analyte-to-IS) versus the spiked concentrations of catalpol. Calibration graphs were obtained using the least square method. Linearity was assessed by linear regression analysis with a weighting factor of $1/x^2$, and deviations of these back-calculated concentrations from the spiked concentrations were set within ±15% (±20% for the lower limit of quantitation). The correlation coefficient and deviation reflected the assay performance over the concentration range.

2.5.4. Precision and accuracy

Intra- and inter-day precision and accuracy were assessed from results of six replicates of the low, medium and high QC samples (20, 500, 16,000 ng/mL) on three consecutive days. The assay precision was calculated by using a one-way analysis of variance (ANOVA) and expressed as the relative standard deviation (RSD) for each QC sample. The assay accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed as the relative error (RE). The acceptable intra- and inter-day precision and accuracy are required to be within ±15%.

If samples are found to exceed the upper limit of quantitation (20,000 ng/mL), it will be diluted with blank plasma and then re-analyzed. Therefore, it is necessary to investigate the precision and accuracy of diluted sample. Blank plasma spiked with 80,000 ng/mL, six replicates, were diluted five times with blank plasma and then treated using the same sample pretreatment method, which were analyzed and compared with the spiked concentration. The acceptable precision and accuracy are required to be within ±15%.

2.5.5. Lower limit of quantitation

The lower limit of quantitation (LLOQ) of the assay were assessed as the lowest concentrations on the calibration curve that can be quantitatively determined with acceptable precision less than 20% and accuracy within ±20%. The LLOQ was established based on six replicates independent of QC samples at 10 ng/mL.

2.5.6. Recovery

The recovery of catalpol from plasma were determined by comparing the peak areas obtained from these prepared plasma samples described above with those obtained from direct injection of standard solutions without preparation at the same concentrations. Experiments were performed at the three QC concentration levels, in triplicate.

2.5.7. Stability

The catalpol stability experiments were investigated by comparing the measured concentrations of triplicate low, medium and high QC samples with the spiked concentrations. The 50 μL of working solution was evaporated to dryness at 40 °C under a gentle stream of nitrogen, and then added 50 μL of blank rat plasma. Subsequently the mixture was vigorously vortex-mixed for 30 s, and then stored according to the following four storage conditions: (1) stability of catalpol in plasma during sample preparation was assessed by detecting samples after storage for 4 h at room temperature. (2) For freeze–thaw stability, the plasma samples were determined through three freeze (−20 °C)–thaw (room temperature) cycles. (3) To evaluate the stability of the treated plasma samples in the auto-sampler, QC samples were prepared and placed in the auto-sampler at 4 °C for a period of 12 h, and then injected for analysis. (4) The long-term stability was performed by assaying the plasma samples after 30 days of storage at −20 °C. All the samples were analyzed together with calibration curves that were freshly prepared. The analyte was considered stable when the percentage deviation was within ±15%.

2.6. Pharmacokinetic study

To show the applicability of the method, we used it to quantitate catalpol in rat plasma for a preclinical pharmacokinetic study of catalpol. Six Wistar rats (200 ± 20 g), male and female, were purchased from Institute of Radiation Medicine, Chinese Academy of Medical Sciences, Tianjin, China. Before orally administered a single dose of catalpol (50 mg/kg) the rats were fasted for 12 h but with access to water, and were further fasted for 2 h after administration. Blood samples of 100 μL were collected in heparin containing tubes from the epicanthic veins of rats by capillary tube before drug administration (0 h) and at 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after oral administration. The blood samples were immediately centrifuged at 6000 × *g* for 5 min at room temperature. The plasma samples were stored at −20 °C until analysis. The pharmacokinetic parameters were calculated for each subject by the DAS 2.0 software (Drug and Statistics, Mathematical Pharmacology Professional Committee of China, Shanghai, China). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were directly obtained from the experimental data. The elimination rate constant (K_e) was calculated by linear regression of the terminal semi-log plot of plasma concentration versus time, and $t_{1/2}$ was calculated as $0.693/K_e$. The area under the curve (AUC_{0-t}) was calculated using the linear trapezoidal rule from zero to the last plasma drug concentration. $AUC_{0-\infty}$ was calculated as $AUC_{0-t} + C_t/K_e$, where C_t is the last detectable plasma concentration.

