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DETERMINATION OF ACETYLSALICYLIC ACID AND ITS MAJOR METABOLITES IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Tran Thai-Son[1], Marcus Öhman[2]

(1) Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam

(2) Department of Chemistry, Karlstad University, SE-651 88 Karlstad, Sweden

Abstract

Objectives: This study describes an assay applied to determine the pharmacokinetic parameters of acetylsalicylic acid following oral administration of 1000 mg of this drug in human. **Materials and method:** In the assay, a reversed-phase high-performance liquid chromatography method (column: Chromtech C8 3µm silica, 100×4.6 mm I.D.; mobile phase: acetonitrile/water: (5/95 v/v) buffer phosphate 0.05M pH 2.15 and acetonitrile/water: (25/75 v/v) buffer phosphate 0.05M pH 2.15; gradient mode; and UV detection at 230 nm) was used to simultaneously quantify acetylsalicylic acid and its main metabolites, including salicylic acid, gentisic acid, and salicyluric acid in urine, which was deproteinized with acetonitrile and centrifuged. **Results:** The method allows the determination of acetylsalicylic acid, salicylic acid, salicyluric acid and gentisic acid in urine as low as 13, 27.4, 21 and 9.3 µg/ml respectively with good precision (better than 5%). **Conclusions:** A fast and easy to perform reversed-phase HPLC method has been developed for the simultaneous determination of acetylsalicylic acid and its major metabolites in urine, in which no time-consuming extraction procedure is needed, simple mobile phases are used, and good precision and accuracy are achieved. The method can be further optimized and improved to be used to study the pharmacokinetic of acetylsalicylic acid.

Keywords*: Acetylsalicylic acid, high-performance liquid chromatography.*

1. BACKGROUND

Acetylsalicylic acid (ASA), commonly known as aspirin, is one of the most widely used therapeutic agents. It is effective as an analgesic, anti-inflammatory and antipyretic drug. ASA is also the principal medication used as a treatment of chronic rheumatoid arthritis, rheumatic fever, and osteoarthritis. In addition, it is indicated that low-dose ASA is beneficial as an antithrombotic agent employed in stroke prevention. Recently, it has been proposed that preparations of ASA in various matrices can be used topically for pain relief in skin infections. The ability of inhibiting the prostaglandin and thromboxane production primarily accounts for the effectiveness of ASA. In this process, ASA blocks irreversibly cyclooxygenase (COX) enzyme which catalyses the conversion of arachidonic acid to endoperoxide compounds. Further, ASA may be effective at preventing certain types of cancer, particularly colorectal cancer [4], [9].

In human body, ASA is rapidly hydrolyzed to salicylic acid (SA) which is also an active substance similar to its parent drug. SA undergoes further metabolism to various conjugated compounds, including salicyluric acid (SUA), diverse acyl and phenolic glucuronides, and to hydroxylated metabolites such as gentisic acid (GA) [1], [9].

For years, many analytical methods, including fluorometry, colorimetry, and chromatography have been employed to determine ASA, its metabolites and degradation products in biological fluids and pharmaceutical preparations [5]. In pharmacokinetic investigations of ASA, an easy and rapid method for analyses of ASA and its metabolites in biological fluids is needed.

Several approaches for this purpose have been developed based on high-performance liquid chromatography (HPLC) [2], [3], [7], [8]. Where reversed-phase HPLC technique with UV-Vis detection is one method of choice.

The present study describes a simple reversed-phase HPLC method developed for the simultaneous determination of ASA and its major metabolites in human urine.

2. MATERIALS AND METHOD

2.1. Reagents and chemicals

ASA, SA, GA, and SUA were obtained from Sigma-Aldrich, Acros and Kebo Lab respectively, acetonitrile (Sigma-Aldrich, HPLC grade), ortho phosphoric acid (85%, p.a.) from E. Merck. Water was purified through a Milli-Q water purification system (Millipore, Eschborn, Germany). The stock solutions of ASA, SA, GA, and SUA (10 mM) were prepared in acetonitrile/water (50/50 v/v). All solutions were stored at 5°C.

