

Research Article

Simultaneous Quantification of Aspirin, Its Metabolite Salicylic Acid, and Salvianolic Acid B in Human Plasma Using UPLC-MS/MS

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Salvianolic acid B is the main active ingredient in salvianolate injection, which is produced by extracting danshen, the most commonly used Chinese herbal medicine for cardiovascular treatment. Clinically, salvianolate injection and aspirin are commonly combined to treat coronary heart diseases in patients with stable angina. To support clinical studies on drug-drug interactions (DDIs) between salvianolate injection and aspirin, a rapid and sensitive UPLC-MS/MS method for the simultaneous determination of aspirin (acetylsalicylic acid), its metabolite salicylic acid, and salvianolic acid B in human plasma was developed. The analytes and internal standard were extracted from the acidified plasma by liquid-liquid extraction with ethyl acetate and then separated by gradient elution with acetonitrile/0.5% formic acid in water on a C18 column. Salvianolic acid B, acetylsalicylic acid, and salicylic acid were quantified in multiple-reaction monitoring mode with negative ion electrospray ionization. The method was fully validated according to the current regulatory guidance for bioanalysis. Calibration curves in the range 5–6000 ng/mL, all with correlation coefficients greater than 0.99, were established using linear regression models for salvianolic acid B and acetylsalicylic acid and a quadratic model for salicylic acid. The validated method was successfully used to measure salvianolic acid B, acetylsalicylic acid, and salicylic acid concentrations in human plasma samples from 16 patients to observe the pharmacokinetic changes caused by DDIs.

1. Introduction

Salvianolate injection, which is prepared by extracting danshen, the most widely used Chinese medicinal herb, is used clinically to treat coronary heart disease in patients with stable angina [1]. A systematic review and meta-analysis have revealed that salvianolate injection in combination with Western medicine could improve the total therapeutic rate and electrocardiogram effective rate [2] as well as reduce the hospitalization time of patients with coronary heart disease [3]. Pharmacological studies have indicated that salvianolic injection could protect cardiomyocytes by inhibiting TGF- β 1/Smad2/3 and TXNIP/NLRP3 inflammatory signaling pathways [4]. Furthermore,

salvianolic acid B (Sal B), the major component of salvianolate injection, could inhibit human platelet activation via PDE inhibition and P2Y₁₂ antagonism [5]. In addition, Sal B could reduce the inflammatory reaction, even if the platelets have already been activated [6].

Clinically, salvianolate injection is often used in combination with aspirin (acetylsalicylic acid; ASA) to treat cardiovascular disease. Clinical research has shown that danshen preparations combined with ASA significantly relieve the symptoms of angina pectoris [7] and reduce the damage to the gastric mucosa caused by ASA and the risk of gastric bleeding [8]. Furthermore, laboratory studies have revealed a potential pharmacokinetic interaction between danshen and ASA [9, 10].

In particular, the combined use of salvianolate injection and ASA may improve the efficacy and reduce the toxicity by changing the pharmacokinetic behavior. Therefore, clinical research on the drug-drug interactions (DDIs) between salvianolate injection and ASA is necessary to guide the rational clinical use of this medication.

To conduct pharmacokinetic research on DDIs between salvianolate injection and ASA, a simple and sensitive detection method is required. In biological samples, ASA and its metabolite salicylic acid (SA) are often quantified together with other cardiovascular drugs. A reported combined detection method for aspirin and clopidogrel uses protein precipitation combined with liquid-liquid extraction to process plasma samples; however, the complexity of these operations may increase the experimental error [10, 11]. In addition, liquid-liquid extraction using methyl *tert*-butyl ether has been used in combined detection methods for aspirin with pravastatin [12] or with atorvastatin [13]. For acidification before liquid-liquid extraction, a relatively mild organic acid, such as 1% formic acid (FA), is typically used to reduce the ionization of ASA and SA in plasma [12]. However, Sal B usually requires a strong acid such as hydrochloric acid as an acidifying agent [14]. In addition, when redissolving the extraction residue, acetonitrile (ACN) [11] or a mixed solution with a high proportion of ACN, such as ACN/H₂O (80 : 20, v/v, containing 0.5% acetic acid) [10] or ACN/H₂O (80 : 20, v/v, containing 5 mM ammonium acetate buffer) [12], is typically used for ASA and SA. In contrast, a solvent mixture that contains methanol (MeOH), such as MeOH/H₂O (50 : 50, v/v), is necessary for Sal B [15]. Therefore, none of the currently available biological sample analysis methods are suitable for the simultaneous detection of ASA, SA, and Sal B. Chemical properties should also be considered when choosing an internal standard. Chloramphenicol, which is an acidic compound with a low baseline in the plasma matrix, has been reported as an internal standard for the *in vivo* analysis of Sal B [16, 17]. Moreover, in view of the inherent problems associated with the bioanalysis of Sal B, ASA, and SA, including a lack of stability and high polarity [18], establishing a suitable bioanalytical method is challenging. LC-MS/MS is considered a gold standard for biological analysis with high throughput, sensitivity, specificity, small sample volume requirements, and suitability for analyzing large numbers of clinical samples. However, to date, no bioanalytical UPLC-MS/MS method has been reported for the simultaneous analysis of Sal B, ASA, and SA.

