

istics of steroidal saponins, such as a high boiling point, a high polarity, and a relatively high molecular weight. SAR lacks UV absorbance and shows a low response in mass spectrometry. Although several methods for the measurement of SAR, including HPLC-ELSD (evaporative light scattering detection)^[8] and thin layer chromatography (TLC), have been previously reported^[9], the sensitivity of such methods is very poor and can not reach the level required of PK assays.

Immunoassay is a potential tool for the analysis of natural products in complex matrices because of its high determination sensitivity, short analysis time, and simple operation procedures. In recent years, immunoassay was frequently applied in the quantitative determination of various natural products, such as sotalol in rat serum^[10], 20(S)-protopanaxatriol^[11], and ginsenoside Rg3 in ginseng^[12], aconitine-type alkaloids in *Aconiti Radixes*^[13] and Plumbagin in *Plumbago zeylanica*^[14].

In previous studies, our laboratory obtained polyclonal antibodies against ruscogenin and glycyrrhizin^[15, 16]. In the current work, we report the preparation of a polyclonal antibody against SAR and its application in the development of a sensitive, accurate, and specific immunosorbent assay for the determination of SAR in rat plasma.

Materials and methods

Chemicals

N,N'-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS) and Freund's complete adjuvant (FCA) were purchased from Sigma (St Louis, MO, USA). Tetramethylbenzidine (TMB) was obtained from Amresco Chemical Co (Solon, OH, USA). Bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Roche (Florence, USA). Goat anti-rabbit IgG horseradish peroxidase (IgG-HRP) was obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania, USA). SAR (purity >98%) was purchased from Wuhu Delta Medical Technology Co Ltd (Wuhu, China). All chemicals and solvents were of analytical grades. A 96-well polystyrene immunoplate was purchased from Corning Costar Corp (New York, USA).

The media for ELISA included the following components: phosphate-buffered saline (PBS, 0.02 mol/L phosphate buffer, pH 7.2, containing 0.15 mol/L NaCl); coating buffer (0.05 mol/L carbonate-hydrogen carbonate, pH 9.6); blocking buffer (0.02 mol/L PBS containing 0.5% gelatin); washing buffer (PBS-T, 0.02 mol/L PBS containing 0.05% Tween 20).

Animals

Two male rabbits (2.5 kg) were obtained from the Qinglong Mountain Animal Breeding Ground of the Nanjing Jiangning District (Nanjing, China, Certificate No SCXK (Su) 2007-0008). Six male Sprague-Dawley rats (clean grade, 7–9 weeks old and weighing 250–280 g) were obtained from the Comparative Medicine Center of Yangzhou University (Yangzhou, China, Certificate No SCXK (Su) 2007-0001). Protocols for the animal studies were approved by the Regulations of Experimental Animal Administration, State Committee of Science and Technology of the People's Republic of China.

Preparation of the immunogen for sarsasapogenin

SAR was coupled to BSA based on a method reported by Naar with slight modifications^[17]. Fifty milligrams of SAR and 1 g of succinic anhydride were added to 1 mL pyridine and refluxed at 100 °C for 9 h. The reaction was monitored by thin layer chromatography (TLC) using a chloroform:methanol:water mixture (8:1:0.1, *v/v/v*) as the developing solvent. The solution was extracted three times with chloroform (15 mL for each extraction). The combined extracts were washed three times with water (25 mL for each wash). The succinylated SAR (SAR-HS) was further purified by Sephadex LH-20 (chloroform:methanol=1:1).

Succinylated SAR was covalently attached to BSA by an active ester method (Figure 2)^[18]. The active ester of SAR-HS was prepared by stirring 10 mg SAR-HS, 19 mg DCC, and 13 mg NHS in 1 mL dimethylformamide for 5 h at room temperature. The solution was added dropwise to 10 mL phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4) containing 30 mg BSA and incubated at 4 °C for 12 h with constant stirring to produce SAR-HS-BSA. The reaction mixture was put into a dialysis bag and dialyzed successively using deionized water for 72 h at 4 °C. Finally, the solution was lyophilized and stored at -20 °C before use. The coating antigen was prepared according to the conjugation protocol described above using SAR-HS and OVA.

