Journal of Chromatography, 584 (1992) 221–228
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6619

Simultaneous high-performance liquid chromatographic determination of salicylates in whole blood, plasma and isolated erythrocytes

J. Klimeš, J. Sochor and M. Zahradníček

Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy, Charles University, Heyrovského 1203, 501 65 Hradec Králové (Czechoslovakia)

J. Sedláček

Department of Pathological Physiology, Faculty of Medicine, Charles University, Šimkova 870, 500 00 Hradec Králové (Czechoslovakia)

(First received July 13th, 1992; revised manuscript received October 12th, 1992)

ABSTRACT

A method using liquid–liquid extraction has been developed for the isolation of acetylsalicylic acid and its metabolites, salicylic, gentisic or possibly salicyluric acids, from whole blood, isolated erythrocytes and plasma. Methylene chloride proved to be the best of the organic solvents tested. For whole blood and isolated erythrocytes it was necessary to carry out haemolysis prior to their extraction. The high-performance liquid chromatographic conditions for the quantitation of acetylsalicylic acid and its metabolites from samples of whole blood, erythrocytes and whole plasma were optimized. Separation was performed using reversed-phase chromatography on Separon SGX C_{18} and ultraviolet detection at 236 nm. A mixture of methanol–water (80:100, v/v) was the mobile phase, acidified with perchloric acid to pH 2.5.

INTRODUCTION

Salicylates, analgesic–antipyretics with an antiphlogistic and an antirheumatic effect, which have been employed in therapy for a number of years, are still among the most widely used groups of drugs. In the investigation of their pharmacokinetics, an irreplaceable role is played by high-performance liquid chromatography (HPLC), which enables sensitive, selective and simple analysis of salicylates in various biological fluids. The major metabolites of acetylsalicylic acid (ASA) are salicylic acid (SA), gentisic acid (GA) and salicyluric acid (SU). Reversed-phase chromatography for the determination of salicylates in urine has been described [1–8]. The salicylates have most frequently been analysed in plasma [1,4–16] or serum [4,17,18] with an ultraviolet detector. We previously [19] elaborated a HPLC method to quantify SA and GA in plasma with detection in the visible region, after prior conversion to a complex by reaction with Fe^{3+} ions which had been added to the mobile phase. Salicylates from plasma and serum are isolated by deproteination [4–6,17], liquid–liquid extraction [7,9,11–15,18,19] or solid–liquid extraction [16].

Correspondence to: Dr. J. Klimeš, Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy, Charles University, Heyrovského 1203, 501 65 Hradec Králové, Czechoslovakia.

0378-4347/92/$05.00

The problems associated with the analysis of salicylates in whole blood, and particularly those for isolated erythrocytes, have been elaborated to a far lesser extent.

In the whole blood samples, ASA and SA were simultaneously determined after extraction into a mixture of ethyl acetate–butyl chloride from whole rat blood [20]. Stability of ASA and SA was examined in whole blood and plasma [10]. In mouse and rat blood [21] only SA and its metabolites after deproteination of samples were determined.

The same procedure of chromatography and isolation was employed in the analysis of SA in erythrocyte samples [22]. ASA was analyzed in erythrocytes in an *in vitro* study [23] by means of HPLC after prior extraction into a benzene–ethyl acetate mixture.

Pharmacokinetic studies of both acids, ASA and SA, or their metabolites, were performed by the HPLC method primarily in plasma [4–9,11,12,14,15,24] after administration of ASA. In whole blood only SA was investigated alongside its metabolites in rats and mice [21] after administration of sodium salicylate.

The aim of the present study was to develop an extraction procedure to isolate ASA and its metabolites from three different biological matrices — whole blood, isolated erythrocytes and plasma. Optimizations of the analytical procedures were intended to assay salicylates in the above-mentioned biological matrices using unified HPLC chromatographic conditions.