In this work, we developed a UPLC-MS/MS method for the simultaneous determination of Sal B, ASA, and SA in human plasma. In addition, the applicability of the validated method to a clinical pharmacokinetic study of the changes caused by DDIs between salvianolate injection and ASA was investigated.

2. Materials and Methods

2.1. Chemicals and Materials. Sal B (lot P10J9F52565, purity > 98%), ASA (lot SJ0714YA14, purity > 98%), SA (lot J22S6J3607, purity > 98%), and chloramphenicol (IS; lot HM0328NA14, purity > 97%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai,

China). The chemical structures of Sal B, ASA, SA, and IS are shown in Figure 1.

HPLC-grade ACN, HPLC-grade MeOH, and Optima LC/MS-grade FA were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ, USA). High-purity water was obtained using a Milli-Q Ultrapure Water purification system (Millipore, Burlington, MA, USA). Blank plasma samples were obtained from healthy volunteers with EDTA used as an anticoagulant.

Salvianolate injection (lot 17090623, 200 mg + 5% glucose injection, in 250 mL, iv) was purchased from Shanghai Green Valley Pharmaceutical Co., Ltd. (Shanghai, China). Aspirin enteric-coated tablets (lot BJ38708, 100 mg) were purchased from Bayer Healthcare Co., Ltd. (Beijing, China).

2.2. UPLC-MS/MS Conditions. A UPLC system (consisting of an Acquity Binary Solvent Manager and an Acquity Sample Manager, Waters, Milford, MA, USA) coupled with an MS/MS system (Triple Quad™ 5500, AB Sciex, Framingham, MA, USA) was applied for the determination of ASA, SA, and Sal B in biosamples. The data were collected and processed using the Analyst software (version 1.6.2, AB Sciex, Framingham, MA, USA).

Chromatographic separation was achieved using an Acquity UPLC™ BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters, Wexford, Ireland) at 50°C. The mobile phase consisted of 0.5% FA in water (solvent A) and ACN (solvent B). The gradient elution program was as follows: 0–0.3 min, 95–75% A; 0.3–1.3 min, 75–70% A; 1.3–2.1 min, 70–30% A; 2.1–2.5 min, 30% A; 2.5–2.7 min, 30–95% A; and 2.7–4.0 min, 95% A. The flow rate was 0.4 mL/min, and the injection volume was 2 μL.

The mass spectrometer was operated in negative ion electrospray ionization mode. The analytes were detected using the multiple-reaction monitoring (MRM) mode with *m/z* transitions at 717.2 → 519.1 for Sal B, 179.1 → 137.0 for ASA, 136.8 → 65.0 for SA, and 321.0 → 152.0 for IS. The optimized instrumental conditions were as follows: gas source temperature, 400°C; ion spray voltage, 4000 V; entrance potential, 10 V; collision cell exit potential, 17 V; curtain gas, 15 psi; gas 1, 18 psi; and gas 2, 5 psi. The collision energies and declustering potentials for the analytes are listed in Table 1.

2.3. Preparation of Standard Solutions. To prepare the primary stock solutions (1 mg/mL), ASA and SA were accurately weighed and dissolved in ACN, whereas Sal B was dissolved in MeOH. The Sal B, ASA, and SA stock solutions were mixed (1 : 1 : 1, v/v/v) and serially diluted with MeOH/H₂O (80 : 20, v/v) to obtain standard working solutions and quality control (QC) solutions. The IS stock solution was prepared in ACN and diluted with FA/H₂O (50 : 50, v/v) to obtain a working solution at a concentration of 50 ng/mL. All solutions were stored at 4°C.

2.4. Preparation of Calibration Standards and Quality Control Samples. The calibration standards and QC samples were prepared by spiking 40 μL of blank human plasma with 10 μL of a working solution. The Sal B, ASA, and SA calibration standards were prepared in plasma at concentrations of 5,