3. Results and discussion

3.1. Chromatographic conditions

We investigated multiple chromatographic conditions in order to get appropriate retention time and better resolution and sensitivity. Diamonsil C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Dikma Technologies, China) was finally selected for the chromatographic separation. As far as the mobile phase was concerned, the mobile phase consisting of methanol and 10 mM ammonium formate (30:70, v/v) was found to be optimal for this study. The use of methanol led to lower background noise than acetonitrile. The addition of 10 mM ammonium formate was to get better peak shape and to enhance the ionization. The chromatographic conditions provided symmetric peak shape, good sensitivity (10 ng/mL) and a short runtime for catalpol and IS. Under the optimized conditions, no significant endogenous interference was found.

3.2. Mass spectrometric conditions

By investigating the full-scan mass spectra of catalpol, we found that the signal intensity in the positive mode was much higher than that in the negative ion mode. Thus, all detection was carried out using the predominantly positive ion mode. The full-scan positive ion mass spectrum showed that both catalpol and IS were ammonia adduct molecular ion ($[M+NH_4]^+$) of *m*/*z* 380.1 and *m*/*z* 364.3, respectively. After fragmentation in the collision cell, the most abundant and stable product ions (Fig. 1) were at *m*/*z* 183.0 for catalpol and at *m*/*z* 167.0 for IS, respectively. The CID parameters were optimized to enhance the highest response and specificity using the SRM mode comprising the precursor and product ions. The most suitable mass spectrometric conditions were determined by optimizing all the parameter of the mass spectrometer such as collision energy, argon collision gas pressure, sheath gas and auxiliary gas pressure, spray voltage and capillary temperature to obtain much higher and stabler response.

3.3. Internal standard

For selecting the ideal internal standard, a similar processing method and a suitable retention time are of significant importance. Several compounds were tried, and aucubin was finally adopted as internal standard because of similarity to the analyte in structure, chromatographic behavior, mass spectrometric conditions (ionization) and recovery.

3.4. Sample preparation

A great number of samples need to be analyzed in pharmacokinetic study. Thus, a simple, rapid and economic sample preparation is necessary and critical. It is unadvisable for the plasma samples consisting of catalpol to be pretreated by liquid–liquid extraction using organic solvents, because the catalpol is a highly hydrophilic, poor lipophilicity compound, which renders it extremely difficult to extract from aqueous biological medium. However, protein precipitation was used for the sample preparation after comparing the liquid–liquid extraction, and methanol was chosen as precipitation agent. The protein precipitation is more advisable and advantageous in the present work, because it could not only ensure less endogenous interference, adequate recovery and high sensitivity, but also perform simple.