2.2. Chromatography

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a solvent delivery system LC-10ADvp, a UV/Vis detector SPD-10A (230 nm), and Orchrom Ver 1.0 integrated software (quantitation was based on peak areas). Separation was performed using a prepacked stainless-steel column (100x4.6 mm I.D.) filled with Chromtech C8 3µm silica. The assay used two mobile phases with gradient separation: (A) acetonitrile/water (5/95 v/v), buffer phosphate 0.05 M pH 2.15; (B) acetonitrile/water (25/75 v/v), buffer phosphate 0.05 M pH 2.15. The injector with 20 µL loop was used, and the flow rate was 1.5 mL/min. The gradient program was set up as follows: 0 – 4 min: %B: 0-50%. 4 – 8 min: %B: 50%

The retention times (back-pressure 160 bar) were: 4.3 min (GA), 5.4 min (SUA), 7.0 min (ASA), 7.9 min (SA).

2.3. Collection and storage of samples

Twenty urine samples from one male volunteer were collected into plastic bottles within 48 hours. The samples were stored in freeze (-18°C) until the assay. A blank sample of urine was taken before ingestion of the preparation containing acetylsalicylic acid. The pH of the urine samples (including the blank) was measured.

2.4. Sample preparation

Frozen urine samples were thawed in a warm-water bath. An amount of 2 mL of urine was added to a test tube containing 2 mL of acetonitrile for deproteinization [5]. Centrifuge the tube on the Rotafix 32A machine (Hettich Zentrifugen, Germany) at 4000 rpm (2900 G) in 10 minutes. After centrifugation, the proteins were precipitated and at least 2 mL of the supernatant was transferred to a new tube then 20 µL of this was injected into the chromatography system. The procedure was applied in the same way to the blank.

2.5. Validation

Standard samples were prepared by appropriate dilution of the stock solutions with acetonitrile/water (50/50 v/v) to obtain concentrations 0.05, 0.25, 0.5, 0.75, 1.0 mM, and stored at 5°C until assay. Limit of detection (LOD) and limit of quantification (LOQ) were determined using "3α rule" [6]. The precision and accuracy of the determination of substances were performed by spiking blank urine with known amounts of each analytes (0.1 mM and 0.25 mM).

3. RESULTS AND DISCUSSION

A simple reversed-phase HPLC method was developed for the simultaneous determination of ASA and its major metabolites in urine. Figure 1 shows typical chromatograms of separation. SUA, ASA and SA are well separated from endogenous urine components, and can be determined quantitatively. In the assay, the centrifugation was done at 4000 rpm (2900 G).

Centrifugation speed didn't affect the separation of ASA and metabolites from urine components considerably, but if this was done at G < 900, the impact would be significant (Figure 2). The separation process was optimized with mobile phase's flow rate of 1.5 mL/min, pH buffer of 2.15 and using gradient elution. With these conditions, the analytes were separated effectively from each other in a relatively short time course. Four standard curves with five different concentrations (range from 0.05 to 1 mM) were obtained with good linearity (Figure 3). The linear regression equations of the standard curves and respective correlative coefficients are indicated in Table 1. Limit of detection (LOD) and limit of quantification (LOQ) are indicated in Table 2. The method of determination has a good accuracy and precision with the recovery (Re) of 87-99.3% and the RSD of 1.2-3.9% (Table 3)

