DEVELOPMENT OF AN HPLC-UV METHOD FOR QUANTITATIVE DETERMINATION OF ACETYLSALICYLIC ACID AND ITS MAIN METABOLITE

V. Yu. Belov,[1] S. V. Kursakov,[1] V. I. Sevast'yanov,[1, 2] E. N. Antonov,[3] S. É. Bogorodskii,[3] and V. K. Popov[3, *]

An HPLC-UV method for determination of acetylsalicylic acid and its main metabolite, salicylic acid, in a model solution and in rabbit blood plasma was developed. Plasma samples were prepared by salting out. Chromatographic analysis was performed in isocratic mode over a Hypersil BDS C18 column using mobile phase MeCN—H$_2$O (pH 2.5, 30:70) with detection at 230 nm. The limit of quantitation for acetylsalicylic and salicylic acids in the model solution was 0.05 µg/mL; in blood plasma, 0.2 µg/mL. The developed method was applied to the development of new acetylsalicylic-acid dosage forms based on biocompatible polymer carriers, including pharmacokinetic studies after i.m. implantation.

Keywords: acetylsalicylic acid, salicylic acid, HPLC, blood plasma, salting out, biocompatible polymer carrier, pharmacokinetics.

Acetylsalicylic acid (ASA) is a drug with anti-inflammatory, antipyretic, and analgesic activity [1]. The ability of ASA to exhibit antiaggregation activity and to inhibit spontaneous and induced platelet aggregation [2] is responsible for its wide use to prevent clot formation in patients with cardiovascular diseases, acute cardiac failure, and ischemic infarct [3, 4]. However, oral administration of ASA causes local irritation of mucous membranes in the gastrointestinal tract. Prolonged use can lead to stomach and duodenum ulceration [5].

Therefore, much attention has recently been paid to improving already existing ASA preparations and to create new prolonged-action dosage forms [6]. Transdermal therapeutic systems [7] and injectable forms of the drug substance (DS) encapsulated in biocompatible polymer carriers [8] are examples of such drugs.

Supercritical CO$_2$ (sc-CO$_2$) is an environmental friendly solvent for drugs that is used in technologies that readily plasticize amorphous and partially crystalline polymers and are among the most promising methods for DS encapsulation [8, 9]. This can avoid the use of toxic organic solvents (Me$_2$CO, CHCl$_3$, etc.) and high (100°C) temperatures that are required to form polymer microparticles of various sizes [10]. Moreover, sc-CO$_2$ can rather effectively remove soluble toxic impurities (unreacted monomers and low-molecular-mass oligomers, polymerization initiators, plasticizers, etc.), which helps to increase the biological safety of the manufactured dosage forms [11].

Development and trials of new drugs and their dosage forms presupposes reliable monitoring of the concentrations of the drug and its metabolites in model media and biological specimens.

A unified method for quantitative determination of ASA and its main metabolite, salicylic acid (SA), in various specimens does not currently exist. The main problems with sample preparation and determination of ASA and its metabolites are the instability of ASA, its low molecular mass, and the high polarity of the determined compounds. Nonspecific cholinesterase inhibitors such as CaF$_2$ [12] was added and

the sample pH and temperature were lowered [13, 14] to prevent ASA hydrolysis in the samples.

Samples containing ASA are prepared using protein precipitation [13, 14], liquid- [15, 16] and solid-phase extraction [17], and combinations of these methods. Quantitative determination methods for ASA and SA consist in most instances of HPLC with UV detection [14, 15]. However, they are typically insufficiently selective and have low levels of extraction of the determined compounds. Existing methods for HPLC-MS determination of ASA and SA [12, 18, 19] have not been widely used because of the complicated and costly equipment, special requirements for analyte purity, etc. The choice of one analytical method or another is often a compromise between fast and simple sample preparation vs. the required determination selectivity and sensitivity.

The present work focused on development of a quantitative determination procedure for these acids in a model solution and blood plasma using HPLC. The selectivity of the method for studying the release kinetics of ASA and SA *in vitro* and *in vivo* from biocompatible polyester carriers was also checked.