EXPERIMENTAL

Apparatus

A liquid chromatograph consisting of a Varian (Palo Alto, CA, USA) Model 8500 high-pressure pump was used. A 10-μl loop (Laboratory Instruments, Prague, Czechoslovakia) was employed to spread the sample on to a Separon SGX C_{18} glass column, 5 μm, 150 mm × 3.2 mm I.D. (Tessek, Prague, Czecholovakia). Detection was at 236 nm by a Varichrom (Varian) UV–VIS detector. An SP 4100 integrator (Spectra Physics, Santa Clara, CA, USA) calculated the areas of the peaks.

The mobile phase used was a mixture of methanol–water (85:100, v/v); its pH of 2.5 was adjusted with 5% perchloric acid. The flow-rate was set at 0.5 ml/min.

Reagents and chemicals

ASA, SA and sodium gentisate were supplied by Léčiva (Prague, Czechoslovakia), and SU was from Sigma (St. Louis, MO, USA). Methanol, methylene chloride, hydrochloric acid (35%), potassium fluoride and benzanilide (internal standard) were obtained from Lachema (Brno, Czechoslovakia), perchloric acid (70%) was from Merck (Darmstadt, Germany), and Aspegic inj. was from Laboratoire Egic (Amilly, France).

ASA, SA and sodium gentisate were PhBs 4 grade, methanol was HPLC grade and all other chemicals were analytical-reagent grade. Water was doubly distilled.

Standard preparation

Working standards for ASA and SA were prepared by dilutions of 25 mg/ml (in methanol) of the stock standard to 20, 15, 10, 5 and 1 mg/ml. The solutions of SU (1 mg/ml) and the internal standard (1 mg/ml) stock solution were prepared in methanol. The solution of GA (2 mg/ml) was prepared by dissolving sodium gentisate in distilled water, acidified with hydrochloric acid to pH 2.5.

Biological sample

Erythrocytes, plasma and whole blood were obtained from the Department of Pathological Physiology, Faculty of Medicine, Charles University (Hradec Králové, Czechoslovakia). Here also three rabbits were treated intravenously with the Aspegic inj. preparation in a 100 mg/kg dose, and the blood samples were withdrawn in the pharmacokinetic study. Samples were withdrawn 3, 6, 15, 30, 60 and 120 min after drug administration.

The withdrawn heparinized rabbit blood was placed in 10-ml test tubes and to it was added a solution of 50% (w/v) potassium fluoride (50 μl per 5 ml of blood) [25]. A portion of each sample of the withdrawn blood was left for analysis (as

the sample of whole blood), and for the other portion of the blood, the erythrocytes were isolated from the plasma by centrifugation at 1500 *g* for 10 min. The samples of whole blood, erythrocytes and plasma were immediately frozen.

Extraction procedure

Whole blood. A 0.5-ml volume of whole blood was pipetted into a 10-ml glass-stoppered centrifuge test tube, 10 μl of the internal standard solution were added and the sample was haemolyzed by adding 0.9 ml of water. The sample was shaken for 5 min, placed in an ultrasonic bath for 5 min, an left at room temperature for 5 min. Then the sample was acidified with 0.3 ml of hydrochloric acid (3 mol/l) and, after 5 min shaking, 6 ml of methylene chloride were added. After shaking and centrifugation (5 min at 1930 *g*) the methylene chloride (5 ml) was separated and evaporated to dryness under a gentle stream of nitrogen. The dry residue was reconstituted with 50 μl of the mobile phase, and 10 μl were injected into the HPLC column.

Erythrocytes. A 0.5-ml vlume of isolated erythrocytes was pipetted into a 10-ml glass-stoppered centrifuge test tube and 10 μl of a sample of the internal standard were added. After haemolysis, the sample was acidified with 0.4 ml of hydrochloric acid (3 mol/l) and, after 5 min shaking, 6 ml of methylene chloride were added. The sample was shaken again and centrifuged for 7 min. A 5-ml volume of the methylene chloride layer was separated off and the procedure continued in the same manner as described for the whole blood sample.