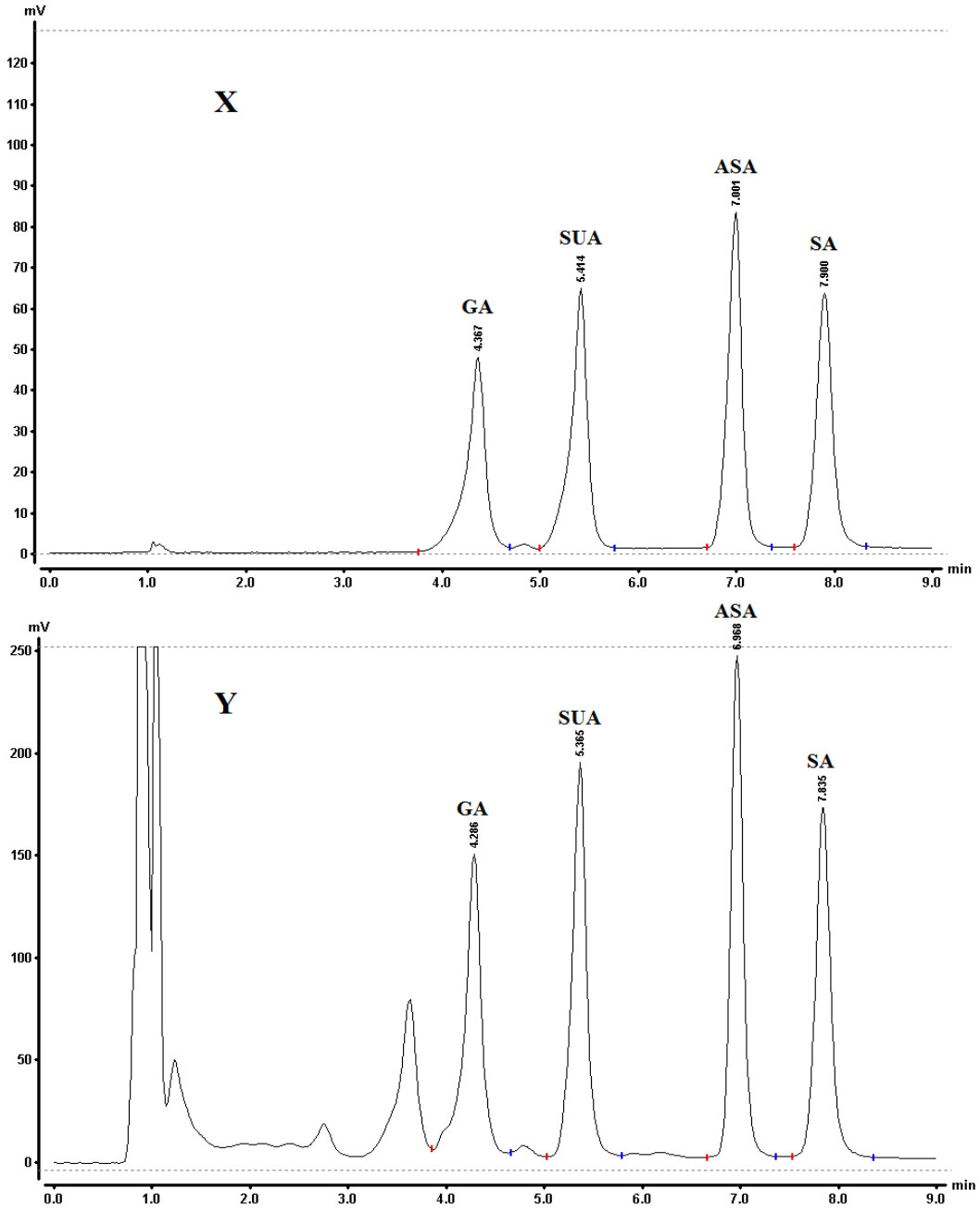

Figure 1. Typical chromatograms of the separation. **X**: Standard mixture of 0.1mM each of GA, SUA, ASA, SA in acetonitrile:water (50:50 v/v). **Y**: Mixture of 0.25mM each of GA, SUA, ASA, SA in blank sample of urine. Column: Chromtech C8 3µm silica, 100×4.6 mm I.D.; mobile phase: A: acetonitrile/water: (5/95 v/v) buffer phosphate 0.05M pH 2.15 and B: acetonitrile/water: (25/75 v/v) buffer phosphate 0.05M pH 2.15; loop: 20 µL; gradient mode: 0 – 4 min: %B: 0-50%. 4 – 8 min: %B: 50%; and UV detection (230 nm). Flow rate: 1.5 mL/min.