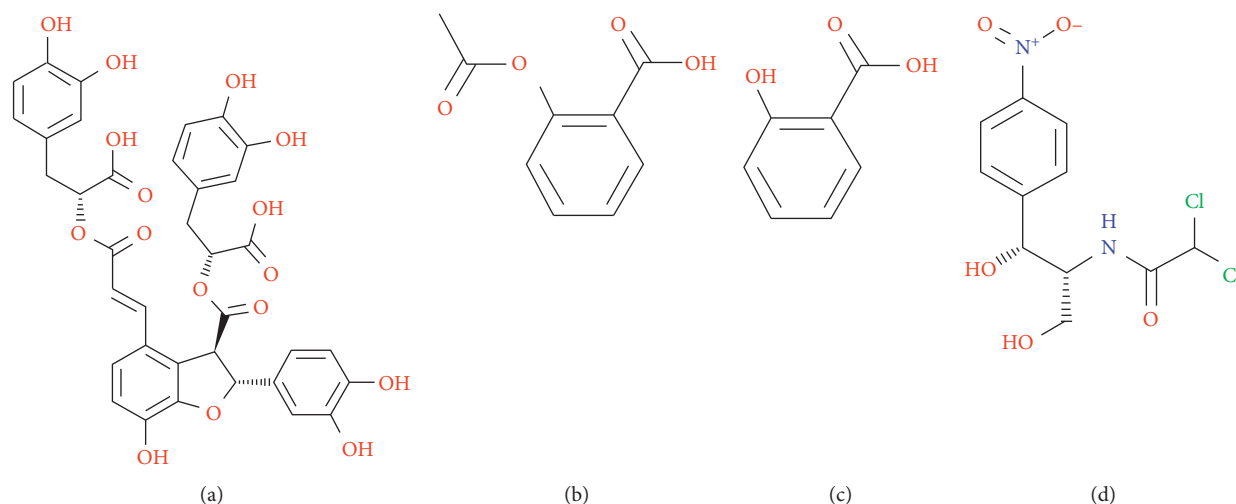


FIGURE 1: Chemical structures of (a) salvianolic acid B, (b) acetylsalicylic acid, (c) salicylic acid, and (d) chloramphenicol.

TABLE 1: MS/MS ion acquisition parameters of Sal B, ASA, SA, and IS.

Components	Transition (m/z)	Collision energy (V)	Declustering potential (V)
Sal B	717.2→519.1	-20.0	-50.0
ASA	179.1→137.0	-10.0	-30.0
SA	136.8→65.0	-25.0	-50.0
IS	321.0→152.0	-21.0	-100.0

10, 50, 300, 800, 2000, 5000, and 6000 ng/mL. The QC samples for Sal B, ASA, and SA were prepared in plasma at four concentrations: 5 ng/mL for lower limit of quantitation (LLOQ), 15 ng/mL for low QC (LQC), 3000 ng/mL for medium QC (MQC), and 4500 ng/mL for high QC (HQC). The calibration standards and QC samples were freshly prepared every day and stored in an ice-water mixture.

2.5. Sample Preparation. A plasma sample (50 μ L) spiked with 20 μ L of IS was vortex-mixed for 30 s. Then, 300 μ L of ethyl acetate was added to the plasma sample. Liquid-liquid extraction was performed by vortexing the mixture for 2 min and then centrifuging at 12000 rpm for 10 min at 4°C. After separation, the organic layer was evaporated at room temperature under a gentle stream of nitrogen. The residue was redissolved in 50 μ L of a MeOH/ACN/H₂O mixture (40 : 40 : 20, v/v/v) and vortex-mixed for 2 min. After centrifugation at 12000 rpm for 10 min at 4°C, 2 μ L of the supernatant was analyzed by UPLC-MS/MS.

2.6. Method Validation

2.6.1. Specificity. The specificity of the method was investigated using six predose plasma samples from different volunteers. A comparison of the chromatograms of blank plasma from six sources with those of blank plasma spiked with the corresponding analytes confirmed that there was no interference from endogenous compounds at the retention times of the analytes. The quantitative criteria can still be satisfied if the peak areas of endogenous compounds

coeluting with the analytes are less than 20% of the peak area of the LLOQ standard and the IS response in the blank does not exceed 5% of the average response in the calibration standards and QCs [17].

2.6.2. Calibration Curve Linearity and Method LLOQ. Calibration curves for Sal B, ASA, and SA were established by adding the working solution of the corresponding analyte to blank human plasma at eight different concentrations and performing measurements on three consecutive days. The curves were constructed using the peak area ratios of the analyte to the IS versus the plasma concentration of the analyte with a weighted least-squares regression model. The correlation coefficient (r) was required to be 0.99 or higher. The LLOQ was defined as the minimum concentration on the calibration curve that could be measured with acceptable accuracy (within $\pm 20\%$) and precision (relative standard deviation (RSD) $\leq 20\%$).

2.6.3. Matrix Effect and Extraction Recovery. The matrix effect (ME) was measured by comparing the peak areas of Sal B, ASA, SA, and IS in pretreated blank plasma from six sources with those of the neat standard solutions at the same concentration. The ratio between the ME of the analyte and the corresponding ME of IS was defined as the IS-normalized ME with RSD $\leq 15\%$. The extraction recovery was calculated using the ratio between the peak area of the analyte in the QC samples and that of the analyte in pretreated blank plasma. Both the recovery and ME were

obtained by six replicate measurements at three QC concentrations (LQC, MQC, and HQC).

2.6.4. Precision and Accuracy. The intraday and interday accuracies were evaluated by the determination of the LLOQ, LQC, MQC, and HQC samples with six replicates at each concentration level. The precision was defined as the RSD between the replicate measurements. The accuracy was calculated as the ratio between the observed concentration and the nominal concentration. The accuracy of the QC concentrations should be within $\pm 15\%$ with RSDs of less than 15%, except for the LLOQ, which requires an accuracy within $\pm 20\%$ with an RSD of less than 20% [19].

2.6.5. Stability. The stabilities of Sal B, ASA, and SA were evaluated using three methods, namely, autosampler stability for 24 h, short-term stability for 4 h, and long-term stability at -80°C for 60 days. To determine the autosampler stability, a blank plasma sample spiked with a QC working solution was kept in the autosampler at 4°C for 24 h and then introduced into the UPLC-MS/MS instrument for analysis. The short-term stability was evaluated by storing at room temperature ($\sim 25^{\circ}\text{C}$) for 4 h. The long-term stability was determined by storing the spiked plasma samples at -80°C for 60 days. Three QC concentrations (LQC, MQC, and HQC) were investigated for each stability measurement, with six parallel replicates for each concentration.

