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QUANTITATIVE ESTIMATION OF SALICYLIC ACID AND ITS METABOLITES BY THIN-LAYER DENSITOMETRY

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SUMMARY

A rapid thin-layer densitometric method for the quantitative determination of salicylic acid and its metabolites in urine or plasma is described. The method is specific and very sensitive. Nanogram quantities of salicylic acid and its metabolites, both free and conjugated, may be estimated. Known metabolites, as well as the newly described gentisuric acid, were estimated quantitatively in urine from a patient treated with aspirin.

INTRODUCTION

Numerous spectrophotometric, fluorimetric or polarographic methods for the estimation of the salicyclic acid and its metabolites have been described[1–11]. Most lack specificity, sensitivity, and require laborious purification and extraction procedures. Several gas–liquid and liquid chromatographic methods for the estimation of the salicylic acid or its metabolites have also been described[11–25], but the complete quantitative separation and determination of all salicylic acid metabolites from the urine or plasma by this technic is difficult. Although many different columns were tried (Sephadex, Dowex, polyamide, DEAE-cellulose, Chromosorb, Carbowax, etc.), the quantitative separation and analysis is usually subject to interference by other metabolites from urine or plasma, with a consequent lack of specificity and sensitivity. From our experience, thin-layer chromatography (TLC) is applicable to the quantitative separation of salicylic acid metabolites from urine or plasma. Various solvent mixtures have been described for the separation of salicylic acid and its derivatives by paper chromatography or TLC[26–32]. We have found that interfering compounds can be extracted from the urine or plasma by an ethyl acetate–diethyl ether mixture at pH 8–9. The next ethyl acetate extract from an acid medium can be used directly for TLC estimations. From many solvents examined, the diethyl ether–butyric acid solvent mixture was found to be very suitable for the separation and quantitative determination of the metabolites of salicylic acid in urine or plasma. Sharp peaks

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and relatively clear background were obtained by using this mixture with silica gel G plates. Reported here is the adaptation of this TLC technic to quantitative estimation of salicylic acid and its metabolites in urine or plasma.

MATERIALS AND METHODS

Urine (10 ml) was alkalinized to pH $\approx$ 8.5 by adding 0.2 *M* NaOH. The urine was then shaken with 14 ml ethyl acetate and 1 ml ether for 2 min. Ten milliliters of the lower layer were transferred and acidified in a separate tube by the addition of 1 ml conc. HCl. The mixture was then extracted by shaking with 14 ml ethyl acetate and 1 ml ether for 3 min. After centrifuging, the clear ethyl acetate layer was separated (10 ml) and precisely 10 μl were spotted on silica gel G TLC plates (HPTLC nanogram glass-supported plates, silica gel 60, without fluorescent indicator, E. Merck, Darmstadt, G.F.R.). The plates were developed by diethyl ether–*n*-butyric acid (10:1, v/v) mixture (for a distance of 10 cm) in a tank for ascending TLC plates. The plates were dried by a fan at 50° and used for quantitative estimation at 325 nm on a Schoeffel TLC densitometer in refractive double beam mode. The standard sample of urine was prepared by the addition of 1 mg gentisic acid, 1 mg salicylic acid, 10 mg salicyluric acid and 1 mg gentisuric acid (all in 100 μl of ethanolic solution added to 10 ml of urine). After addition of these standard amounts the urine was treated in the same manner as the sample urine. The hydrolyzed urine was treated exactly the same way (no standards were added), except that before the second extraction, after the addition of conc. HCl, the urine was boiled 2 h under reflux. After cooling, the hydrolyzed mixture was extracted by 14 ml ethyl acetate and 1 ml ether. As with the non-hydrolyzed urine and the standard samples of urine, the ethyl acetate layer was used for TLC analysis. The blood plasma (1 ml) was deproteinized by 5% trichloroacetic acid (1 ml), centrifuged, and the supernatant was alkalinized (pH $\approx$ 8.5 by adding 1 *M* NaOH). This alkaline solution was then treated in exactly the same manner as the urine. If necessary, a smaller volume of the sample (urine or plasma) may be used (for example, 0.5–1 ml). The volume of the reagents and the volume of the extractant must be changed in the same ratio. The ethyl acetate extract may be evaporated in vacuum to a smaller volume (50–1000 μl) which increases proportionally the sensitivity of the method. It is not recommended to spot more than 10–20 μl of the sample on TLC nanogram plates. The above method is suitable for human urine or plasma collected after a dose of 0.3–10 g of aspirin per adult man.