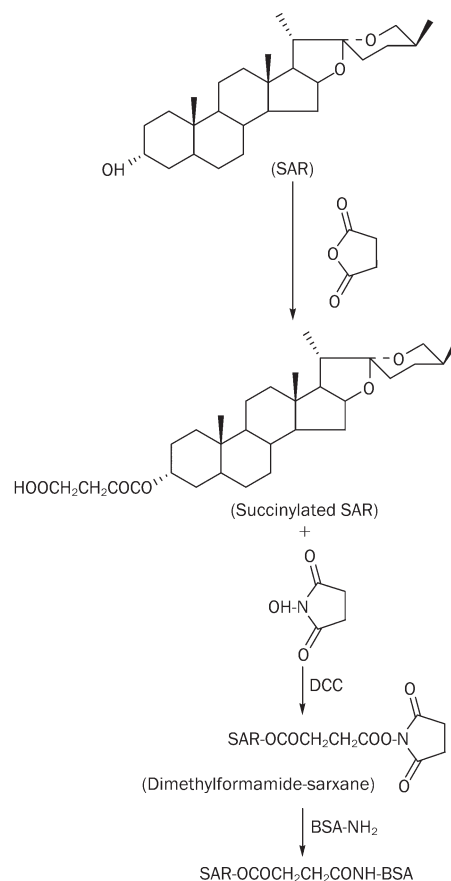


Figure 2. Conjugation of sarsasapogenin to BSA carrier protein.

Antiserum production in rabbits

Two male rabbits were used for the immunization of SAR-HS-BSA. Pre-immunization blood samples were taken. The emulsifying mixture of the conjugate (1 mg) and FCA was injected into each rabbit subcutaneously. The same dose of the conjugate emulsified with incomplete Freund's adjuvant in the same ratio was used as a booster once every 14 d. Blood was collected from the marginal ear vein 1 week after each boost. The blood was centrifuged at 3000 round per minute for 15 min, and the serum was collected and stored at -20 °C. Ten days after the last injection, whole blood was collected from the carotid artery, and the serum was prepared using the same procedure as described above.

ELISA procedure

The wells of a 96-well immunoplate were coated overnight with SAR-HS-OVA (10 µg/mL) dissolved in 50 mmol/L carbonate buffer (pH 9.6). The plate was washed three times with PBS-T and treated with 150 µL of PBS-T containing 0.5% gelatin for 1 h to reduce non-specific adsorption. Fifty microliters of antibody was added to each well followed by the addition of a competitor and incubated for 2 h. The plate was washed three times with PBS-T and incubated with 100 µL of HRP-conjugated anti-rabbit IgG (1:40000 dilution) for 1 h. Subsequently, the plate was washed three times with PBS-T, 100 µL of TMB peroxidase substrate solution was added to each well, and the plate was incubated for 20 min in the dark at room temperature. The reaction was stopped by the addition of 50 µL of 2 mol/L H₂SO₄. The activity of enzyme bound to the plate was spectrophotometrically measured by a microplate reader at 450 nm.

The cross-reactivities (CRs) of polyclonal antibodies (PABs) against various compounds were evaluated and calculated using the method of Weiler and Zenk^[19] as follows:

$$\text{CR(\%)} = \frac{\text{IC}_{50} \text{ for SAR}}{\text{IC}_{50} \text{ for compound under investigation}} \times 100\%$$

Sample preparation

The calibration and quality control samples for SAR were prepared by adding standard SAR to negative control serum. A stock solution of standard SAR prepared in methanol (0.5 mg/mL) was serially diluted with methanol to obtain a series of working solutions. Ten microliters of each working solution was added to 200 µL of negative control serum to prepare the calibration samples spanning a concentration range of 1 ng/mL to 1000 ng/mL, and to prepare the quality control samples at 10, 100, and 500 ng/mL. All the samples were mixed on a vortex mixer for 30 s before application to the microtiter plate. The separated real rat serum samples obtained from the pharmacokinetic study were added to the microtiter plate for analysis.