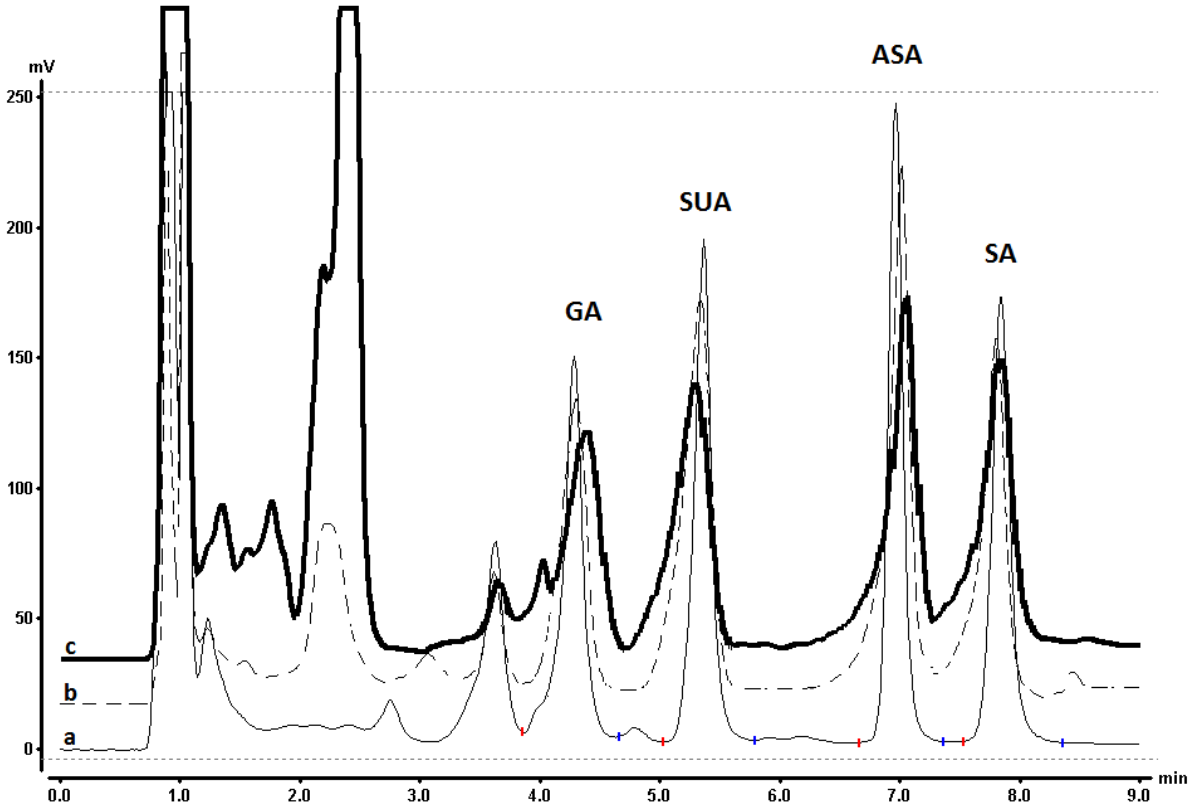

Figure 2. Chromatograms of mixtures of 0.25mM each of GA, SUA, ASA, SA in blank sample of urine prepared by centrifugation at a speed of (**a**) 4000 rpm (2900 G), (**b**) 2200 rpm (900 G), and (**c**) 2000 rpm (700 G). Column: Chromtech C8 3µm silica, 100×4.6 mm I.D.; mobile phase: A: acetonitrile/water: (5/95 v/v) buffer phosphate 0.05M pH 2.15 and B: acetonitrile/water: (25/75 v/v) buffer phosphate 0.05M pH 2.15; loop: 20 µL; gradient mode: 0 – 4 min: %B: 0-50%. 4 – 8 min: %B: 50%; and UV detection (230 nm). Flow rate: 1.5 mL/min.