Plasma. A 0.5-ml volume of plasma sample was pipetted into a 10-ml glass-stoppered centrifuge test tube, 10 μl of a solution of the internal standard were added and the sample was acidified gradually with 0.9 ml of hydrochloric acid (0.1 mol/l) and, after shaking, with another 0.2 ml of hydrochloric acid (3 mol/l). After 5 min shaking, 6 ml of methylene chloride were added. Then the procedure continued in the same manner as described for the whole blood sample.

Preparation of the standard curve

Calibration standards were prepared by adding 10 μl of the appropriate working standard and 10 μl of the internal standard to 0.5 ml of whole blood, 0.5 ml of plasma or 0.5 ml of erythrocytes. Five calibration concentrations of ASA (20, 100, 200, 300 and 400 μg/ml), five calibration concentrations of SA (100, 200, 300, 400 and 500 μg/ml) and five calibration concentrations of GA (2, 6, 16, 30 and 40 μg/ml) were used for the standard curves in each matrix. Sample extraction was carried out as described above.

Concentrations of ASA, SA and GA were calculated from the linear regression equation of the calibration curve constructed by plotting the peak areas of ASA, SA and GA against the peak area of the internal standard.

RESULTS AND DISCUSSION

An isolation technique was developed and the HPLC conditions were optimized for the simultaneous determination of ASA, SA and GA in the samples of whole rabbit blood, isolated erythrocytes and plasma. Immediately after blood withdrawal, a solution of potassium fluoride must be added to the samples to prevent the action of cholinesterases which hydrolyze ASA to SA *in vitro* [5,14,25]. This measure prevents the undesirable hydrolytic process of ASA prior to the separation of erythrocytes from plasma.

Under the chromatographic conditions described in Experimental, GA, ASA, SA and the internal standard were completely separated with retention times of 3.0, 5.3, 8.4 and 14.2 min, respectively. No interfering peaks of the retention times of GA, ASA, SA and the internal standard were seen in blank samples of any matrix, only the peak for SU (retention time, 4.0 min) interfered with the peaks of the residues from whole blood, erythrocytes and plasma. SU determination was not pursued further. Figs. 1 and 2 show the representative chromatograms of blank (A), control (B) and dosed (C) rabbit whole blood and erythrocyte samples, respectively.

In order to increase the efficacy of the extraction procedure, it was necessary to carry out the

