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Development of a non-derivatization high-performance liquid chromatography method with resonance Rayleigh scattering detection for the detection of sisomicin in rat serum

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ABSTRACT

A resonance Rayleigh scattering (RRS) detection approach was developed to detect sisomicin (Siso) in rat serum following chromatographic separation. The detection principle is based on the enhancement of RRS intensity of ion-association complex formed from aminoglycosides and pontamine sky blue (PSB) used as molecular recognition probe. The high-performance liquid chromatography (HPLC) coupled with RRS detection scheme was implemented post-column by mixing a PSB solution with the column eluent prior to detection. The RRS signal was detected by fluorescence detector at $\lambda_{ex} = \lambda_{em} = 365$ nm. Separation and detection conditions were optimized. Siso and etimicin (Eti) chosen as the internal standard (IS) were separated on a C_{18} reversed phase column with the mobile phase consisting of a ternary mixture of 20 mM sodium acetate aqueous solution–methanol (92:8, v/v) containing 0.22% TFA (v/v). The limit of detection (S/N = 3) for Siso was 18 ng. A calibration curve ranged from 25 ng to 700 ng shown to be linear. The presented method was applied for the determination of Siso in rat serum and used for the pharmacokinetics study of Siso in rat.

1. Introduction

Sisomicin (Siso) was a compound known as aminoglycoside antibiotics, which have a very broad antimicrobial spectrum, extending from gram-positive cocci to gram-negative bacilli. The compound binds to mammalian RNA [1], bacterial ribosomes and inhibits the protein synthesis by microorganisms, which results in a rapid, concentration-dependent bactericidal action. However, the clinical use of aminoglycosides is limited to life-threatening infections because of their ototoxic and nephrotoxic side effects. Therefore, monitoring aminoglycoside concentration is very important in the side effects studies as well as studies concerning the clinical efficacy and pharmacokinetics of this drug.

Siso contains no chromophore in molecular structure, consequently fluorescence and UV detection are impossible. The traditional high-performance liquid chromatography (HPLC) methods measure Siso after derivatizing the free amino group with fluorescent reagents [2–8]. It is necessary to purify the samples after extraction in most detection methods. Thus, the accuracy of the methods is affected by selective adsorption of the purification columns or unstable derivatization. Therefore, numerous efforts have been made to seek simple methods for the analysis of aminoglycosides. In the past decade, HPLC methods with electrospray ionization/ion-trap tandem mass spectrometry [9–13], pulsed electrochemical detection (PED) [14–17], evaporative light scattering detection (ELSD) [18–23], chemiluminescence detection (CLD) [24] and based on a ligand displacement reaction non-derivatization fluorescence detection [25] for gentamicin, tobramycin, neomycin, kanamycin, framycetin, streptomycin, etimicin, netilmicin, spectinomycin, amikacin, paromomycin and isepamicin have been investigated, respectively.

Resonance Rayleigh scattering (RRS) is a special elastic scattering produced when the wavelength of Rayleigh scattering (RS) is located at or close to its molecular absorption band [26]. It forms new spectral characteristics and provides new information concerning molecular structure, size, form, charge distribution, state of combination and so on. Due to its sensitivity, simplicity and low cost, it has been applied to the determination of proteins [27,28], nucleic acid [29] and pharmaceuticals [30,31]. Recently, RRS as a detection technique has been successfully incorporated with flow injection analysis (FIA) [32,33], CE [34] and HPLC [35], which indicates that the analyte can form an ion-association complex with

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molecular recognition probe, and produce strong scattering signal in mobile medium, high voltage electric field and under appropriate pressure. Nevertheless, no reference on the analysis of aminoglycoside antibiotics by HPLC with RRS detection was reported to date.

The aim of this work was to develop a rapid and simple chromatographic method with a direct sample introduction (no derivatization) for the determination of Siso. Therefore, an HPLC method with RRS detection was optimized and validated for the quantification of Siso. And the method was used for the pharmacokinetics study of Siso in rat.

2. Experimental

2.1. Chemicals and solutions

Siso sulfate (548 IU/mg) and etimicin (Eti) sulfate (586 IU/mg) were purchased from China's National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Pontamine sky blue (PSB) was purchased from Chroma Company (Switzerland). Methanol and sodium acetate anhydrous (99%), were of HPLC grade purity. Trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were of analytical grade purity. Ultrapure water was obtained from a Milli Q plus 185 purification system (Bedford, MA, USA) and used throughout the work.