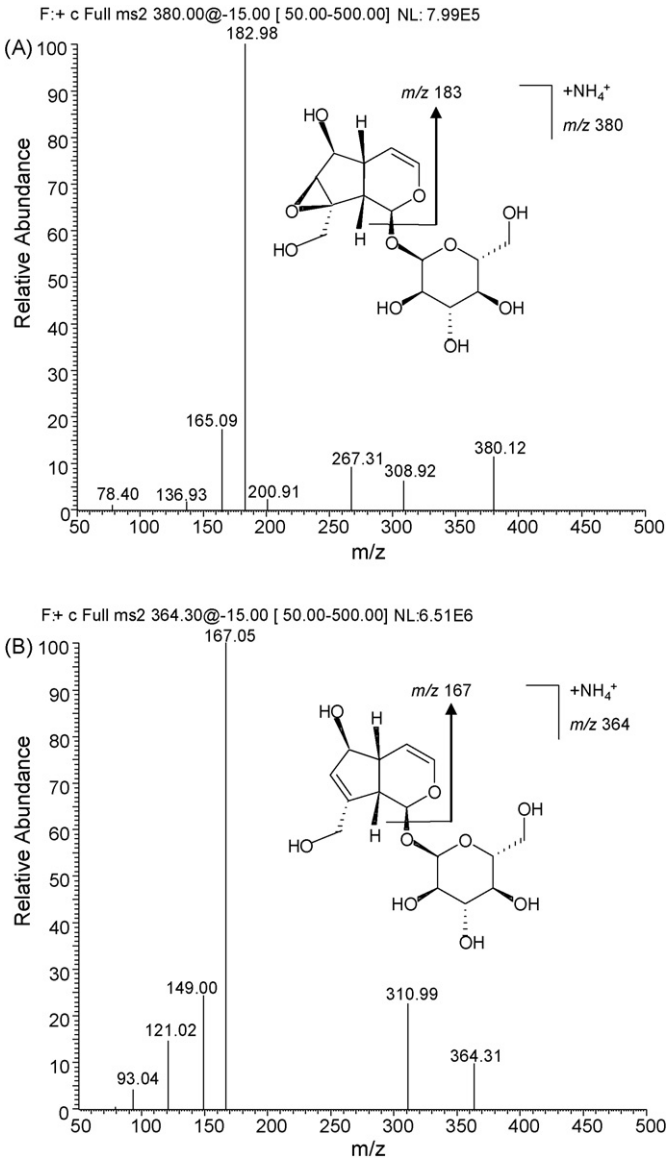

Fig. 1. Product ion mass spectra of $[M+NH_4]^+$ of (A) catalpol and (B) aucubin (IS).

3.5. Method validation

3.5.1. Selectivity and specificity

The selectivity of the method was assessed by comparing chromatograms of blank plasma, spiked plasma and rat plasma after oral administration of catalpol. The retention times were 4.4 and 5.0 min for catalpol and IS, respectively (Fig. 2). As shown in the figures, there was no significant endogenous peaks that could interfere with the analyte and IS, and a stable baseline was maintained throughout. The results indicated that the method exhibited good specificity and selectivity and was applicable to plasma samples for the pharmacokinetic study.

3.5.2. Matrix effect

The ion suppression/enhancement in signal ranged from −5.1 to 13.0% for the three QC levels, indicating that the matrix effect on the ionization of analyte is not obvious under these conditions.

3.5.3. Linearity and lower limit of quantitation

The calibration curves were ranged from 10 to 20,000 ng/mL. The typical regression equations obtained by least squared