2.7. Application to a Pharmacokinetic Trial. In the randomized, open-labeled, parallel-grouped, single-center clinical trial, 16 coronary heart disease patients were randomly allocated into three arms. Participants in the aspirin group were given aspirin (100 mg QD), participants in the salvianolate injection group were given a salvianolate injection (200 mg + 5% glucose injection, 250 mL, iv), and participants in the combination group were given aspirin (100 mg QD) and salvianolate injection (200 mg + 5% glucose injection, 250 mL, iv). For each group, the treatment was administered for 10 consecutive days. Blood samples were collected before dosing on days 8, 9, and 10 and at 5 min (aspirin group only), 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h after dosing on day 10. The plasma was separated by centrifugation at 4500 rpm for 10 min at 4°C and then stored at -80°C until analysis. The validated UPLC-MS/MS method was successfully applied to determine the concentrations of Sal B, ASA, and SA in the plasma samples, which allowed the observation of pharmacokinetic changes due to DDIs between salvianolate injection and ASA [20]. The trial was registered on October 9, 2017 (ClinicalTrials.gov, NCT03306550). The pharmacokinetic analysis was performed using the Phoenix WinNonlin 7.0 software (Pharsight, a Certara Company, Mountain View, CA, USA).

3. Results and Discussion

3.1. Optimization of the UPLC-MS/MS Conditions. It has been reported that Sal B, ASA, and SA all exhibit higher

signal intensities in negative ion mode than in positive ion mode [10–15]. Through Q1 screening, high-intensity and stable peaks were found for the deprotonated molecular ions $[\text{Sal B-H}]^{-}$, $[\text{ASA-H}]^{-}$, $[\text{SA-H}]^{-}$, and $[\text{IS-H}]^{-}$. After the precursor ions were further fragmented with medium-intensity collision energy, the two products with the strongest ion signals were selected for each analyte, and the declustering potential and collision energy for each ion pair were optimized separately. Finally, the mass transitions with strong signals were identified (m/z 717.2 \rightarrow 519.1 for Sal B, m/z 179.1 \rightarrow 137.0 for ASA, m/z 136.8 \rightarrow 65.0 for SA, and m/z 321.0 \rightarrow 152.0 for IS).

ASA generated $[\text{SA-H}]^{-}$ fragment ions during the in-source collision-induced dissociation process, so that the chromatographic peak of SA was detected at the retention time of ASA. To avoid affecting the accurate quantification of SA, the ASA and SA chromatographic peaks were separated using an optimized gradient elution method, as described in Section 2.2. As a result, the resolution factor between the two chromatographic peaks was greater than 1.5. Furthermore, lowering the mobile phase pH significantly improved the tailings of the Sal B and SA chromatographic peaks. In particular, 0.1% FA in water and 0.5% FA in water were tested as solvent A, and the latter provided better peak shapes than the former.

Because blank plasma contains a certain amount of SA (from food sources) [21], reducing the gas source temperature, ion spray voltage, and SA collision energy improved the baseline. As shown in Figure 2, the signal-to-noise ratios for Sal B, ASA, SA, and IS meet the specificity requirements and can be used for sample analysis.

3.2. Sample Preparation. Liquid-liquid extraction and protein precipitation are the most common methods for plasma processing. Owing to the first-pass effect, the plasma concentration of ASA after oral administration is low [22]. Thus, the use of the organic solvent protein precipitation method would dilute the sample, making the signal strength of the LLOQ insufficient to meet the quantitative requirements. Therefore, we chose the liquid-liquid extraction method to extract the analytes. Generally, liquid-liquid extraction is preferred over protein precipitation because cleaner samples are produced, which may minimize the ME and increase the instrument performance.

When redissolving the nitrogen-dried residue, we found that using an inappropriate combination of solvents led to an unqualified standard curve with $r < 0.99$, which may be related to the solubility of the analytes. First, ACN/ H_2O ratios from 80 : 20 (v/v) to 20 : 80 (v/v) were investigated. As the ACN content increased, the ASA and SA curves gradually improved, but most of the concentration points in the Sal B curve remained scattered. When MeOH was added to the solvent mixture instead of ACN, the standard curve of Sal B became linear. Then, a combination of MeOH/ H_2O (80 : 20, v/v) was considered, but the ASA curve became nonlinear. Finally, MeOH/ACN/ H_2O (40 : 40 : 20, v/v/v) was selected as the solvent mixture to redissolve the nitrogen-dried residue.

