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Journal of Chromatography B, 833 (2006) 231–235
JOURNAL OF CHROMATOGRAPHY B
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Determination of rutin deca(H-) sulfate sodium in rat plasma using ion-pairing liquid chromatography after ion-pairing solid-phase extraction

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Received 31 October 2005; accepted 2 February 2006
Available online 2 March 2006

Abstract

Rutin deca(H-) sulfate sodium (RDS) is one of the most important drug candidates, which possesses very good activity as inhibitor of the complement system of warm-blooded animals and human immunodeficiency virus (HIV). In order to understand RDS metabolism and disposition, an ion-pairing coupled with solid-phase extraction technique (IP-SPE) was developed to extract RDS from rat plasma sample. Tetrabutyl ammonium bromide (TBAB) buffer (0.2 M, pH 8.0) was used as the ion-pairing extraction reagent and LC-18 was used as SPE sorbent. In addition, an ion-pairing HPLC method was established for the specific determination of RDS. A reversed phase C_8 column was used for the separation of RDS and nitrendipine (internal standard). The mobile phase was composed of 10 mM phosphate buffer solution containing 25 mM TBAB–acetonitrile (52:48, v/v, pH 7.5). The calibration curve was linear from 0.3 to 30 nmol/mL. The analytical recovery from rat plasma was found to be $97.9 \pm 4.1\%$ ($n = 15$). LOD and LOQ for RDS in plasma were calculated to be 0.12 nmol/mL and 0.30 ± 0.024 nmol/mL (R.S.D. = 8.2%, $n = 5$), respectively. The intra- and inter-day precision was less than 9.2%. The assay was applied to a preliminary pharmacokinetic study in three male rats after those received a single intravenous bolus via caudal vein of 12 μmol/kg RDS.

Keywords: Rutin deca(H-) sulfate sodium; Ion-pairing solid-phase extraction; Ion-pairing chromatography; Plasma

1. Introduction

Rutin deca(H-) sulfate sodium (RDS, Fig. 1), a polysubstituted flavone, is one of the most important drug candidates, which possesses very good activity as an inhibitor of the complement system of warm-blooded animals and can be used in the therapeutic treatment of certain immunological diseases [1,2]. It may also be useful in the treatment of transplant rejection [3]. Recently, it was found that this compound also possesses good activity as an inhibitor of human immunodeficiency virus (HIV) [4].

At present, there is no data available regarding its disposition in different tissues or organs [2]. One reason is that it is difficult to extract RDS from biological fluids due to its high polarity. Another is that RDS has a very short retention time when reversed phase chromatography is used and may co-elute with the endogenous substances in the plasma.

Ion-pairing (IP) reagents have been known and used for their ability to change selectivity and increase retention of polar compounds on reversed-phase (RP) analytical columns [5–12]. Typical IP reagents contain a nonpolar portion, such as a long chain aliphatic hydrocarbon, and a polar portion, such as an acid or base. On the one hand, the polar portion of the IP reagent interacts with the charged group of the analyte, forming an "ion-pairing". On the other hand, the nonpolar portion of the IP reagent interacts with the RP media. Retention of an analyte may be enhanced by increasing the concentration of the IP reagent or by increasing the carbon chain length of the IP reagent [5].

A method for determining of RDS in raw materials has been reported by us earlier using ion-pairing high-performance liquid chromatography (IP-HPLC) [13]. As the concentrations of RDS prepared for analysis from raw materials are much higher than those in plasma, but the method cannot be used directly to

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doi:10.1016/j.jchromb.2006.02.012

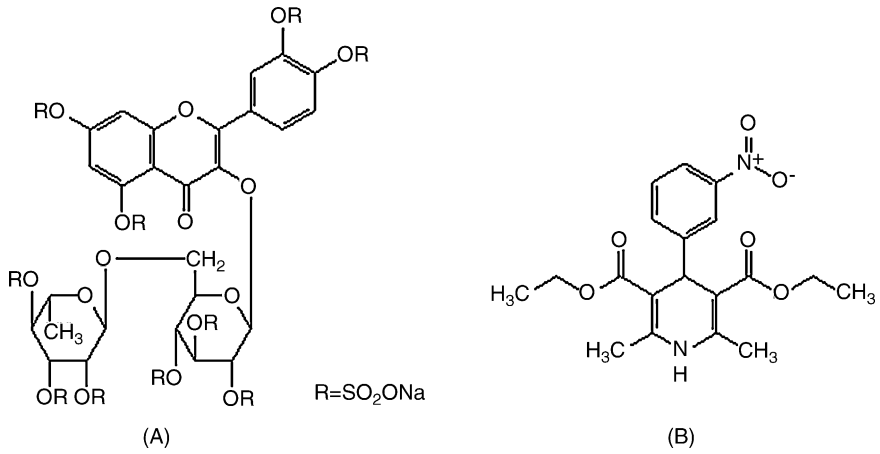

Fig. 1. Chemical structures of RDS (A) and nitrendipine (I.S.) (B).

measure plasma levels of RDS, due to inadequate sensitivity or interference by endogenous components in plasma.