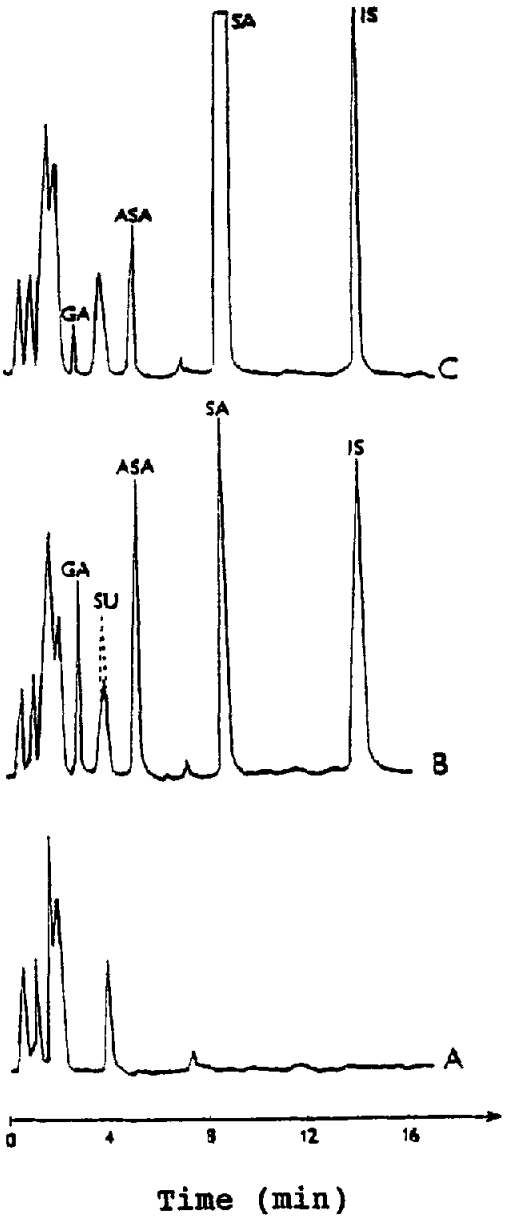

Fig. 1. Typical chromatograms for ASA, SA, GA, SU and the internal standard (IS) in rabbit whole blood. (A) Blank sample; (B) control sample spiked with the standard solution of ASA (6 μg/ml) and its metabolites (and IS); (C) 30-min sample from a rabbit given a single dose of 100 mg/kg ASA.

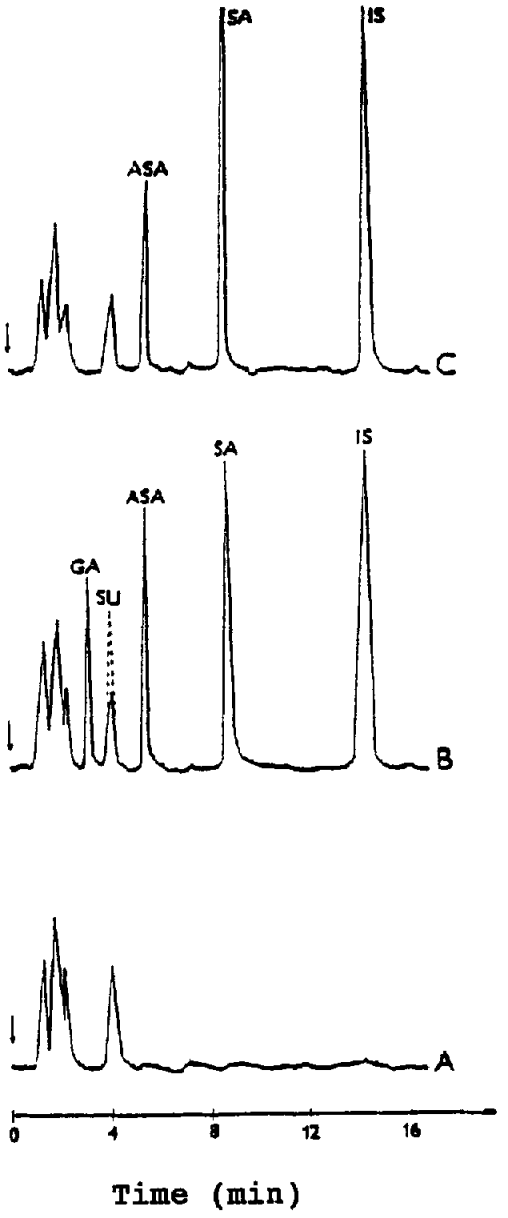

Fig. 2. Typical chromatograms for ASA, SA, GA, SU and the internal standard (IS) in rabbit erythrocytes. (A) Blank sample; (B) control sample spiked with the standard solution of ASA (10 μg/ml) and its metabolites (and IS); (C) 30-min sample from a rabbit given a single dose of 100 mg/kg ASA.

haemolysis of erythrocytes in the samples of whole blood and erythrocytes, and afterwards to perform the extraction of the drugs to be determined into methylene chloride, which was employed primarily because little of the residues from the erythrocyte and from whole blood are exracted into it (less than, *e.g.*, into diethyl ether). In order to prevent undesirable sublimation of

TABLE I

RECOVERY OF ASA, SA AND GA IN RABBIT WHOLE BLOOD, ERYTHROCYTES AND PLASMA

Biological material	Recovery (mean ± S.D., n = 6) (%)		
	ASA	SA	GA
Whole blood	98.9 ± 1.8	70.9 ± 1.5	50.3 ± 3.7
Erythrocytes	90.7 ± 4.2	61.7 ± 3.8	–
Plasma	100.7 ± 1.6	80.1 ± 1.4	67.2 ± 3.3

the drugs [8,14,20], after separation the methylene chloride layer was evaporated to dryness by a stream of nitrogen. The values of the extractive efficiency are listed in Table I.