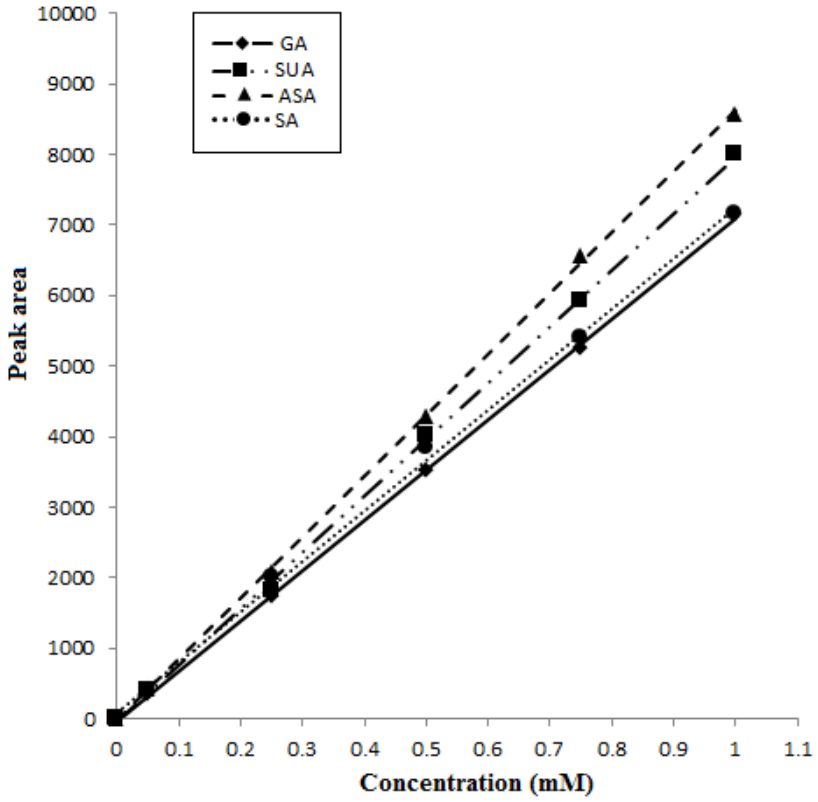

Figure 3. Standard curves of substances

Table 1. Linear regression equations of the standard curves

	GA	SUA	ASA	SA
Equation	y = 7125.3x - 26.749	y = 8012.9x - 44.181	y = 8642.6x - 13.117	y = 7140.1x + 96.084
R^2	0.9998	0.9994	0.9997	0.998

Table 2. Limit of detection (LOD) and limit of quantification (LOQ) of the method

		GA	SUA	ASA	SA
LOD	mM	0.0198	0.035	0.024	0.066
	mg/L	3.1	6.9	4.1	9.0
LOQ	mM	0.060	0.108	0.072	0.199
	mg/L	9.3	21.0	13.0	27.4

Table 3. Accuracy and precision of the method

Study	Analyte	Added (mM)	Found (mM)				RSD (%)	Re (%)
			Time 1	Time 2	Time 3	Mean		
I	GA	0.1	0.09	0.095	0.091	0.092	2.2	92.4
	SUA	0.25	0.236	0.251	0.243	0.243	3.0	97.4
	ASA	0.1	0.098	0.010	0.095	0.098	2.2	97.9
	SA	0.25	0.224	0.242	0.233	0.233	3.9	93.1
II	GA	0.25	0.228	0.242	0.238	0.236	3.1	94.3
	SUA	0.1	0.099	0.100	0.098	0.099	1.2	99.3
	ASA	0.25	0.232	0.247	0.237	0.238	3.2	95.4
	SA	0.1	0.087	0.089	0.085	0.087	2.4	87.0

The method was applied for the determination of ASA and its metabolites in 20 urine samples collected from one male volunteer within 48 hours. Results in Table 4 indicate that the method failed to quantify GA but the remainders (there is no peak of GA in Figure 4). This may due to either the degradation of GA during sample collection and preparation or the occurrence of this metabolite in urine with the concentration lower than its LOQ. The results also indicate that SUA is the main metabolite of ASA with the total amount excreted within 24 hours is about 515.2 mg; but in first 70 minutes, SA is the dominant (with 70.7 mg excreted in urine in comparison with 60.5 mg of SUA). The average urinary excretion rates of

the substances plotted against the average times (t*) (Figure 5) indicate the almost complete elimination of ASA and metabolites within 24 hours after a 1000 mg oral dose of ASA. In the plot, SUA-curve is different from the others with the highest excretion rate achieve at about 2.2 hours after administration. This can be explained by the time it takes for the conjugation of SA with glycine to form SUA as mentioned previously. The impact of urine's pH on substance excretion is obviously, in which acidic pH will lower the excretion rate of acidic compounds. This can explain for the excretion rate of SUA at 3.6 hours after administration, which is lower than those after that (Figure 5).