Siso and Eti standard was directly dissolved in water to prepare stock solution of 1 mg/ml. Britton–Robinson (B–R) buffer solutions with different pH values were prepared by mixing 0.04 M H_3PO_4, H_3BO_3, HAc and 0.2 M NaOH solution at different ratios. The PSB solution was prepared by dissolving PSB standard in B–R buffer solution (pH = 6.4) to give a solution of 5.0×10^{-5} M. The mobile phase consisted of 20 mM sodium acetate solution, 8% methanol (v/v) and 0.22% TFA (v/v), and pH value of the solution was adjusted to 3.5 with sodium acetate and TFA. The Siso and Eti work solutions were prepared by diluting a stock solution with mobile phase before being injected. The mobile phase and PSB solution have to be filtered through a 0.45 μm pore-size filter and degasificated.

2.2. Apparatus and HPLC procedure

The instrumental set-up is given in Fig. 1. This system was modified from Shimadzu LC-10AT binary high pressure gradient system (Shimadzu, Kyoto, Japan) consisting of a RF-10AXL fluorescence detector fitted with a 10-μL detection flow cell for RRS detection. Pump B was employed to deliver PSB probe solution (0.1 ml/min) at post-column via a T-shaped interface. A stainless steel reacting tube (200 cm × 0.25 mm i.d.) was required between the T-shaped interface and the detector to form aminoglycoside–PBS ion-association complex. Separation of the aminoglycosides was achieved with the use of a Sino Chrom ODS column (250 mm × 4.6 mm i.d., 5 μm particle sizes, Elite, China). All mobile phases and post-column reagent

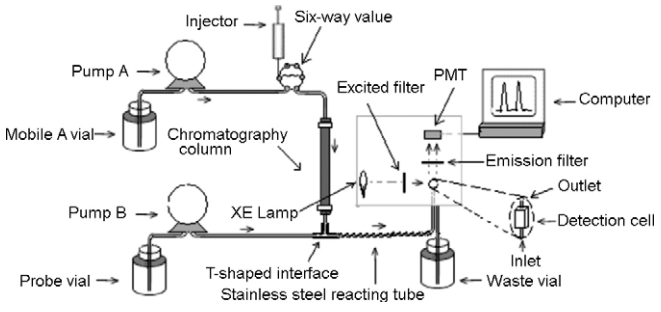

Fig. 1. Schematic diagram of HPLC–RRS system.

were filtered through a 0.45-μm pore-size membrane filter prior to use. A CLASS-VP 5.03 chromatographic workstation (Shimadzu, Kyoto, Japan) was used to acquire and process chromatographic data. LS55 fluorescence spectrophotometer (PerkinElmer, USA) was used for the static RRS spectra studies. The pH value was measured with a PHSJ-4A pH meter (Leici, Shanghai, China).

A 20 μL of sample solution was injected and separated in chromatogram column. The separated components were associated with PSB to form an ion-association complex in reacting tube. The RRS signal was measured at $\lambda_{ex} = \lambda_{em} = 356$ nm. The flow rate of pump A was 0.5 ml/min, and that of pump B was 0.1 ml/min.

2.3. Sample preparation

A 100 μL of serum sample was placed into 500 μL centrifuge tube, and 20 μL of 50 μg/ml Eti (internal standard, IS) and 70 μL of 10% TCA was added. The mixture was vortex-mixed for approximately 1 min, then allowed to stand for 15 min to deproteinize, and the precipitate was removed by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a 500 μL centrifuge tube, and mobile phase was added to give a final volume of 200 μL. The solution was vortexed and kept at 4 °C. The serum samples were filtered through 0.45-μm membrane filter and a 20 μL aliquot was injected into the HPLC system.

The standard working solutions were prepared by spiking Siso in blank rats serum to give final concentrations of 1.25, 5, 10, 15, 25, and 35 μg/ml of Siso and 3 of them (5, 15 and 25 μg/ml) were used as quality control (QC) samples. The serum samples spiked with Siso were treated as above mentioned procedure. All the solutions were stored at 4 °C and were brought to room temperature before use.