EXPERIMENTAL PART

Drug substances of ASA and SA (Shandong Xinhua Pharmaceutical Co., Ltd., China) were used in the work.

MeCN (for HPLC), H_3PO_4 (chemically pure), HCl (chem. pure), Na_2SO_4 (chem. pure), NaCl (chem. pure), and H_2O (for chromatography) were used to prepare and analyze samples.

Stock ASA and SA standard solutions were prepared by dissolving weighed substances in H_2O (pH 2.5, regulated by adding conc. H_3PO_4). Working standard solutions were pre-
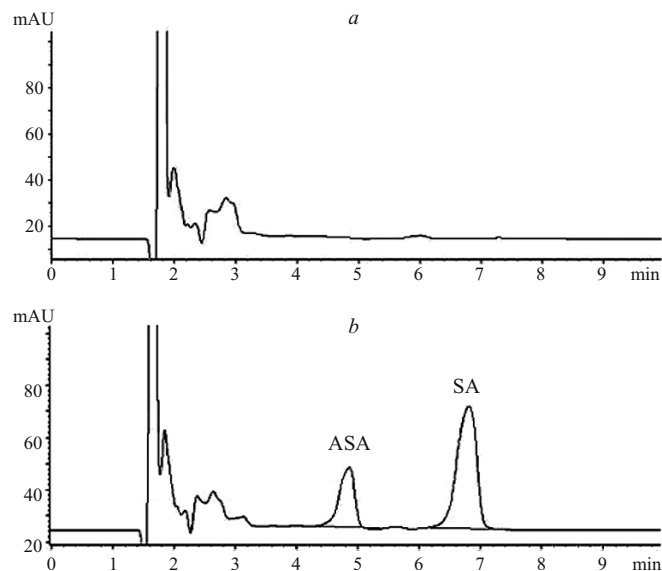
Fig. 1. Chromatograms of pure plasma (*a*) and plasma with added standard solutions of ASA and SA (20 μg/mL) (*b*).

pared by diluting stock standard solutions with H_2O (pH 2.5).

Model solution was prepared by dissolving NaCl in H_2O to a concentration of 0.9 mass%. Model and standard solutions were stored in a Pozis KhF-400 pharmaceutical refrigerator (Russia) at 2 – 8°C.

Rabbit blood plasma samples were stored in a Sanyo MDF-U5412 biomedical freezer (Japan) at –34 ÷ -38°C and were thawed to room temperature before sample preparation.

ASA and SA release kinetics from biocompatible polymer carriers into model solution and blood plasma were studied using finely disperse powders (20 – 50 μm particle size)

TABLE 1. Studied Sample-Preparation Methods for Blood-Plasma Containing ASA and SA