Table 4. Determination of substances in samples

No.	Time (hour)	t (hour)	t* (hour)	Amount substance in urine M (mg)				M/t (mg/h)				pH
				GA	SUA	ASA	SA	GA	SUA	ASA	SA	
0	0	0	0	-	-	-	-	-	-	-	-	~ 7
1	1.2	1.2	0.6	-	60.5	34	70.7	-	50.4	28.3	58.9	~ 7
2	2.2	1	1.7	-	84.9	-	51.9	-	84.9	-	51.9	~ 7
3	4.9	2.7	3.6	-	41.6	-	-	-	15.4	-	-	~ 5
4	8.2	3.3	6.6	-	140.8	-	-	-	42.7	-	-	~ 7
5	9.4	1.2	8.8	-	61.1	-	-	-	50.9	-	-	~ 6
6	10.3	0.9	9.9	-	37.5	-	-	-	41.7	-	-	~ 6
7	12.3	1.9	11.3	-	50.3	-	-	-	26.5	-	-	~ 7
8	14.4	2.2	13.4	-	17.4	-	-	-	7.9	-	-	~ 7
9	23.1	8.7	18.8	-	21.1	-	-	-	2.4	-	-	~ 6
10	25.5	2.4	24.3	-	-	-	-	-	-	-	-	~ 6
11	26.4	0.9	26	-	-	-	-	-	-	-	-	~ 7
12	28.7	2.3	27.6	-	-	-	-	-	-	-	-	~ 7
13	29.9	1.2	29.3	-	-	-	-	-	-	-	-	~ 6
14	32.6	2.7	31.3	-	-	-	-	-	-	-	-	~ 5
15	34.5	1.9	33.6	-	-	-	-	-	-	-	-	~ 5
16	36.8	2.3	35.7	-	-	-	-	-	-	-	-	~ 6
17	38.1	1.3	37.5	-	-	-	-	-	-	-	-	~ 7
18	39.1	1	38.6	-	-	-	-	-	-	-	-	~ 7