2.4. Animals and pharmacokinetic study

Sprague–Dawley (SD) rats (half male and half female, $n = 6$, 280–330 g, Grade II, Certificate No. SCXK GUI 2007-0001) were purchased from the Experimental Animal Center of Guilin Medical University. The rats were fasted overnight before administration of drug with free access to water. Dose of 30 mg/kg Siso was injected intramuscularly. Blood samples (0.3 ml) were collected from tail vein according to the time schedule, including a blank blood sample just prior to dosing and blood samples at 10 min and 30 min, 1.0, 1.5, 2.0, 3.0, 4.0 h after drug administration. Serum was separated by centrifugation at 4000 rpm for 10 min and stored at −20 °C until assay. The pharmacokinetic data analysis was performed by using Drug and Statistics 2.0 (DAS 2.0) published by Mathematical Pharmacology Professional Committee of China (Shanghai, China).

3. Results and discussion

3.1. Chromatographic separation

Due to strong hydrophilicity of aminoglycosides, hydrophilic SinoChrom ODS column was utilized to make retention easy. In order to assure appropriate and adequate ionization of aminoglycosides, pH of the mobile phase was adjusted to 3.5 with sodium acetate and TFA. To achieve an efficient separation of Siso and Eti, the effect of separation parameters such as the compositions of the mobile phase, the concentration of sodium acetate and TFA in mobile phase and the flow rate of mobile phase was investigated in detail.

A mixture solution of water–methanol in the range of 90:10–95:5 (v/v) was examined for the mobile phase. The results showed that a well-pleasing separation was obtained by using the mobile phase consisting of 92% (v/v) water and 8% (v/v) methanol.

Sodium acetate and TFA adding into the mobile phase can improve the peak shape and enhance the resolution; therefore the concentrations of sodium acetate and TFA in mobile phase in the ranges of 10–50 mM and 0.1–0.3% (v/v) were also examined. It was noted that the separation efficiency increases with the augment of concentrations of sodium acetate and TFA. But higher concentration of sodium acetate will bring the pollution of separation column, thus a mobile phase containing 0.22% TFA (v/v) and 20 mM sodium acetate was considered optimal.

The flow rate of mobile phase from 0.4 ml/min to 0.7 ml/min was also optimized. The results indicated that the retention times of Siso and Eti decrease with increase of the flow rate. After the flow rate was more than 0.6 ml/min, the resolution was less than 1.5. Considering both the analysis time and the resolution, a flow rate of 0.5 ml/min was used for further experiment.

Based on the results mentioned above, the optimum separation conditions were as follows: the mobile phase was a mixture of 20 mM sodium acetate aqueous solution–methanol (92:8, v/v) containing 0.22% TFA (v/v). The flow rate of mobile phase was 0.5 ml/min. And the column temperature was fixed at 30 °C. Under this optimum condition, the baseline separation was achieved and the resolution was more than 1.5. The retention time of Siso and the IS was approximately 16.5 min and 17.5 min, respectively. For the precision, a standard solution containing 6.0 μg/ml of Siso and 5.0 μg/ml of Eti was separated seven times. The results showed that the relative standard deviations (RSDs) of the retention times and peak area were lower than 2.5 and 3.2%, respectively.

3.2. Choice of molecular probe and RRS spectra

The molecular probe combines with analyte to from ion-association complex, which can produce strong RRS signal. In this work, three probes, i.e., PSB, titan yellow and trypan red were chosen to determine which probe combines with Siso to produce the strongest RRS signal. It was found that the highest sensitivity was obtained when PSB was used as the molecular probe. PSB is an acid bisazo dye in weakly acidic or near-neutral medium, which can be dissociated into two sulfonic groups with negative charges, while Siso dissociated with positive charges because of the protonation of amidocyanogen (Fig. 2A and B). Under this condition, when PSB was mixed with Siso, PSB and Siso reacts with each other to form a 1:1 ion-association complex. The possible structure of the complex was illustrated in Fig. 2C [36]. The RRS spectra of Siso, PSB and Siso–PSB ion-association complex were measured by fluorescence spectrophotometer at $\lambda_{ex} = \lambda_{em}$ with synchronous scanning, and the results were shown in Fig. 3. As can be seen in Fig. 3, free PSB and Siso produce very weak RRS signals. When the PSB and Siso react with each other to form the ion-association complex, the RRS signal can be enhanced markedly. The maximum wavelength of RRS was located at 356 nm. Therefore, 356 nm was chosen as the excitation and emission wavelengths in presented method.