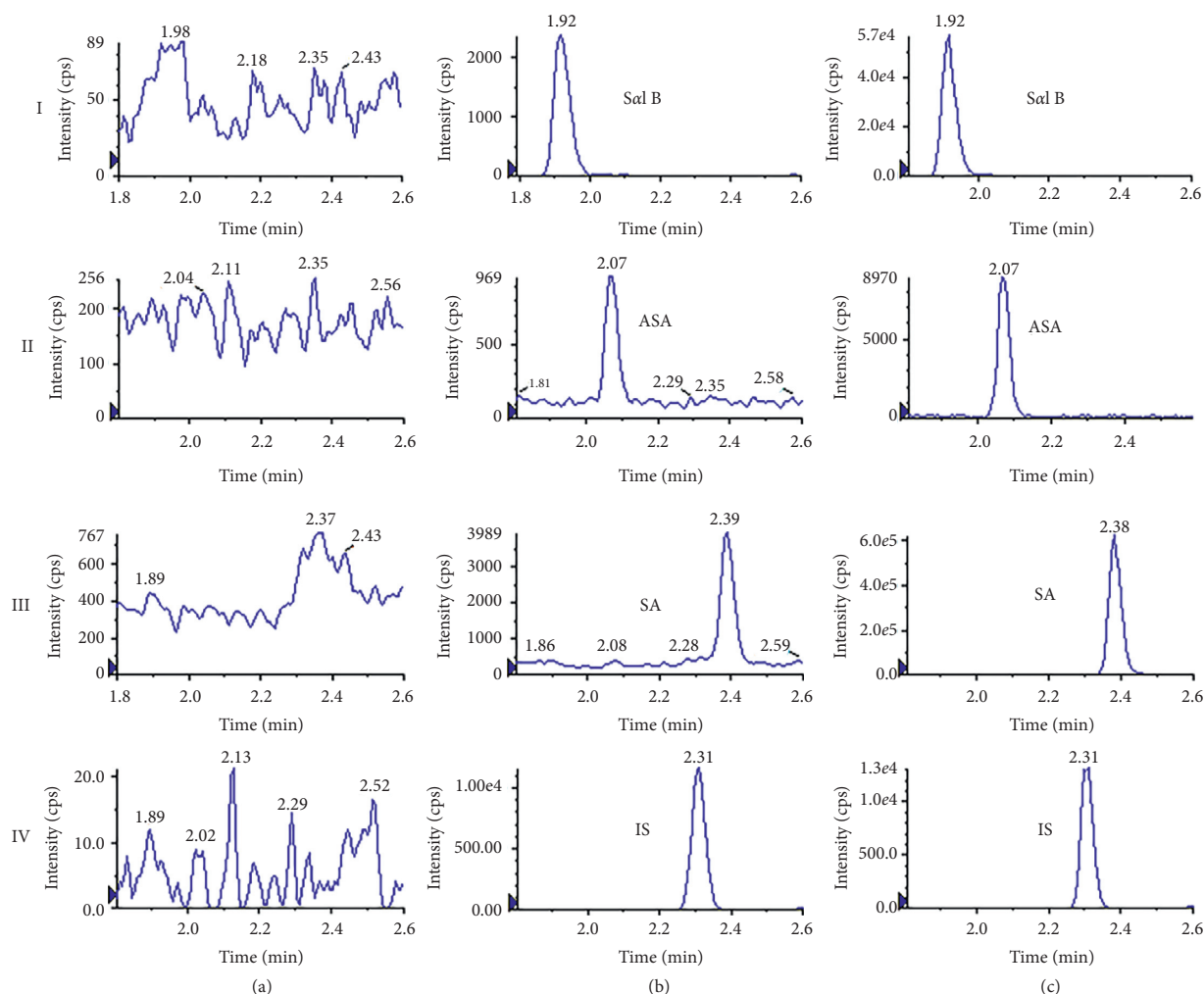


FIGURE 2: Representative MRM chromatograms of Sal B, ASA, SA, and IS in plasma. (a) Blank plasma. (b) LLOQ sample (Sal B, ASA, and SA at 5 ng/mL; IS at 20 ng/mL). (c) Plasma sample 4 h after administration.

3.3. Method Validation

3.3.1. Selectivity. Typical chromatograms for blank plasma, the LLOQ sample, and an actual plasma sample are shown in Figure 2. The retention times were 1.92 min for Sal B, 2.07 min for ASA, 2.39 min for SA, and 2.31 min for IS. These results demonstrated that, under the optimized experimental conditions, no interference peaks were induced by endogenous compounds at the retention times of any of the analytes, and the signal-to-noise ratios of each analyte in the LLOQ samples were greater than 10, which meets the acceptability criteria for quantitative biological analysis.

3.3.2. Calibration Curve Linearity and Sensitivity. Calibration standard curves for Sal B, ASA, and SA in the range 5–6000 ng/mL were established using eight concentrations. The three standard curves for each analyte in Table 2 are the accompanying standard curves for the three batches of precision and accuracy validation. The fitting of the Sal B and ASA data

using a least-squares linear model with a weight of $1/x^2$ gave regression correlation coefficients in the range 0.9986–0.9991 for Sal B and 0.9959–0.9972 for ASA. In contrast, the SA data were fitted with a $1/y$ weighted least-squares quadratic model, which gave regression correlation coefficients in the range 0.9988–0.9999. The LLOQs of Sal B, ASA, and SA met the accuracy requirement within $\pm 20\%$, and the accuracies of the other concentration points were within $\pm 15\%$.