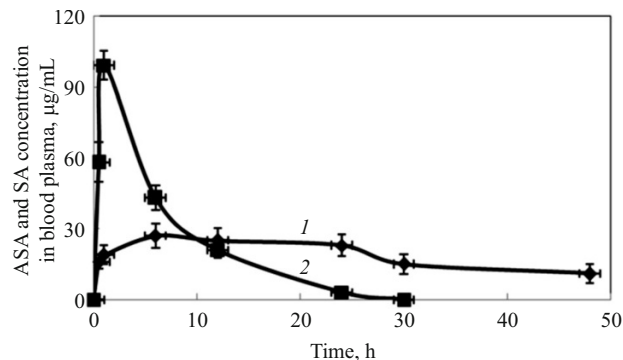| Sample preparation method	Description	Advantages and disadvantages
Protein precipitation	Protein precipitation by MeCN; organic acids; and Na, Mg, and Pb salts. Variation of precipitant volume and nature.	Simple and rapid preparation. Contamination of analyte by endogenous substances; insufficient selectivity for ASA and SA extraction.
Freezing	Sample treatment with MeCN followed by freezing of the aqueous layer.	Simple preparation. Contamination of analyte by endogenous substances; low selectivity and degree of extraction for ASA and SA.
Salting out	Sample treatment with MeCN and saturated inorganic-salt solution. Variation of salting-out agent amount and nature.	Simple and rapid preparation.
Liquid—liquid extraction	Extraction of determined compounds by organic solvents followed by concentration of the analyte. Variation of the extract volume, nature, and polarity.	Ability to concentrate analyte. Large time and reagent losses; insufficient selectivity.
Solid-phase extraction	Isolation of determined compounds from blood plasma in reversed-phase, normal-phase, and ion-exchange modes using Chromabond C_{18} Hydra, HILIC, CN, SiOH, and XTR cartridges (Macherey-Nagel, Germany). Variation of solution composition and volume during conditioning, rinsing, and elution.	Purity of obtained analyte. High consumable costs; insufficient ASA and SA extraction.
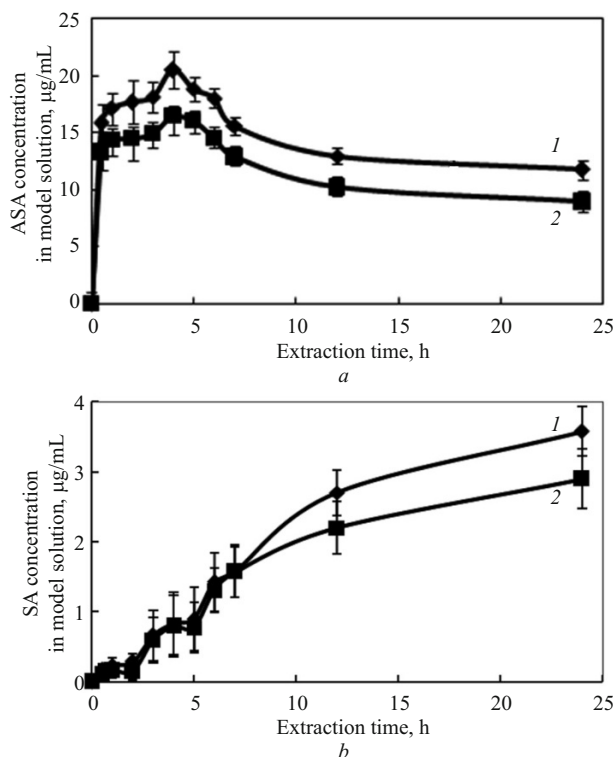
Fig. 2. Kinetics of ASA release (a) and SA formation (b) from amorphous D, L-polylactide PURASORB PDL02 (1) and amorphous polylactoglycolide PURASORB PDLG02 (2) containing ASA (10 mass%) ($n = 3$).

Fig. 3. Average pharmacokinetic curves for ASA (1) and SA (2) after a single i.m. implantation in rabbits of amorphous polylactoglycolide PURASORB PDLG02 at a dose of 10 mg/kg ($n = 3$).

of D,L-polylactide PURASORB PDL02 and polylactoglycolide PURASORB PDLG02 {intrinsic viscosity 0.2 dL/g (in the CGS system), PURAC biochem BV, Netherlands, containing ASA (10 mass%) and prepared by the particles from gas-saturated solutions (PGSS) method [9]}. The PGSS

method is based on plasticizing (reducing the viscosity) polymers by reacting them with sc-CO_2 [10]. CO_2 (ultrahigh purity, GOST 8050-85) was produced by Balashikha Oxygen Plant (Balashikha, Moscow Oblast) and was used without further purification.

Quantitative determination used an Agilent 1200 liquid chromatograph with a UV-detector, autosampler, and column thermostat. Data processing was performed by Chem Station software (USA). A Multi-Vortex V-32 stirrer (Latvia), MPW-250 laboratory centrifuge (Poland), LAB-PU-01 shaker (Russia), Vibra-AF-R220CE Laboratory analytical balance (Japan), and variable volume pipettors (0.5 – 10, 5 – 50, 20 – 200, and 100 – 1,000 μL, Russia) were used to prepare samples.