3.3. Optimization of post-column variables

According to the detection scheme, PSB probe solution was pumped at post-column and mixed with the eluted Siso and Eti to form ion-association complexes for RRS detection. To obtain high

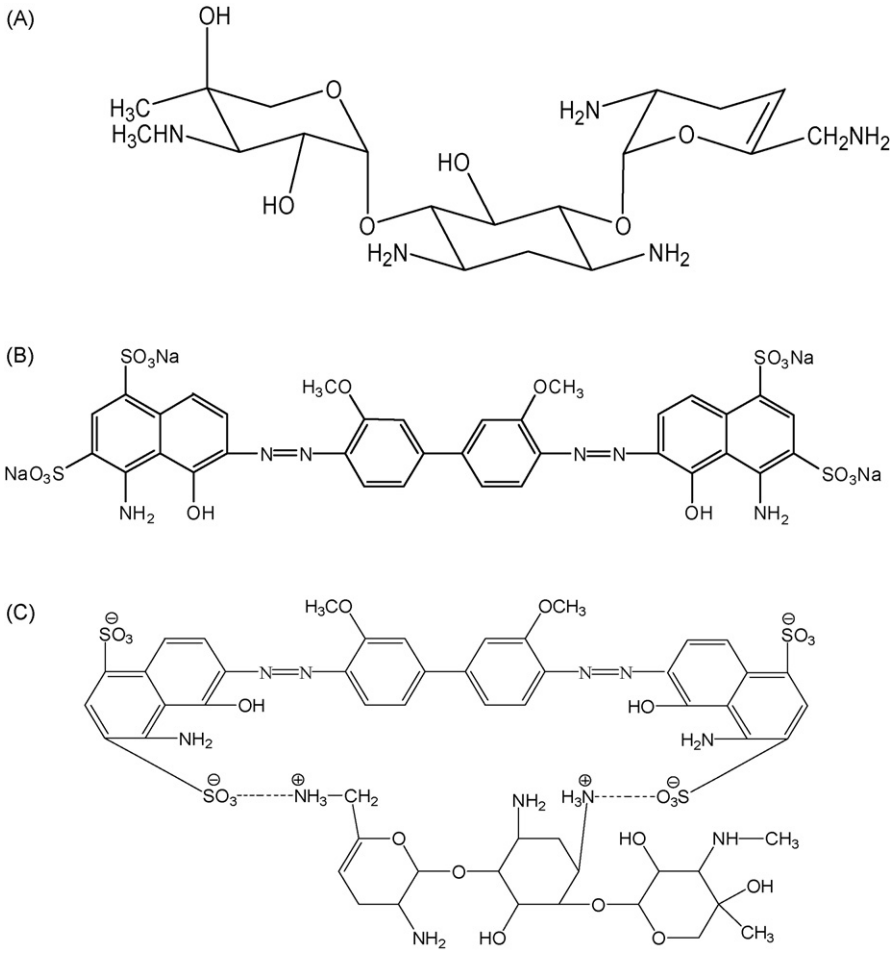

Fig. 2. Molecular structures of Siso (A), PSB (B) and Siso–PSB ion-association complex (C).

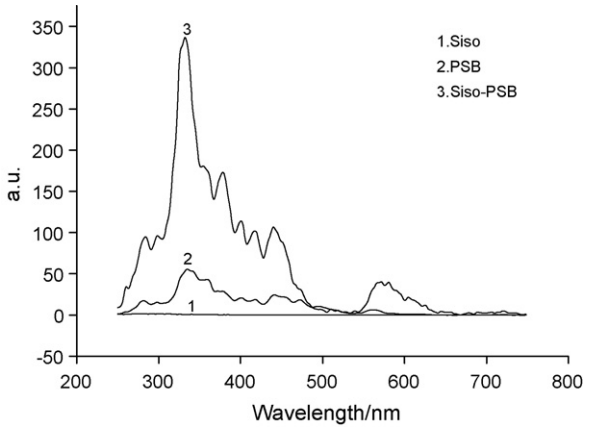

Fig. 3. RRS spectrum of Siso (1), PSB (2) and Siso–PSB ion-association complex (3).

detection sensitivity, a series of experiments were performed to optimize the post-column variables including the length of reacting tube, the pH of probe solution, PSB concentration and flow rate.

The length of reacting tube was evaluated in the range of 145–540 cm. The RRS intensity of Siso and Eti increased with increasing reaction tube length up to 200 cm, where the maximum of RRS signal was observed. The further increase in the length of reacting tube led to a decrease in the RRS intensity. On the other hand, with increasing reaction tube length from 145 to 200 cm, the chromatography peak width was slightly changed from 1.50 min to 1.53 min for Siso and 0.92–0.94 min for Eti, which demonstrated that the chromatography peak was not broadened by the use of longer reaction tube. According to the results, optimal length of reacting tube was selected to be 200 cm.