Pharmacokinetic evaluation

Rats were intragastrically administered with 100 mg/kg SAR.

Blood samples were collected at 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h after administration. Serum was separated by centrifugation at 8000 round per minute for 5 min. The separated serum was stored at -20 °C prior to use.

Results

Production of antigen

SAR-HS was obtained as a white powder and showed a positive Liebermann-Burchard reaction. The ESI-MS spectrum of SAR-HS showed the [M+H]⁺ ion peak at 517.4, which corresponds to the [M+H]⁺ ion peak at 417.4 of SAR and suggests that a succinyl group was introduced to SAR. In the IR spectrum, the carbonyl bond nature of the strong absorption band at 1731.43 cm⁻¹ and 1706.21 cm⁻¹, and the characteristic peaks of spirostanol-type steroids at 984.74, 910.12, 895.55, and 851.15 cm⁻¹, revealed that a nomadic carboxy group might exist in the spirostanol steroid SAR-HS.

SAR-HS-BSA

In the IR spectrum, the similar absorption bands of SAR-HS-BSA and BSA of 3600–3200 cm⁻¹ and 1700–1600 cm⁻¹, respectively, represented the characteristic amino acid bonds and suggested that a BSA functional group was introduced to SAR-HS. The fact that the characteristic peaks of spirostanol-type steroids at 985.91, 926.51, 898.14, and 866.29 cm⁻¹ were observed in the IR spectra of SAR-HS-BSA, while absent in BSA, further confirmed the introduction of BSA to SAR-HS and indicated the preparation of an intact antigen.

Production of antibodies against sarsasapogenin

Rabbits inoculated with the SAR-HS-BSA provided antisera suitable for detection. The curves of different concentrations of anti-SAR sera against absorbance showed that the titer reached 32000 after 2 monthly boosters.

Assay linearity and sensitivity

After the antigen coating concentration and the dilution of antibody were optimized by the checkerboard method, a competitive ELISA method was developed for the determination of SAR in rat plasma. *B* and *B*₀ were the percentage of binding in the presence and absence of SAR, respectively, and *C* was the concentration of SAR. The sigmoidal curve of *B*/*B*₀ values against lg(*C*) was developed, and, after logit transformation, a calibration curve of logit (*B*/*B*₀) and lg(*C*) was obtained (Figure 3). The competitive ELISA was proven to be linear (*R*²=0.9811) within a range (IC₂₀-IC₈₀) of 2.4–760 ng/mL in rat samples.

Assay specificity

Cross-reactivity is an important factor in judging the quality of an antibody and its usefulness. Because there are some structural analogs in the plants of Chinese medicines, it was extremely important to evaluate the specificity of anti-SAR serum. The results are shown in Table 1. Although there was a structural similarity between SAR and ruscogenin, diosgenin, and 25 (*R,S*) ruscogenin 1-*O*-[β-*D*-glucopyranosyl

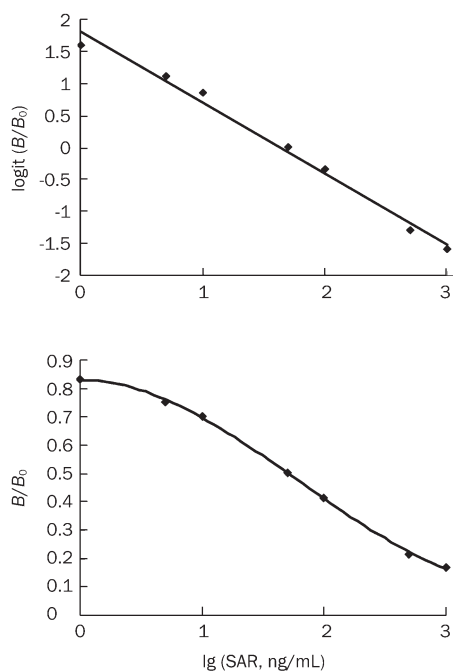


Figure 3. The standard curve of sarsasapogenin by ELISA. B and B_0 are the percentages of binding in the presence and absence of sarsasapogenin, respectively. The logit-log plot is obtained from $\ln[(B/B_0)/(1-B/B_0)]$. Coating antigen concentration: 10 $\mu\text{g/mL}$; rabbit anti-sarsasapogenin antibody dilution: 1/1000; peroxidase-labeled anti-rabbit IgG dilution: 1/40000.