19	46.5	7.4	42.8	-	-	-	-	-	-	-	-	~ 6
20	48	1.5	47.3	-	-	-	-	-	-	-	-	~ 6

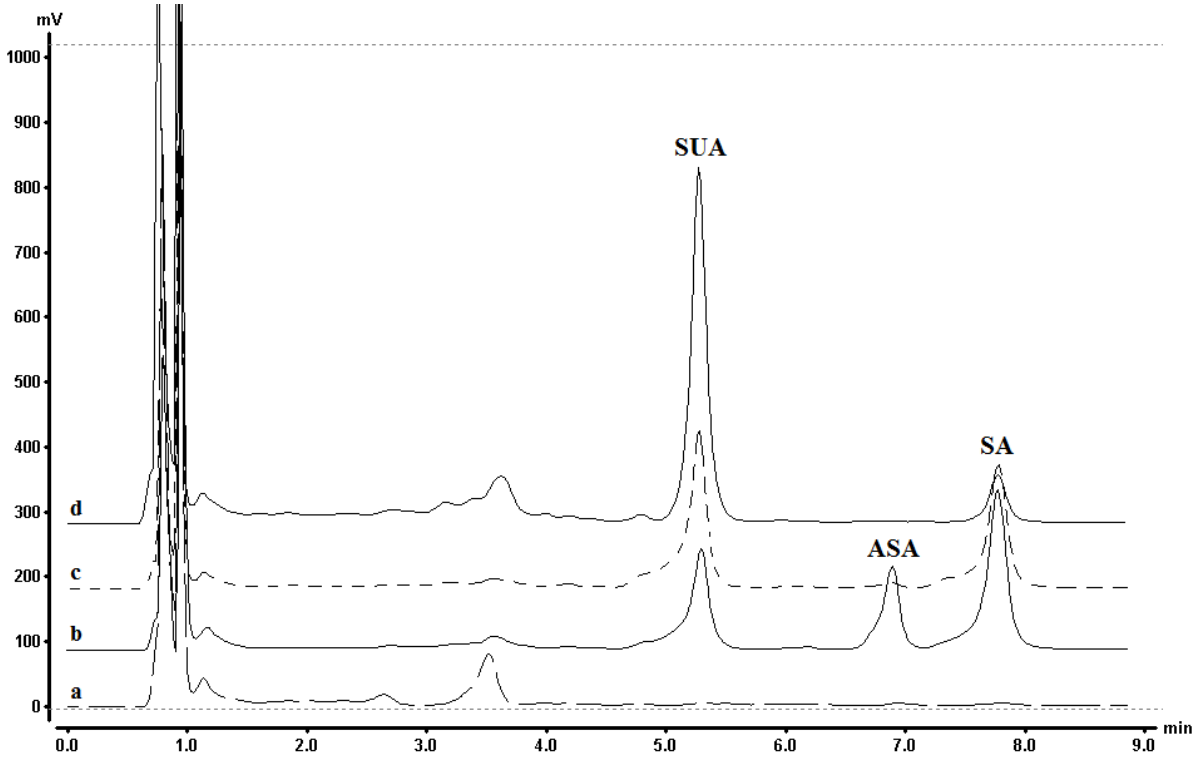

Figure 4. Chromatograms of urine samples (a) before, (b) 1.2h after, (c) 2.2h after, and (d) 8.2h after oral administration of a 1000mg dose of ASA. Column: Chromtech C8 3µm silica, 100×4.6 mm I.D.; mobile phase: A: acetonitrile/water: (5/95 v/v) buffer phosphate 0.05M pH 2.15 and B: acetonitrile/water: (25/75 v/v) buffer phosphate 0.05M pH 2.15; loop: 20 µL; gradient mode: 0 – 4 min: %B: 0-50%. 4 – 8 min: %B: 50%; and UV detection (230 nm). Flow rate: 1.5 mL/min.

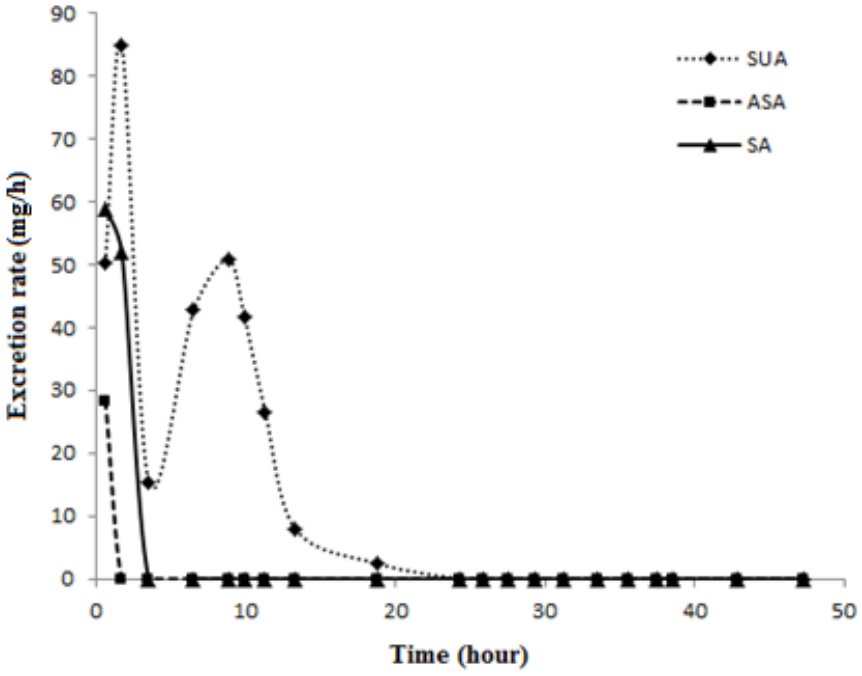

Figure 5. Urinary excretion rate of ASA and its metabolites

4. CONCLUSIONS

In this study, a fast and easy to perform reversed-phase HPLC method has been developed for the simultaneous determination of ASA and its major metabolites in urine, in which no time-consuming extraction procedure is needed, simple mobile phases are used, and good precision and accuracy are achieved. This method failed to quantify GA, but it can be further optimized and improved to be used to study the pharmacokinetic of ASA.

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