Owing to the ion-association reaction pH dependent, the effects of buffer pH for ion-association reaction on RRS intensity were also investigated. It was found that pH of the probe solution affected mainly RRS intensity in the range of 3.4–7.3 because the reaction was favored at weakly acidic or near-neutral pH. The maximum the peak area was obtained when pH value of probe solution was at 6.4, so a probe solution of pH 6.4 was selected for the post-column ion-association reaction.

The effects of PSB solution concentration in the range of 5.0×10^{-7} M to 2.0×10^{-5} M and PSB flow rate in the range of 0.06–0.14 ml/min on the RRS intensity were investigated. The results indicate that the RRS intensity of the antibiotics first increased and then decreased with the increase of PSB concentration and PSB flow rate, and the maximum was attained at 5.0×10^{-6} M and 0.10 ml/min, respectively.

According to the experiment results described above, the best post-column conditions were confirmed as follows: 200 cm reacting tube, 5.0×10^{-6} M PSB solution at pH 6.4 and the flow rate of 0.10 ml/min.

The chromatograms obtained from the analysis of a blank rat serum sample and a serum sample spiked with Siso (6.0 μg/ml) and Eti (5.0 μg/ml) are shown in Fig. 4. As can be seen, the peaks for aminoglycosides were not interfered by endogenous substances in rat serum which caused no RRS signals under the optimized detection conditions. It was obvious that the method can be used for the determination of Siso in serum sample.

3.4. Method validation

The quantification was carried out by means of the signal ratio of Siso to internal standard (Eti). Five-point calibration

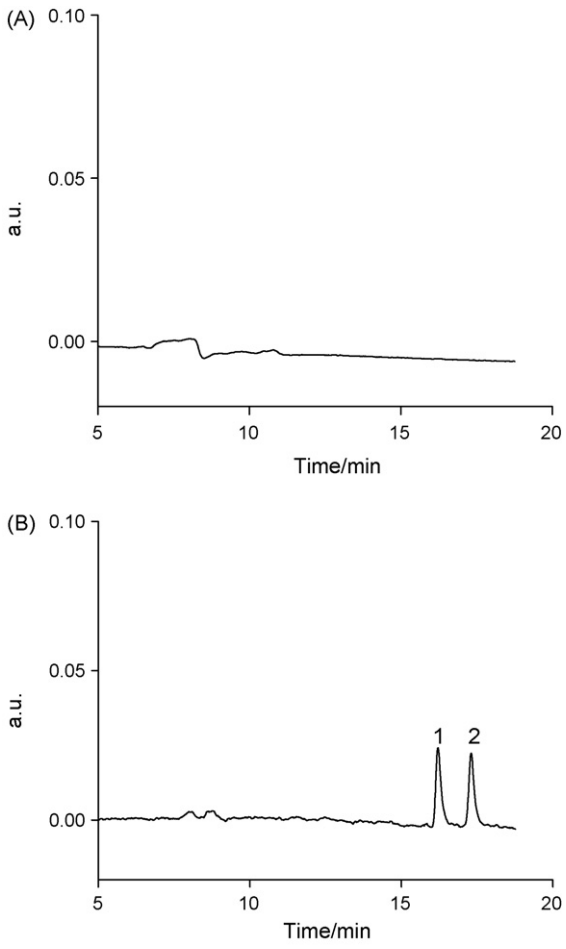

Fig. 4. Chromatogram of a blank serum sample (A), a blank rat serum sample spiked with Siso (6.0 μg/ml) and Eti (IS, 5.0 μg/ml) (B). Chromatographic and detection conditions: isocratic elution was used with a mobile phase consisting of 20 mM sodium acetate solution, 8% methanol (v/v) and 0.22% TFA (v/v) at 0.5 ml/min; PSB probe solution: 5×10^{-6} M (pH 6.4) at 0.1 ml/min; $\lambda_{ex} = \lambda_{em} = 365$ nm. Peak identification: 1, Siso; 2, Eti (IS).

curves were prepared with Siso standard working solutions at mass ranging from 25 ng to 700 ng while keeping Eti mass constant at 100 ng. Peak areas ratio were used for the calculation. Linear regression analysis of the results yielded the following equation: $Y = 0.0245X - 0.5625$, $r = 0.9990$, where Y is the peak area ratio of the Siso to Eti, X is mass of Siso (ng), and r is the correlation coefficient. From the calibration curves, the limit of detection was estimated to be 18 ng for Siso (signal/noise = 3).