For pharmacokinetic studies, it is required to develop a suitable sensitive HPLC method that allows measurement of low concentration of RDS in biological matrices. In this study, an analytical method using ion-pairing liquid chromatography (IP-LC) after ion-pairing solid-phase extraction (IP-SPE) was developed and applied to study RDS pharmacokinetics in rat plasma.

2. Experimental

2.1. Chemicals and reagents

Rutin deca(H-) sulfate sodium was kindly donated by Dr. Yong-Zhou Hu (Department of Medicinal Chemistry, Zhejiang University, China). Internal standard (I.S.), nitrendipine was purchased from Chengdong Chemical Raw Materials Factory (Jintan, China). Triethylamine (TEA) and the ion-pairing reagent, tetrabutyl ammonium bromide (TBAB) were of analytical grade and purchased from Wulian Chemical Factory (Shanghai, China). HPLC-grade acetonitrile was purchased from Tedia Company (Fairfield, USA). Potassium dihydrogen phosphate was purchased from Huzhou Chemical Factory (Huzhou, China). Plasma used for calibration curve and validation of the assay was obtained from male Sprague–Dawley rats (Laboratory Animal Center of Zhejiang University, Hangzhou, China).

2.2. Instrumentation

Chromatographic assay was carried out on a Shimadzu integrated HPLC system LC-2010C liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an UV detector, autosampler and column oven. Instrument control, data collection and processing were performed by Shimadzu Class-VP software version 6.12. All chromatographic separations in this study were performed using an XDB-C_8 analytical column (150 mm × 4.6 mm I.D., particle size 5 μm, Agilent, USA), connected with a C_{18} guard column (20 mm × 4.6 mm I.D., particle size 5 μm).

2.3. Chromatographic conditions

The buffer solution of the reversed-phase ion-pairing HPLC assay was composed of 10 mM potassium dihydrogen phosphate and 25 mM TBAB that was adjusted to pH 7.5 (Mettler DELTA 320 pH meter, Shanghai, China) with TEA. This solution was then filtered through a 0.45-μm-filter membrane and degassed using an ultrasonic bath (KQ2200B, Kunshang, China) before use. The mobile phase consisted of the buffer solution–acetonitrile (52:48, v/v), mixed on-line at a flow rate of 1.0 mL/min. The temperature of the column oven was maintained at 25 °C. Each sample was injected into the column at a volume of 20 μL. The detection wavelength was set at 326 nm.

2.4. Stock and working solutions

Stock solution of RDS was prepared in water to yield 600 nmol/mL and stored at 4 °C. Prior to use, the stock solution was further diluted with water to obtain working solutions of concentrations between 3 and 300 nmol/mL. An appropriate dilution of the working solution with drug free rat plasma gave a final concentration range between 0.3 and 30 nmol/mL. The quality control samples (1.2 and 6.0 nmol/mL) for stability test were prepared in the same manner.

TBAB buffer (0.2 M) containing nitrendipine (I.S., 14.0 nmol/mL), as the ion-pairing reagent, was prepared with 8.06 g of TBAB dissolved in 1 L of 10 mM potassium dihydrogen phosphate and adjusted to pH 8.0 with TEA.

2.5. Sample preparation

The samples to be analyzed were removed from the freezer and thawed. Calibration standards, quality control samples, and unknown samples were pipetted into 1.5 mL microcentrifuge tubes. Solid-phase extraction cartridge columns (Supelclean™ LC-18, 1 mL, 100 mg, SUPELCO, Bellefonte, PA, USA) were activated before use by washing successively with 2 mL of methanol and 2 mL of water. 0.2 mL TBAB buffer (0.2 M, pH 8.0) containing I.S. was added to 0.2 mL plasma sample. The mixture was passed slowly through the cartridge column under

mild vacuum without allowing the cartridge column to run dry. The cartridge column was then washed with 0.5 mL of water and drained completely after the wash. 0.2 mL of methanol was applied to each column to elute RDS and I.S. from the cartridge column. After vortex-mixed for 30 s, 20 μL of eluate was injected directly into the HPLC system.