Rabbits (Soviet Chinchilla, ~2,500 g) were used as experimental animals and were obtained from the nursery at OPKh Manikhino. The number of animals was sufficient for forming representative experimental groups and statistical processing of the results. The ability to replace and exclude animals from the experiment due to force major circumstances was also provided.

All manipulations with animals were performed according to rules adopted by the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (ETS 123, Strasbourg, 1986).

Sample preparation. Blood plasma (500 μL) was placed into a 2-mL microcentrifuge tube, treated with MeCN (500 μL) and anhydrous Na_2SO_4 (500 mg), stirred on the Multi-Vortex for 2 min and centrifuged at 13,000 rpm for 10 min. The supernatant liquid (400 μL) was transferred to an HPLC tube. Model solution was filtered through a nylon filter (0.45 μm) before chromatography.

Chromatography. The chromatographic separation was performed in isocratic mode over a Hypersil BDS C18 column (150 × 3.0 mm, 5 μm, USA) with a precolumn (8 × 4 mm) packed with the same sorbent. The mobile phase was MeCN—H_2O (pH 2.5, regulated by adding conc. H_3PO_4) (30:70). The mobile phase was filtered and degassed

TABLE 2. Main Chromatographic Analytical Parameters for Plasma with Added Standard Solutions of ASA and SA ($C = 20$ μg/mL, $n = 6$, $p = 0.95$)

Parameter	Found	
	ASA	SA
Retention time, min	4.83	6.78
Capacity coefficient	2.0	3.2
Peak width at half-height, min	0.29	0.36
Number of theoretical plates calculated at peak half-height	1539	1966
Height of an equivalent theoretical plate (HETP), μm	97.5	76.3
Peak asymmetry coefficient	1.7	1.6
Separation (selectivity) coefficient	1.6	
Resolution	2.3	

under vacuum before use. The mobile-phase flow rate was 1 mL/min; column thermostat, 25°C. Detection was made at 230 nm. The injected sample volume was 20 µL. The retention time of ASA was ~4.8 min; of SA, ~6.8 min; chromatography time, 12 min.

Statistical processing of the experimental results used the Microsoft Excel program.

RESULTS AND DISCUSSION

Various sample-preparation techniques were studied to determine the optimum method for blood plasma containing ASA and SA. Table 1 presents the results.

Salting-out, which gave the best selectivity, simplicity, speed, and cost for preparing plasma samples, was selected based on the combined results. The degrees of extraction of ASA and SA from blood plasma were 95.8 and 98.1%, respectively.

Reversed-phase, ion-pair, and normal-phase chromatography were used for samples containing ASA and SA. The efficiencies of the determinations in the various chromatography modes were compared using columns with the same shapes and sorbent-particle sizes.

Reversed-phase chromatography over a Hypersil BDS C_{18} column was the most suitable chromatography mode for ASA and SA according to the experiments. The conditions are given below. Figure 1 shows chromatograms of pure plasma and plasma with added standard solutions of ASA and SA.

Table 2 presents the main chromatographic analytical parameters over the Hypersil BDS C_{18} column that characterized the efficiency and selectivity of the separation under the selected conditions and were calculated from chromatograms of plasma with added standard solutions of ASA and SA.

Selectivity is the main factor influencing the choice of chromatographic separation conditions. It is a summed characteristic of the physicochemical interactions in the system and is a key factor affecting the resolution [20]. The separation coefficient of the proposed chromatographic system was adequate for complete separation of the peaks for ASA and SA. The resolution and obtained chromatograms confirmed that the ASA and SA peaks were baseline separated.

The developed method was used to study new forms of ASA based on biocompatible polymer carriers. ASA and SA were determined quantitatively in model solution and blood plasma using calibration curves for the dependence of the concentration on the peak area of the determined compounds.

Calibration curves for ASA and SA were constructed in the concentration range 0.05 – 5 µg/mL for model solution and 0.2 – 10 µg/mL for blood plasma. The dependences obtained in these ranges were linear.