To determine method precision and accuracy, three serum samples spiked with Siso at 5, 15 and 25 μg/ml (QC sample) were analyzed seven times each within the same day and in different days, respectively. Intra- and inter-day reproducibility data for Siso is presented in Table 1. The reproducibility data and recoveries of Siso at different concentrations are shown in Table 2. Relative standard deviations (RSDs) were all below 5.3% for intra-day precision and inter-day precision.

Table 1
Intra- and inter-day reproducibility data.

Added (μg/ml)	Intra-day RSD (%, $n = 7$)	Inter-day RSD (%, $n = 7$)
5.0	2.3	5.3
15.0	1.7	3.6
25.0	2.1	4.5

Table 2
Accuracy and precision of Siso added to serum ($n = 5$).

Drug	Added (μg/ml)	Found (μg/ml)	Recovery (%)	RSD (%)
Siso	5.0	4.8	96.0	4.1
	15.0	15.1	100.7	2.8
	25.0	25.5	102.0	2.6

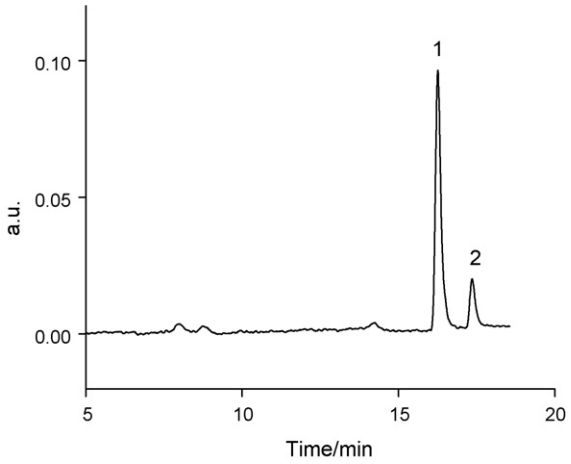

Fig. 5. Chromatogram of a rat serum sample at 1.0 h after intramuscular injection of 30 mg/kg Siso. Chromatographic and detection conditions were as in Fig. 4.

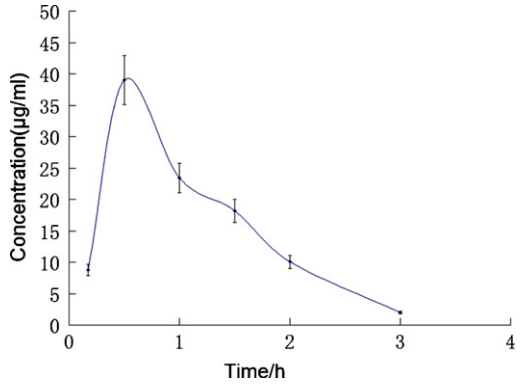

Fig. 6. Mean serum concentration–time profiles of Siso after intramuscular injection of 30 mg/kg to healthy SD rats.

3.5. Application of the method in pharmacokinetic studies

The described method was successfully applied to pharmacokinetic study of Siso in rats. The serum samples from six SD rats at different time after intramuscular administration of 30 mg/kg Siso were analyzed. A typical chromatogram is shown in Fig. 5. Serum concentrations of Siso in rat were detectable at least 3 h after intramuscular administration and the mean serum concentration–time curve is shown in Fig. 6. The main pharmacokinetic parameters of Siso were calculated from the detection datum using DAS 2.0. After intramuscular administration of 30 mg/kg Siso, the mean values of T_{max} and C_{max} were 0.5 h and 39.00 (34.28–43.08) μg/ml, respectively. The elimination half-life ($t_{1/2}$) of Siso was 0.56 (0.45–0.69) h. The AUC_{0-4} and $AUC_{0-\infty}$ values obtained were 47.10 (43.78–51.51) and 48.78 (45.06–53.19) μg h/ml, respectively.

4. Conclusion

An HPLC–RRS method was developed for the determination of Siso. The use of RRS in HPLC is demonstrated to be suitable to detect the substances which are not fluorescing and not UV absorbed. Simple modification from a commercial HPLC instrument allows direct analysis of Siso in rat serum for the pharmacokinetics study without any derivatization step. The present method showed better selectivity and repeatability and can be used for the determination of Siso in biological samples.

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