Quantitation was based on least-squares linear regression analysis of concentration (x) *versus* peak-area ratio (y). Calibration curves were linear in the range of concentrations necessary for the determination of the drugs in their pharmacokinetic study. Their regression equations and correlation coefficients are given in Table II. The regression equation for GA is not shown as no GA was detected in erythrocytes during a 2-h pharmacokinetic study.

Both within-day and day-to-day precision and accuracy of standard curves were examined. Within-day precision was calculated from the analysis of six samples in each matrix of four concentrations of each compound. Day-to-day reproducibility was investigated during a six-week period. Measured concentrations and coefficients of variation (C.V.) are presented in Tables III–V; the C.V. values were all less than 5%.

The detection limit for both ASA and SA was 20 ng/ml in whole blood and plasma (or 60 ng/ml in erythrocytes). The detection limit for GA was 50 ng/ml in whole blood and plasma.

In a 2-h pharmacokinetic study the samples of whole blood, isolated erythrocytes and plasma were analyzed and the amounts of ASA, SA and GA determined. The observed blood erythrocyte and plasma levels of ASA, SA and GA (see Fig. 3) with a simultaneous examination of the distribution of drug between erythrocytes and plasma from three laboratory rabbits are presented. Fig. 4 compares the concentrations obtained by adding the concentrations found in erythrocytes and plasma (related to 1 ml of withdrawn blood with regard to the haemotocrit value) and the concentration found in whole blood. The comparison shows that 3 min after administration ASA is already evenly distributed between erythrocytes and plasma and its level decreases very rapidly in both matrices. 30% of released SA is demonstrated in erythrocytes and 70% in plasma. GA was not demonstrated in erythrocytes in the course of a 2-h pharmacokinetic study.

The papers dealing with the determination of salicylates in plasma preferred liquid–liquid extraction to deproteination. Diethyl ether [11,24],

TABLE II

REGRESSION EQUATION AND CORRELATION COEFFICIENTS OF ASA, SA AND GA IN WHOLE BLOOD, ERYTHROCYTES AND PLASMA

Compound	Whole blood	Erythrocytes	Plasma
ASA	$y = 0.0150x + 0.0077$ ($r = 0.9999$)	$y = 0.0141x + 0.7100$ ($r = 0.9874$)	$y = 0.1937x + 0.0421$ ($r = 0.9975$)
SA	$y = 0.0184x + 0.1920$ ($r = 0.9833$)	$r = 0.1594x - 0.1010$ ($r = 0.9975$)	$y = 0.0212x + 0.1058$ ($r = 0.9878$)
GA	$y = 0.0015x + 0.0001$ ($r = 0.9994$)	–	$y = 0.0041x + 0.0010$ ($r = 0.8993$)

TABLE III

ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF ASA, SA AND GA IN SPIKED WHOLE BLOOD