2.6. Assay validation

2.6.1. Specificity

To demonstrate the specificity of the method, blank plasma samples and plasma samples from rats that had been prescribed RDS were used. Representative chromatograms were generated to show that the extraneous peaks were resolved from the peaks of RDS and I.S.

2.6.2. Calibration curve

Aliquots of 180 μL of blank rat plasma were spiked with 20 μL working solutions of RDS (3, 6, 12, 30, 60, 120 and 300 nmol/mL) to yield spiked plasma concentrations corresponding to 0.3, 0.6, 1.2, 3.0, 6.0, 12.0 and 30.0 nmol/mL for RDS. 0.2 mL TBAB buffer (0.2 M) containing I.S. (14 nmol/mL) was added to each calibration standard sample. After vortex-mixed for 1 min, the mixtures were extracted and assayed as mentioned above. The calibration curve (weighted regression line) was constructed by linear least-squares regression analysis plotting of peak area ratios (RDS/I.S.) versus the drug concentrations.

2.6.3. Sensitivity

Six independent blank samples were measured once each. Limit of detection (LOD) was expressed as the analyte concentration corresponding to the sample blank value plus 3 standard deviations. Limit of quantification (LOQ) was expressed as the analyte concentration corresponding to the sample blank value plus ten standard deviations.

2.6.4. Accuracy, precision and extraction recovery

Accuracy and precision of the method were estimated by assaying five replicate plasma samples at three different concentrations. The average precision was defined as the percentage of relative standard deviation (R.S.D.) of five standards at three different concentrations on the same day. Inter-day variability was estimated from the analysis of the five standards on five separate days.

The extraction recovery of RDS after the ion-pairing solid-phase extraction was assessed at three concentrations, 1.2, 6.0 and 30.0 nmol/mL. The values of extraction recovery of RDS and I.S. were calculated in five replicates by comparing observed peak areas in extracted samples to those of unprocessed standard solutions containing an equivalent amount of RDS or I.S., respectively.

2.6.5. Stability

The freeze–thaw stability of RDS in plasma was evaluated over three freeze–thaw cycles. Quality control samples in triplicate at the levels of 1.2 and 6.0 nmol/mL were immediately frozen at −20 °C, and thawed at room temperature three consecutive times. After that, the samples were processed and assayed. The stability of RDS in quality control samples that were stored at room temperature for 24 h and stored at −20 °C for 4 weeks was also assessed. The mean values of RDS were compared with the initial ones, which were assayed immediately after the preparation of quality control samples. The stability was expressed as a percentage of the initial value.

2.7. Pharmacokinetics study in rats

The assay was applied to a single dose (12 μmol/kg) pharmacokinetic study in rats. Three male Sprague–Dawley rats were obtained from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The study protocol complied with the Institutional Guidelines on Animal Experimentation of Zhejiang University. After a single intravenous bolus administration via caudal vein, blood samples of about 0.5 mL were collected via caudal vein in 1.5 mL microcentrifuge tubes (containing sodium heparin) at 10, 45, 60, 90, 120, 240, 480 and 720 min. The blood samples were centrifuged at 3000 × *g* for 10 min at room temperature. Then the plasma was transferred to separate plasma tube and stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of HPLC separation

Initially, a mobile phase consisting of 25 mM tetramethyl ammonium chloride or tetraethyl ammonium bromide and acetonitrile (pH 7.5) was tested. Under these conditions, the retention time of RDS was around 3 min and was not suitable for the analysis of biological samples due to potential interferences of endogenous substances in plasma. The variation of either the percentage of acetonitrile or the pH value of the mobile phase did not lead to a significant change in the retention of RDS. Conversely, separation improved markedly when TBAB was used as an ion-pairing reagent. The explanation might be that the long alkyl groups of tetraalkylammonium increased the interaction between the RDS and the C_8 stationary phase, which improved the selectivity in the reversed-phase HPLC column [14].

The potassium dihydrogen phosphate buffer used in the mobile phase allowed the control of both the pH value (at 7.5) and the ionic strength. It could also increase the affinity of the ion-pairing forms to column C_8 particles. When potassium dihydrogen phosphate concentration reached 10 mM, an improvement in the baseline separation was observed for RDS, I.S. and endogenous substances.

3.2. Optimization of SPE

It is known that the efficiency of SPE depends on the type of sorbent, the sample volume and its pH, the content of organic modifier and the volume of the elution solvent. Moreover, the enrichment of multiply charged compounds on hydrophobic extraction materials always requires the addition of salts or ion-

pairing reagents to the samples to increase the solute–sorbent interaction [15,16]. This fact was confirmed in our experiments.