SA has a high protein binding rate [23]. Therefore, when SA is added to blank plasma, some of it binds to plasma proteins, resulting in a low concentration of free SA. Because the amount of albumin in blank plasma is limited, this phenomenon has a much greater effect on the detection of low-concentration SA than on that of high-concentration SA, which is speculated to contribute to the nonlinearity of the SA calibration curve. On the other hand, when the concentration or the ion intensity generated from a target compound exceeds a certain level, mass spectrometers will exhibit a nonlinear response owing to ionization saturation, detector saturation, or both [24]. We found that the SA

TABLE 2: Calibration curves and correlation coefficients of Sal B, ASA, and SA.

Components	Equation	Correlation coefficient (<i>r</i>)
Sal B	$y = 0.0499x + 0.0457$	0.9991
	$y = 0.482x + 0.0697$	0.9988
	$y = 0.0436x + 0.0595$	0.9986
ASA	$y = 0.0211x + 0.00794$	0.9959
	$y = 0.0213x - 0.00818$	0.9972
	$y = 0.0187x + 0.00661$	0.9966
SA	$y = -5.98e - 007x^2 + 0.012x + 0.236$	0.9999
	$y = -6.67e - 007x^2 + 0.013x + 0.277$	0.9999
	$y = -4.99e - 007x^2 + 0.0124x + 0.259$	0.9988

calibration curve was significantly bent at two points near the quantitative upper limit, even if it was constructed using a neat solution with the standard working solutions instead of plasma samples. A similar but less pronounced phenomenon was observed for the ASA curve but not for the Sal B curve. Furthermore, the signal strength of SA was lower than that of ASA or Sal B at the same concentration, which indicated that the nonlinear curve of SA was not attributable to detector saturation and more likely originated from ionization saturation. Thus, at high concentrations, SA was not completely ionized during the ionization process, which could be related to the adjustments made to the mass spectrometry parameters to reduce non-drug-derived SA signals in the plasma samples. However, as shown in Table 2, the regression correlation coefficients of the SA standard curves for the three batches were greater than 0.99; thus, these effects and the nonlinearity of the calibration curve do not affect the efficacy of the developed analytical method.

3.3.3. Extraction Recovery and Matrix Effect. The extraction recovery data indicated that the extraction procedure was efficient for Sal B, ASA, and SA at three QC levels. At concentrations of 15, 3000, and 4500 ng/mL, the average extraction recoveries were $83.3 \pm 4.3\%$, $85.3 \pm 4.3\%$, and $87.2 \pm 4.9\%$ for Sal B; $97.9 \pm 5.2\%$, $96.3 \pm 2.6\%$, and $93.2 \pm 1.8\%$ for ASA; and $93.1 \pm 4.3\%$, $94.6 \pm 1.3\%$, and $95.2 \pm 2.1\%$ for SA. As shown in Table 3, the MEs were in the range 99.7–103% for Sal B, 97.3–102% for ASA, and 96.7–106% for SA, which indicated that there were no significant MEs. After normalization of the ME data with the IS, the ME values of each analyte were reduced, which was related to the enhancement of the IS signal by the matrix. The RSDs of the IS-normalized MEs ranged from 3.75% to 4.55%, which demonstrated that the MEs of the analytes at three concentrations were stable.

3.3.4. Precision and Accuracy. The intraday and interday precisions and accuracies of Sal B, ASA, and SA were determined by analyzing four QC concentrations from three batches with six replicates for each concentration. As shown in Table 4, the intraday and interday accuracies were within $\pm 8.7\%$ and $\pm 8.5\%$ for Sal B, $\pm 4\%$ and $\pm 5.2\%$ for ASA, and $\pm 11\%$ and $\pm 8.9\%$ for SA, respectively. The intraday precisions of Sal B, ASA, and SA were less than 5.31%, 6.19%, and 8.52%, respectively, and the interday precisions were less

than 7.56%, 8.54%, and 9.59%, respectively. The results indicated that the precision and accuracy met the acceptability criteria for biological sample analysis.

3.3.5. Stability. The stability results are summarized in Table 5. QC samples at three concentrations of Sal B, ASA, and SA were stored at room temperature ($\sim 25^\circ\text{C}$) with an acidifying agent (20 μL of FA/ H_2O (50 : 50, v/v) containing IS) for 4 h or in a freezer at -80°C for 60 days before analysis. The accuracies of the QC samples were within $\pm 15\%$, which indicated that the analytes were stable in plasma under these storage conditions. Considering that ASA and SA may be degraded by biological enzymes in plasma, we also investigated the stability of the QC samples at room temperature ($\sim 25^\circ\text{C}$) without the acidifying agent for 4 h. The results showed that approximately 40% of ASA was metabolized by plasma esterase to produce SA, demonstrating that acidified plasma inhibits analyte degradation. Therefore, in clinical operation, whole blood should be processed immediately after collection and stored at -80°C , and acidifying agents should be added to the thawed plasma as soon as possible to prevent analyte degradation. Furthermore, the QC samples were stored in an autosampler at 4°C for 24 h before analysis. The obtained accuracies also met the acceptability criteria, indicating that the analytes were stable in the autosampler before the analysis was performed.