Compound	Concentration added (μg/ml)	Within-day (n = 6)		Day-to-day (n = 13)	
		Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)
ASA	60	61.5 ± 0.6	1.0	60.4 ± 0.6	1.0
	140	141.0 ± 1.6	1.1	141.8 ± 1.9	1.3
	300	298.1 ± 3.9	1.3	298.8 ± 6.0	2.0
SA	150	148.4 ± 1.8	1.2	152.0 ± 2.2	1.5
	250	248.0 ± 4.6	1.9	247.7 ± 3.8	1.5
	450	447.2 ± 6.0	1.3	453.0 ± 7.0	1.6
GA	5	5.0 ± 0.1	2.0	4.8 ± 0.2	4.2
	10	9.9 ± 0.2	2.0	10.2 ± 0.4	3.9
	20	20.0 ± 0.6	3.0	19.8 ± 0.8	4.0

ethyl acetate [7,12], chloroform [8], methylene chloride [14] or mixtures of solvents [9,15] were used to extract salicylates from plasma. Most studies used UV detection, the sensitivity of the HPLC assay as a rule being about 500–100 ng/ml [7,8,12,14,15]. A limit of detection of 50 ng/ml has been reported in one paper [9], in which salicylates were analyzed in plasma and urine using extraction into a mixture of diethyl ether–hexane and UV detection at 234 nm. The best sensitivity, 2 ng/ml, was achieved by Tebbett *et al.* [18], who analyzed ASA and SA in serum. In the present paper, a limit of detection of 20 ng/ml was found for plasma as well as for whole blood, a very complicated biological matrix. However, in the present paper determination levels of salicylates were higher due to the administration of 100 mg ASA per kg of the rabbit. This was purposely required by our collaborators from the Faculty of Medicine with regard to their other pathophysiological studies.

TABLE IV

ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF ASA AND SA IN SPIKED ERYTHROCYTES

Compound	Concentration added (μg/ml)	Within-day (n = 6)		Day-to-day (n = 13)	
		Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)
ASA	50	52.4 ± 1.4	2.7	49.5 ± 1.1	2.2
	150	147.8 ± 2.2	1.5	147.7 ± 4.1	2.8
	350	348.0 ± 2.6	0.8	351.9 ± 4.6	1.3
SA	150	148.2 ± 2.4	1.6	148.4 ± 2.4	1.6
	250	252.7 ± 3.6	1.4	249.6 ± 4.0	1.6
	450	454.0 ± 4.4	1.0	448.5 ± 7.9	1.8

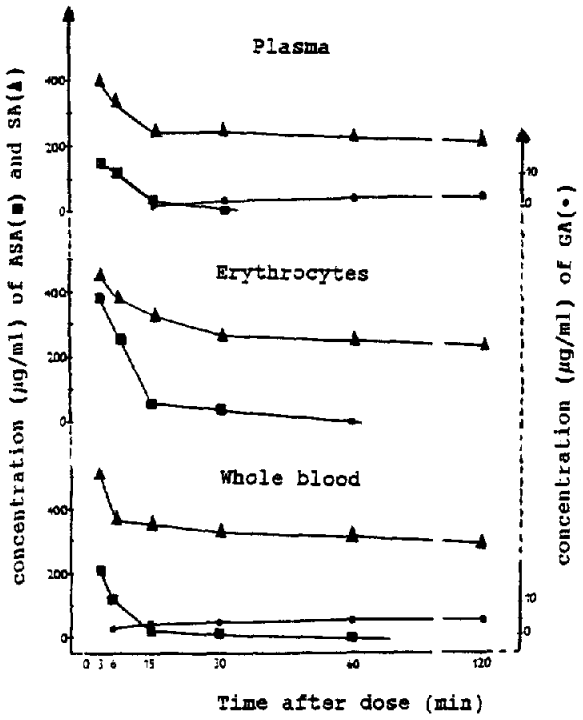

Fig. 3. Concentration of ASA (■), SA (▲) and GA (●) in rabbit following intravenous administration of Aspegic inj.

CONCLUSION

The results obtained provide a more detailed picture of the composition of salicylates in blood between erythrocytes and plasma and contribute to a more objective view of the results of the analysis as dependent on the isolated biological matrix. It can be generally stated that whole blood provides more objective information about drug levels than plasma or serum do, though it is a more complicated biological matrix. The error arising from the bonding of the drug to erythrocytes is thus eliminated. The methodology developed will be further used to determine the immediate blood levels of salicylates in laboratory rabbits, in which other physiological and pathophysiological parameters influenced by salicylates will be investigated.