The extraction of RDS from plasma samples is difficult because of its high polarity. Therefore, we studied the SPE behavior of RDS using various polymeric sorbent materials. TBAB was added to the plasma samples as ion-pairing reagent when LC-18, ODS, DSC-18 and LC-NH_2 cartridges were employed to extract RDS, while no ion-pairing reagent was added when LC-Si and LC-SAX cartridges were used. Among the investigated SPE cartridges (all from Supelco), the highest recoveries were obtained with LC-18. ODS and DSC-18 cartridges showed very similar extraction recoveries, whereas no retention of RDS was achieved on LC-NH_2, LC-Si (normal-phase extraction) and LC-SAX (ion-exchanger) cartridges. When the relative costs of different RP SPE cartridges were taken into consideration, LC-18 cartridge was chosen for the extraction and purification of R.S.D.

In addition to the different cartridges, the pH effect of the plasma was studied. RDS is a highly polar compound. Therefore, the plasma samples should be alkalinized before SPE in order to form "ion-pairing" to enhance the hydrophobicity. The pH values of plasma samples were adjusted to 5, 6, 7, 8, 9, and 10 with TEA. The highest recoveries were obtained at pH > 8. In order to reduce the damage to the analytical column by higher pH value, pH 8 was chosen [17].

3.3. Optimization of assay validation

3.3.1. Specificity

Representative chromatograms of (Fig. 2A) rat blank plasma, and (Fig. 2B) rat blank plasma spiked with RDS (6 nmol/mL) and the I.S. were obtained using the above chromatographic conditions. No endogenous peaks in plasma were found to interfere with RDS or I.S. The RDS and I.S. were well resolved with good symmetry and the retention time was 8 and 11 min, respectively. Fig. 2C shows the chromatogram of plasma sample obtained at 4 h after rats treated with RDS.

3.3.2. Calibration curve

The relationship between RDS concentrations (y) and peak area ratio (x) of RDS to I.S. was linear from 0.3 to 30.0 nmol/mL. The regression equation was shown as follows: $y = 0.3944x - 0.1093$, $R = 0.9997$.

3.3.3. Sensitivity

The LOD of RDS in plasma was calculated to be 0.12 nmol/mL. The LOQ was 0.30 ± 0.024 nmol/mL (extraction recovery $62.2\% \pm 8.2\%$, $n = 5$).

3.3.4. Accuracy, precision and extraction recovery

The accuracy of the assay was assessed by evaluating the analytical recovery. As shown in Table 1, the average analytical recovery of RDS from plasma samples was $97.9 \pm 4.1\%$ ($n = 15$).

The intra- and inter-day precisions of the assay were determined by assaying five samples of drug-free plasma containing known concentrations of RDS. As described in Table 1, the intra- and inter-day R.S.D. (%) was less than 9.2%