Table 1. Cross-reactivity of anti-sarsasapogenin antiserum against sarsasapogenin.

Compound	Cross-reactivity (%)
SAR	100
Ruscogenin	23
Diosgenin	22
25 (R,S) Ruscogenin 1-O- $[\beta\text{-D}\text{-glucopyranosyl}$ (1 \rightarrow 2)] $[\beta\text{-D}\text{-xylopyranosyl}$ (1 \rightarrow 3)]- $\beta\text{-D}\text{-fucopyranoside}$	26
Diammonium glycyrrhizinate	<0.01
Notoginsenoside R1	<0.01

(1 \rightarrow 2)] $[\beta\text{-D}\text{-Xylopyranosyl}$ (1 \rightarrow 3)]- $\beta\text{-D}\text{-fucopyranoside}$, all of which had spiroketal structures, the differences in C-1, C-5, and C-6 reduced the affinities of the polyclonal antibodies to these components. No cross-reactivity was found for diammonium glycyrrhizinate and notoginsenoside R1, both of which lack spiroketal structures. Therefore, spiroketal structures were regarded as antigenic determinants that play an important role in the generation of antiserum.

Assay precision and accuracy

The precision and accuracy of the ELISA method were further validated based on the quality control samples at a concentration of 10, 100, and 500 ng/mL. As shown in Table 2, the accu-

Table 2. Precision and accuracy of ELISA for sarsasapogenin in rat plasma.

	Added (ng/mL)	Estimated (ng/mL) ^a	Recovery (%) ^b	CV (%) ^c
Intra-assay	10	9.1 \pm 0.7	91.0	8.3
	100	92.7 \pm 6.3	92.7	6.8
	500	480.9 \pm 15.1	96.2	3.1
Inter-assay	10	8.9 \pm 1.3	89.0	14.1
	100	89.7 \pm 7.1	89.7	7.9
	500	460.1 \pm 27.8	92.0	6.0

^a Values represent the mean \pm SD of 6 experiments

^b Recovery = measured [SAR]/spiked [SAR] \times 100%

^c Coefficient of variation

racy of intra- and inter-assays for all quality control samples were within a range of 91.0%–96.2% ($n=6$) and 89.0%–92.0% ($n=6$). The coefficients of variation (CV) for intra- and inter-assays were 3.1%–8.3% ($n=6$) and 6.0%–14.1% ($n=6$), respectively.

The LLOQ of the ELISA was defined as the lowest concentration of a validation sample that could be determined with an accuracy and precision of 75%–125% and with a CV of less than 25%. In this assay, LLOQ was determined at 2.4 ng/mL with the precision of intra-assay and inter-assay at 8.2% and 16.0%, respectively; the accuracy of intra- and inter-assays were 88.4% and 84.5%, respectively. These results verified that the developed ELISA method was precise and accurate and that the ELISA could serve as a new analytical method for the determination of SAR in rat plasma.