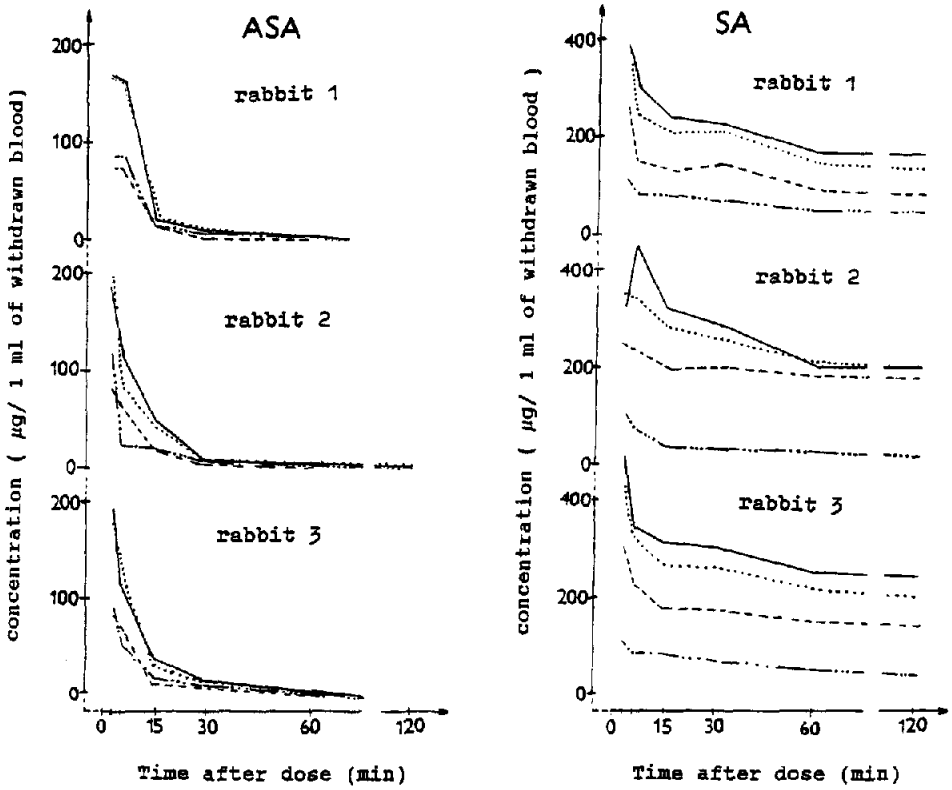

Fig. 4. Distribution of ASA and SA between erythrocytes and plasma in 1 ml of withdrawn blood during 2 h after intravenous administration. (– · · · –) Erythrocytes; (– – –) plasma; (· · · · ·) sum of the levels; (——) level found in the analysis of the whole blood sample.

TABLE V

ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF ASA, SA AND GA IN SPIKED PLASMA

Compound	Concentration added (μg/ml)	Within-day (n = 6)		Day-to-day (n = 13)	
		Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)
ASA	20	21.6 ± 0.3	1.4	20.5 ± 0.5	2.4
	60	59.8 ± 0.5	0.8	59.7 ± 0.6	1.0
	160	161.4 ± 2.1	1.3	162.1 ± 3.8	2.3
SA	150	148.6 ± 1.1	0.7	154.4 ± 1.8	1.2
	250	248.0 ± 1.9	0.8	251.2 ± 2.7	1.1
	400	403.7 ± 3.9	1.0	404.4 ± 8.9	2.2
GA	5	5.0 ± 0.2	4.0	4.8 ± 0.2	4.2
	10	9.8 ± 0.1	1.0	10.0 ± 0.4	4.0
	20	19.6 ± 0.5	2.6	20.2 ± 0.8	4.0

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