The limit of quantitation (LOQ) of ASA and SA was determined based on the linearity of the calibration curve as the minimum concentration at which the relative standard deviation was ≤ 20% [21, 22]. The LOQ of ASA and SA in model solution was 0.05 µg/mL; in blood plasma, 0.2 µg/mL.

The relative error (RE) and relative standard deviation (RSD) of the ASA and SA determination that characterized the accuracy and precision, respectively, of the method were determined on two levels, i.e., intra-day (during one work day) and inter-day (on several work days) [21, 22]. For this, four plasma samples, each of which was diluted beforehand with standard solutions of ASA and SA to concentrations of 0.2, 0.6, 5, and 7.5 µg/mL, were analyzed.

The resulting RSDs and REs met the standards [21, 22] of ≤ 20% for the minimum concentration and ≤ 15% for the others.

ASA release kinetics from biocompatible polymer carriers into model solution

The release kinetics of ASA from biocompatible polymer particles was studied in model solution in order to select the optimum carrier for the active ingredient.

Interference from ASA adsorbed on the carrier particle surface was eliminated by placing polymer (100 mg) containing encapsulated ASA (10 mass%) into model solution (100 mL), which was shaken for 5 min, left to stand for 10 min, shaken again for 5 min, and filtered through a nylon filter (0.45 µm).

The rinsed particles together with the filter were placed into a flask with model solution (400 mL) thermostatted at 37°C. Samples (0.5 mL) were taken after 0.5, 1, 2, 3, 4, 5, 6, 7, 12, and 24 h and were filtered through a nylon filter (0.45 µm) and chromatographed.

Figure 2 shows the ASA release kinetics from D,L-polylactide PURASORB PDL02 and polylactoglycolide PURASORB PDLG02.

ASA was released from polylactide and polylactoglycolide particles via the same mechanism. ASA was actively released from the polymer microparticles for 4 h after the start of the extraction. The greatest ASA concentration increase occurred in the first 30 min. The ASA concentration in model solution started to decrease 4 h after the start of the extraction. This was explained by hydrolysis of ASA to form SA (Fig. 2b).

The mass of ASA released from D,L-polylactide PURASORB PDL02 during the first 4 h was ~25% greater than that released from polylactoglycolide PURASORB PDLG02. This could provide a basis for selecting polylactoglycolide PDLG02 as the carrier for the prolonged-release ASA dosage form. Therefore, pharmacokinetic studies were performed only for ASA encapsulated in polylactoglycolide microparticles.

Pharmacokinetics of ASA encapsulated in biocompatible polymer carrier

The ASA release kinetics from polylactoglycolide PURASORB PDLG02 into blood were analyzed by HPLC according to the following algorithm.

An experimental group of three animals was formed randomly. The group included rabbits with similar appearances so that the individual body mass of each animal did not differ from the mean by >10%.

Amorphous polylactoglycolide PURASORB PDLG02 containing ASA (10 mass%) was implanted into the rabbit femoral muscle at a dose of 10 mg ASA/kg. Blood samples were collected before implantation and 0.5, 1, 6, 12, 24, 30, and 48 h after it. The volume of the collected blood samples was sufficient to obtain 0.5 mL of plasma. The ASA and SA contents in the obtained plasma samples were determined.

Figure 3 shows results from the ASA pharmacokinetic studies.

The ASA blood-plasma concentration increased during the first 6 h, remained at the same level until 24 h, and decreased smoothly to insignificant values. The SA plasma content reached a maximum at 1 h after injection i.m. of ASA encapsulated in a biocompatible polymer carrier.

The obtained results indicated that ASA was released gradually over 24 h after i.m. implantation of ASA encapsulated in a biocompatible polymer carrier.

Thus, a method for quantitative determination of ASA and SA in model solution and blood plasma using HPLC with UV-detection was developed. The analytical range of the method without considering dilution was $0.05 - 5$ µg/mL for model solution and $0.2 - 10$ µg/mL for blood plasma. The degrees of extraction of ASA and SA from blood plasma were 95.8 and 98.1%, respectively. The developed method was demonstrated to be suitable for developing new ASA dosage forms.

ACKNOWLEDGMENTS

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