Application to SAR pharmacokinetics evaluation

The presently established ELISA method was applied to the PK evaluation of SAR in rats for further validation of its reliability. Rats received an intragastric administration of 100 mg/kg SAR. The mean plasma concentration-time curve is illustrated in Figure 4. The PK parameters were calculated using DAS 2.1.1 and are summarized in Table 3. SAR was characterized by a relatively long terminal elimination phase

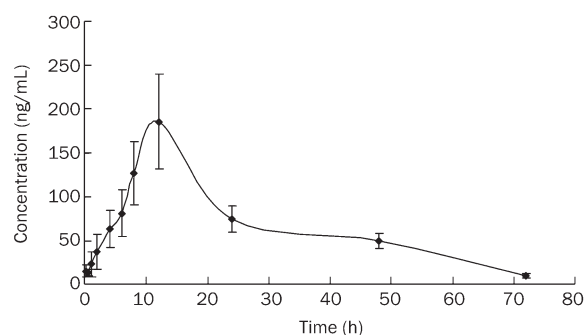


Figure 4. Mean plasma concentration-time profiles of sarsasapogenin after a single oral administration to rats ($n=6$).

Table 3. Pharmacokinetic parameters of sarsasapogenin in 6 rats after an oral dose of 100 mg/kg.

Parameter	Value
AUC _(0-t) (ng/mL·h)	4899.06±953.27
AUC _(0-∞) (ng/mL·h)	5207.95±988.16
t _{1/2} (h)	17.72±2.87
t _{max} (h)	12.00±0.00
MRT _(0-t) (h)	24.90±1.97
MRT _(0-∞) (h)	29.30±3.10
CL/F (L·h ⁻¹ ·kg ⁻¹)	19.80±3.82
C _{max} (ng/mL)	185.72±54.25

half-life (>17 h). These results indicated that the established ELISA method was sufficiently sensitive and reliable for the study of SAR PKs.

Discussion

Because there were no amino or carboxylic acid groups in the skeleton of SAR, we introduced carboxylic acid groups onto the hydroxyl groups at C-3 in the molecule to form a succinylated SAR that made the linkage of BSA possible. This 4-carbon spacer for conjugation between SAR and BSA allowed for the free rotation of the desired epitope.

In general, the antibody specificity to the hapten appears to be determined by the group on the hapten remote from the region of conjugation^[20]. These results suggested that our polyclonal antibody could recognize the structural frame of SAR, thus revealing its specificity for the SAR-type steroid saponin, which is consistent with a previous study^[15].

Precision was used to evaluate the efficiency of our analysis method. As shown in Table 3, the developed ELISA method was precise, according to the intra-assay and inter-assay variations. To validate the accuracy of the ELISA, a recovery experiment was performed; the results were satisfactory.

Because of the strong pharmacological activity of SAR, it was necessary to precisely monitor its concentration in bodily fluids to ascertain a dose for optimal use and the avoidance of side effects. There have been no studies on the PK behavior of SAR. Because our developed ELISA was proven to be sensitive and precise, we used it to monitor SAR in bodily fluids. The results indicated that a delayed t_{max} and long half-life were found for SAR. However, it should be noted that the present ELISA method might not distinguish metabolites from the SAR prototype. Therefore, the results presented here may represent the overall PK characteristics of both the prototype and its metabolites. In this study, 11 time points up to 72 h were sampled and detected using the presently developed and validated ELISA method. In view of the unexpectedly long half-life of SAR in rats, the sampling points covering the elimination phase were probably too few, which might influence the accuracy of the PK parameters.

In conclusion, the present study developed an indirect competitive ELISA method based on a specific polyclonal antibody

to SAR. This ELISA method was validated for the determination of SAR in rat plasma. The antibody obtained was found to be valuable for the assay of the SAR-type steroid saponin due to its high specificity. Detailed validation results suggest that the developed ELISA method is reliable and can be used for the determination of SAR in rat plasma. With an LLOQ at 2.4 ng/mL and a linear range of 2.4–760 ng/mL, this ELISA method was proven to be sufficiently sensitive and satisfactory for the PK study of SAR in rats.

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Author contribution

Jing WANG, Ji-hua LIU, and Bo-yang YU designed the research; Jing WANG performed all the research; Xu GUO assisted in part of the research; Jian ZHANG contributed analytical tools and reagents; Jing WANG and Ji-hua LIU analyzed the data; Jing WANG wrote the paper.

Supplementary information is available at Acta Pharmacologica Sinica's website.

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