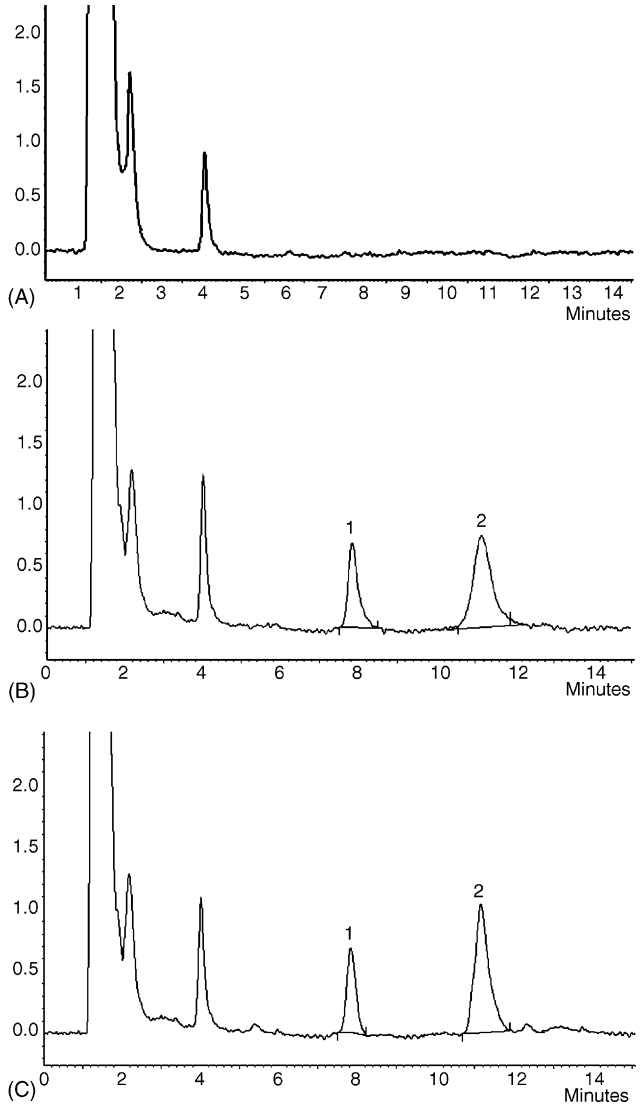

Fig. 2. Typical ion-pairing HPLC chromatograms for (A) rat blank plasma, (B) rat blank plasma spiked with RDS (6 nmol/mL) and the I.S., and (C) rat plasma sample obtained at 4 h after rats received a single intravenous bolus via caudal vein of 12 μmol/kg RDS.

The average extraction recovery of RDS at concentrations of 0.2, 6.0 and 30.0 nmol/mL was $60.35 \pm 3.1\%$ ($n = 15$) (see Table 1). The result suggested that there was no difference in extraction recovery at different concentrations of RDS.

3.3.5. Stability

The stability of RDS under various conditions is described in Table 2. Under all conditions tested, RDS was stable with detected concentrations of at least 93.4% of the initial concentration.

3.4. Pharmacokinetics study in rats

The assay was applied to a preliminary pharmacokinetic experiment in rats. A single bolus dose of 12 μmol/kg RDS was administered intravenously to three male rats via caudal vein. The plasma concentration–time profile is illustrated in Fig. 3.

Table 1
Intra- and inter-day precision, extraction recovery and accuracy of the determination of RDS in plasma ($\bar{x} \pm$ S.D., $n = 5$).

Concentration (nmol/mL)	R.S.D. (%)		Extraction recovery (%)	Accuracy (%)
	Intra-day	Inter-day		
1.2	4.2	9.2	63.6 ± 7.1	95.3 ± 4.3
6.0	6.4	6.8	57.4 ± 6.4	97.1 ± 4.1
30.0	4.8	5.8	60.0 ± 5.9	101.2 ± 3.8

Table 2
Stability of RDS in plasma ($\bar{x} \pm$ S.D., $n = 3$)

Concentration (nmol/mL)	Treatment	Percentage of initial value
1.2	Three freeze–thaw cycles	97.2 ± 10.2
	Stored at room temperature for 24 h	103.1 ± 6.4
	Stored at −20 °C for 4 weeks	98.5 ± 5.9
6.0	Three freeze–thaw cycles	93.4 ± 2.4
	Stored at room temperature for 24 h	98.6 ± 4.6
	Stored at −20 °C for 4 weeks	97.7 ± 3.9

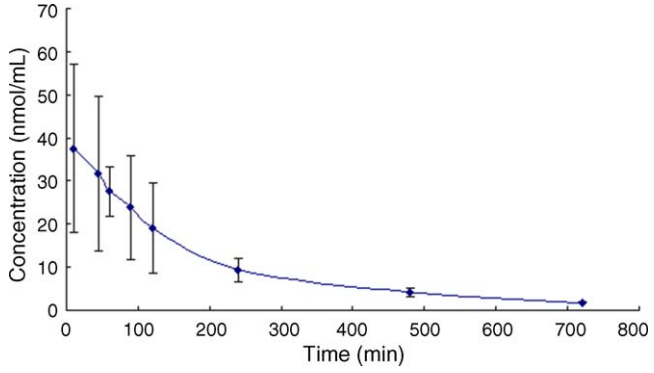

Fig. 3. Mean plasma concentration–time profile of RDS in three male rats after received a single intravenous bolus via caudal vein of 12 μmol/kg RDS. The solid line represents fitted average plasma concentrations using *3p87* software (CPA, China).

The pharmacokinetic parameters of clearance, volume of distribution and terminal half-life were 1.333 mL/min/kg, 240 mL/kg and 125 min, respectively.

4. Conclusion

An analytical method using ion-pairing solid-phase extraction followed by ion-pairing reversed-phase high-performance liquid chromatography (IP-RP-HPLC) for quantitative determination of RDS in rat plasma has been developed and proven to be simple, sensitive, accurate and reproducible. The present method is, to our knowledge, the first bioanalytical method described for the quantitative determination of RDS at low plasma concentrations.

The overall performance of the HPLC method was found to be satisfactory for the purpose of determining the concentration of RDS in plasma samples from pharmacokinetic measurements of RDS in rats.

Acknowledgments

The project was support by the National High Technology Research and Development Program of China (863 Program, #2002AA2Z3114) and Foundation of the Ministry of Education of China (#20040335013).

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