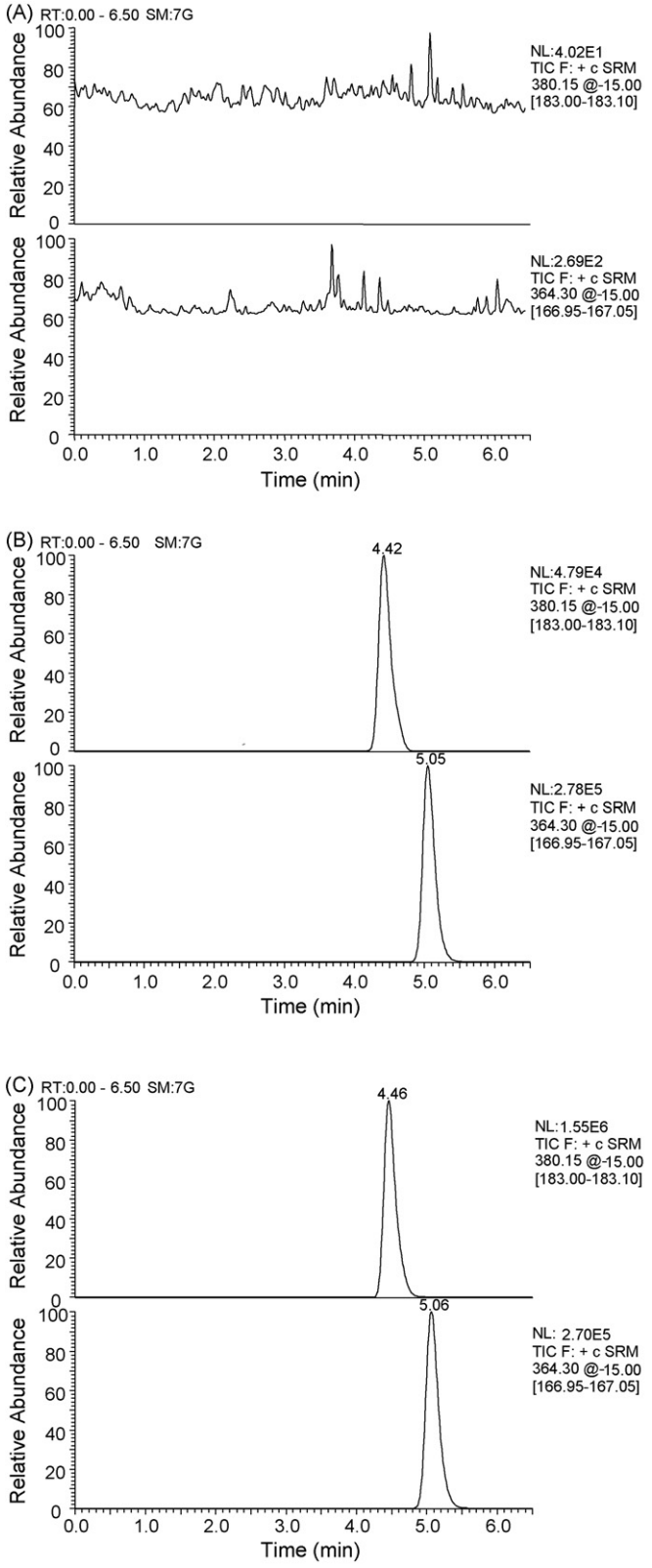

Fig. 2. Typical SRM chromatograms of catalpol and IS: (A) a blank rat plasma sample; (B) a blank plasma spiked with catalpol (500 ng/mL, 4.4 min) and IS (2000 ng/mL, 5.0 min); and (C) a rat plasma sample from 0.5 h after oral administration of 50 mg/kg catalpol spiked with IS.

Table 1
Precision and accuracy of catalpol in rat plasma.

Concentration (ng/mL)		Precision (RSD (%))		Accuracy (RE (%))	
Spiked	Measured (mean ± SD)	Intra-day	Inter-day	Intra-day	Inter-day
10	10.2 ± 1.0	10.2	7.8	1.9	−1.6
20	18.9 ± 1.2	6.3	6.5	−1.2	−5.6
500	491.8 ± 19.3	3.2	7.5	1.1	−1.6
16,000	15,893.5 ± 886.9	2.6	14.6	−4.8	−0.7

regression were $y = 3.535 \times 10^{-4}x + 9.841 \times 10^{-4}$. Where y is the peak area ratios of analyte-to-IS, and x is the concentrations of analyte. The correlation coefficient (r^2) was ≥0.9904 for all calibration curves, and the observed deviation was within ±15% for all calibration concentrations. The results indicated that the calibration curves with a good linearity could describe the concentration versus response relationship adequately.

The LLOQ of catalpol was 10 ng/mL. At LLOQ, the accuracy was within ±1.9% and the precision less than 10.2%, which was sufficient for pharmacokinetic studies of catalpol in rat.

3.5.4. Precision and accuracy

QC samples at three concentrations were analyzed in six replicates for determining the accuracy and precision of this assay. As shown in Table 1, the intra- and inter-day precision were less than 6.3 and 14.6%, and the intra- and inter-day accuracy were within ±4.8 and ±5.6%. All within the acceptable range indicated that the present method has a satisfactory accuracy, precision and reproducibility.

For diluted samples, the precision was less than 8.4%, and the accuracy was within ±9.6%.

3.5.5. Recovery

Under the given set of operating conditions, the recovery of catalpol from the rat plasma was 76.5 ± 5.2% for three QC concentration levels. The recovery was consistent over its calibration range, indicating the extraction efficiency of the current assay is independent of the concentrations in the ranges studied. The recovery of the IS was 78.9 ± 7.3% and was steady throughout.

3.5.6. Stability

The catalpol stability experiments aimed at studying possible conditions that the samples might be exposed to during storing and handling. Results of the stability tests are summarized in Table 2.

Table 2
Stability of catalpol in rat plasma.