3.4. Clinical Sample Analysis. The developed method was applied to measure the concentrations of Sal B, ASA, and SA in the plasma samples of 16 patients. Table 6 shows the mean values calculated at each blood collection time point. Most concentrations of Sal B and SA were within the quantitative range, whereas those of ASA were below the LLOQ, which was related to the spacing of the blood collection time points. The pharmacokinetic parameters are shown in Table 7. The C_{max} , AUC_{0-t} , and $\text{AUC}_{0-\infty}$ values of the analytes in the combined aspirin and salvianolate injection group (A + S group) were lower than those in the aspirin group (AP group) or the salvianolate injection group (SV group), which indicated lower drug exposure in the A + S group. Furthermore, the time required for the SA plasma concentration to reach C_{max} was different between the A + S group and the SV group, suggesting that DDIs changed the absorption or elimination rate of SA. These observations verified that DDIs between aspirin and salvianolate injection affect the pharmacokinetic behavior.

TABLE 3: Extraction recovery and matrix effects of Sal B, ASA, SA, and IS ($n=6$).

Compound	Nominal conc. (ng/mL)	Extraction recovery (mean \pm SD%)	ME (mean \pm SD %)	IS-normalized ME (mean \pm SD%)	RSD% (IS-normalized ME)
Sal B	15	83.3 \pm 4.3	101 \pm 4	92.1 \pm 4.3	3.95
	3000	85.3 \pm 4.3	103 \pm 4	94.0 \pm 2.8	
	4500	87.2 \pm 4.9	99.7 \pm 4.0	91.3 \pm 3.9	
ASA	15	97.9 \pm 5.2	102 \pm 3	93.4 \pm 3.0	3.75
	3000	96.3 \pm 2.6	97.3 \pm 3.2	93.3 \pm 3.1	
	4500	93.2 \pm 1.8	101 \pm 2	92.3 \pm 1.7	
SA	15	93.1 \pm 4.3	106 \pm 8	97.1 \pm 7.0	4.55
	3000	94.6 \pm 1.3	96.7 \pm 2.6	95.0 \pm 2.5	
	4500	95.2 \pm 2.1	96.7 \pm 2.1	95.0 \pm 2.6	
IS	20	107 \pm 6	109 \pm 3	—	

Extraction recovery = (mean peak area of analyte in the plasma matrix)/(mean peak area of analyte in the extracted plasma matrix). ME = (mean peak area of analyte in the extracted plasma matrix)/(mean peak area of analyte in the dilution solution). IS-normalized ME = ((mean peak area of analyte in the extracted plasma matrix)/(mean peak area of analyte in the dilution solution))/((mean peak area of IS in the extracted plasma matrix)/(mean peak area of IS in the dilution solution)). RSD% = (standard deviation/mean) \times 100%.

TABLE 4: Accuracy and precision of Sal B, ASA, and SA.

Nominal conc. (ng/mL)	Intraday ($n=6$)			Interday ($n=18$)		
	Observed conc. (ng/mL)	Precision RSD (%)	Accuracy (RE %)	Observed conc. (ng/mL)	Precision RSD (%)	Accuracy (RE %)
Sal B						
5	4.89 \pm 0.26	5.31	-2.2	4.94 \pm 0.37	7.56	-1.2
15	14.5 \pm 0.5	3.44	-3.3	15.1 \pm 0.8	5.01	0.6
3000	2802 \pm 106	3.77	-6.6	2746 \pm 108	3.92	-8.5
4500	4108 \pm 99	2.42	-8.7	4333 \pm 237	5.46	-3.7
ASA						
5	4.80 \pm 0.28	5.85	-4	4.96 \pm 0.42	8.54	-0.8
15	14.8 \pm 0.9	6.19	-1.3	14.4 \pm 0.8	5.48	-4
3000	3037 \pm 111	3.67	1.2	3157 \pm 147	4.55	5.2
4500	4332 \pm 140	3.22	-3.7	4184 \pm 196	4.70	-7
SA						
5	5.38 \pm 0.46	8.52	7.6	5.14 \pm 0.49	9.59	2.8
15	15.8 \pm 0.8	5.26	5.3	15.7 \pm 1.0	6.29	4.6
3000	2663 \pm 82	3.09	-11	2734 \pm 134	4.90	-8.9
4500	4257 \pm 98	2.30	-5.4	4291 \pm 199	4.64	-4.6

RSD% = (standard deviation/mean) \times 100%. RE% = (mean of observed conc. - nominal conc.)/nominal conc. \times 100%.

TABLE 5: Stability of Sal B, ASA, and SA.

Nominal conc. (ng/mL)	Storage in autosampler at 4°C for 24 h ($n=3$)		Storage in freezer at -80°C for 60 days ($n=6$)		Storage at 25°C for 4 h after acidification ($n=6$)		Storage at 25°C for 4 h without acidification ($n=6$)	
	Observed conc. (ng/mL)	Accuracy (RE%)	Observed conc. (ng/mL)	Accuracy (RE%)	Observed conc. (ng/mL)	Accuracy (RE%)	Observed conc. (ng/mL)	Accuracy (RE%)
Sal B								
15	16.7 \pm 0.6	11	15.9 \pm 0.8	6	15.2 \pm 1.0	1.3	10.1 \pm 1.1	-33
3000	3047 \pm 85	1.6	2920 \pm 63	-2.7	2767 \pm 85	-7.8	2580 \pm 61	-14
4500	4490 \pm 358	-0.2	4438 \pm 179	-1.4	4370 \pm 290	-2.9	3988 \pm 164	-11
ASA								
15	17.0 \pm 0.3	13	14.1 \pm 0.5	-6	15.9 \pm 0.7	6	8.47 \pm 0.6	-44
3000	3357 \pm 90	12	3117 \pm 27	3.9	3260 \pm 96	8.7	1762 \pm 87	-41
4500	4747 \pm 274	5.5	4242 \pm 110	-5.7	4470 \pm 206	-0.7	2767 \pm 179	-39
SA								
15	15.9 \pm 1.3	6	14.8 \pm 1.2	-1.3	16.1 \pm 0.9	7.3	18.3 \pm 0.5	22
3000	2797 \pm 59	-6.8	3138 \pm 86	4.6	2892 \pm 37	-3.6	3767 \pm 78	26
4500	4233 \pm 188	-5.9	4635 \pm 156	3	4293 \pm 91	-4.6	5738 \pm 216	28

RE% = (mean of observed conc. - nominal conc.)/nominal conc. \times 100%.

TABLE 6: Measured concentrations of Sal B, ASA, and SA in patients.

Collection time		Sal B conc. (ng/mL)		ASA conc. (ng/mL)		SA conc. (ng/mL)	
		SV group	A + S group	AP group	A + S group	AP group	A + S group
8th day	Before	<LLOQ	8.27	<LLOQ	<LLOQ	99.8	22.8
9th day	Before	<LLOQ	7.73	<LLOQ	<LLOQ	76.6	24.9
10th day	Before	<LLOQ	12.3	<LLOQ	<LLOQ	69.7	18.5
	5 min	NA	NA	<LLOQ	NA	63.1	NA
	15 min	2078	1059	<LLOQ	<LLOQ	50.3	18.0
	30 min	1274	653	<LLOQ	<LLOQ	51.8	17.3
	45 min	720	436	<LLOQ	<LLOQ	48.6	15.5
	1 h	471	246	<LLOQ	<LLOQ	46.2	18.5
	2 h	211	128	<LLOQ	12.4	27.2	375
	4 h	64.5	43.3	270	239	1725	2157
	8 h	34.0	24.9	22.9	79.2	2229	1740
	12 h	15.9	16.3	104	6.05	1191	640
	24 h	14.9	9.96	<LLOQ	<LLOQ	84.7	57.2

NA represents that the administration group was not collected sample at 5 min after administration.

TABLE 7: Mean of pharmacokinetic parameters of Sal B in the SV group and A + S group and SA in AP group and A + S group (mean \pm SD%).

Parameter	Sal B		SA	
	SV group ($n = 5$)	A + S group ($n = 5$)	AP group ($n = 6$)	A + S group ($n = 5$)
T_{\max} (h)	1.6 \pm 0.1	1.6 \pm 0.1	7.3 \pm 3.0	5.6 \pm 2.2
C_{\max} ($\mu\text{g/mL}$)	2.3 \pm 1.8	1.1 \pm 0.5	3.3 \pm 1.1	2.8 \pm 1.0
$T_{1/2}$ (h)	4.4 \pm 1.5	7.3 \pm 5.3	2.6 \pm 0.8	2.8 \pm 0.8
AUC_{0-t} ($h \times \mu\text{g/mL}$)	3.5 \pm 1.9	1.9 \pm 0.8	24 \pm 7.5	19 \pm 8.9
$\text{AUC}_{0-\infty}$ ($h \times \mu\text{g/mL}$)	3.6 \pm 2.0	2.1 \pm 0.9	24 \pm 7.3	20 \pm 9.0

T_{\max} : time point of maximum plasma concentration; C_{\max} : maximum plasma concentration; $T_{1/2}$: half-life of drug elimination during the terminal phase; AUC_{0-t} : area under the plasma concentration-time curve from zero hours to last time point; and $\text{AUC}_{0-\infty}$: area under the plasma concentration-time curve from zero hours to infinity.

4. Conclusions

A fast, simple, and stable UPLC-MS-/MS method for the simultaneous quantification of Sal B, ASA, and SA in human plasma was developed and validated. The analytes were extracted from acidified plasma by liquid-liquid extraction, separated by gradient elution on a C18 column, and quantified using MRM with negative ion electrospray ionization. The method was successfully applied to the determination of Sal B, ASA, and SA concentrations in human plasma samples from 16 patients to observe the pharmacokinetic changes caused by DDIs between salvianolate injection and ASA. This method is expected to be applicable to follow-up studies in this area as well as other clinical studies of aspirin combined with Chinese medicine preparations containing a Sal B component. Furthermore, this method provides a reference for the development of in vivo quantitative analysis methods for similar compounds to further assist with pharmacokinetic research on the combined application of Chinese and Western medicines.

Data Availability

The research data used to support the findings of this study are included within the Supplementary Materials file.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Supplementary material associated with this report contains the data for method validation, which can be found online. (*Supplementary Materials*)

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