Storage conditions	Concentration (ng/mL)		RE (%)
	Spiked	Measured (mean ± SD)	
Initial concentrations measured for the spiked QC samples	20	19.0 ± 0.8	−4.8
	500	502.3 ± 7.8	0.5
	16,000	15,735.1 ± 670.7	−1.7
At room temperature for 4 h	20	22.7 ± 1.0	13.6
	500	529.5 ± 16.2	5.9
	16,000	16,581.6 ± 197.5	3.6
Three freeze–thaw cycles	20	21.7 ± 0.7	8.4
	500	515.4 ± 12.2	3.1
	16,000	15,073.2 ± 694.8	−5.8
At 4 °C in the auto-sampler for 12 h	20	23.7 ± 0.7	18.4
	500	525.0 ± 16.1	5.0
	16,000	15,783.4 ± 813.8	−1.4
Long-term stability (at −20 °C for 30 days)	20	22.9 ± 1.0	14.5
	500	526.4 ± 4.5	5.3
	16,000	16,263.7 ± 556.9	1.6

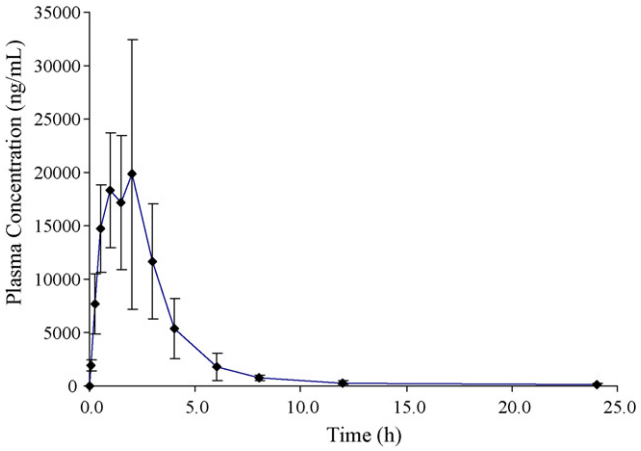

Fig. 3. Mean plasma concentration–time profile of catalpol after oral administration of 50 mg/kg to rats. Each point represents mean ± SD (n = 6).

Table 3
Main pharmacokinetic parameters of catalpol after oral administration of 50 mg/kg to rats (n = 6, mean ± SD).

Parameters	Mean ± SD
$t_{1/2}$ (h)	1.212 ± 0.388
V1/F (L/kg)	1.428 ± 0.681
CL/F (L/h/kg)	0.824 ± 0.317
$t_{1/2Ka}$ (h)	0.53 ± 0.302
$AUC_{0-\infty}$ (ng h/mL)	69,520 ± 22,927
$MRT_{0-\infty}$ (h)	3.273 ± 0.365
T_{max} (h)	1.333 ± 0.408
C_{max} (ng/mL)	23,318 ± 10,468

As be seen that catalpol was stable in rat plasma after being placed at ambient temperature for 4 h, after being stored at −20 °C for 30 days or through three freeze–thaw cycles. On the other hand, treated samples were found to be stable at 4 °C in the auto-sampler for a period of 12 h, indicating that a large number of samples could be treated in each analytical run.

3.6. Pharmacokinetic study

We successfully applied the present method to the pharmacokinetic study of catalpol in six Wistar rats after oral administration of 50 mg/kg. Plasma concentrations of catalpol were determined up to 24 h throughout the study. The mean plasma concentration versus time curves is presented in Fig. 3. The major pharmacokinetic parameters of catalpol were calculated by one-compartment model and demonstrated in Table 3. The pharmacokinetics of catalpol showed a short half-life which was almost less than 1.5 h. The successful application of LC/MS/MS method to pharmacokinetic study of catalpol indicated that the established analytical method was suitable and sufficient for pharmacokinetic study.

4. Conclusions

A sensitive, specific, rapid and simple LC/MS/MS method was developed and validated for the determination of catalpol in biological matrix for the first time. The plasma samples were treated by protein precipitation and followed by chromatography with tandem mass spectrometry detection in runtime of 6.5 min. Furthermore, the method required only 50 μL plasma and 5 μL injection volume with the LLOQ of 10 ng/mL. It was also the first assay that the method was successfully applied to characterize the pharmacokinetics of catalpol in rats after oral administration of 50 mg/kg. The method presented here will be valuable for deter-

mining catalpol plasma concentrations and could be applied in therapeutic drug monitoring of catalpol.

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