Commercially available (highest purity) salicylic acid, salicyluric acid and gentisic acid were used as reference compounds. Gentisuric acid was synthetized as previously described[33] by using a modification of the method of Sheehan and Hess[34], Ito and Neilands[35], and Reio[36]. Gentisuric acid was previously identified as a metabolite of acetylsalicylic acid, (aspirin) in human urine[33].

RESULTS AND DISCUSSION

As is apparent from Fig. 1, separation of the different salicylic acid derivatives added to urine or plasma was accomplished by TLC technique. Although the first alkaline extraction of urine or plasma caused some loss of the compounds (Table I), this step is necessary because the urine or plasma usually contains compounds which

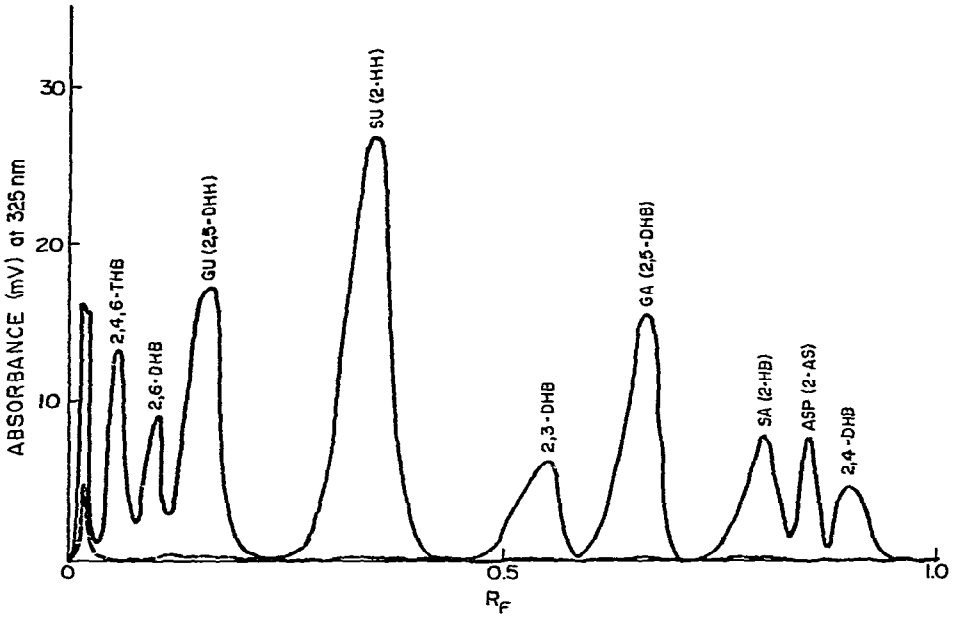

Fig. 1. TLC of control urine (— — —) and urine marked with different benzoic acid derivatives (—). The control urine was treated as described in Materials and methods. The TLC background was clear of interfering spots. The same urine was then marked with benzoic acid derivatives (10 mg/ml of 2-hydroxyhippuric acid, and 1 mg/ml of 2,5-dihydroxyhippuric acid, 2,4,6-trihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2-hydroxybenzoic acid, 2-acetylsalicylic acid, 2,4-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid). The TLC plates were developed and measured on the densitometer at 325 nm as described.

fluoresce in ultraviolet and may interfere with the estimation. These interfering compounds are extracted to ethyl acetate from an alkaline medium. On the other hand, the derivatives of salicylic acid are not extracted under the same conditions, and the partial loss is caused mostly by oxidation (it may be significantly decreased in the presence of nitrogen).

The method of standard addition to the sample used in this work usually does not require precautions about the extraction efficiency. The over-all extraction of the estimated products (without the nitrogen medium) was good (*ca.* 80%).

In urine from an adult patient (83 kg) after a single dose (1.3 g) of aspirin four metabolites and their conjugates were found: salicylic acid (SA), gentisic acid

TABLE I

EXTRACTION EFFICIENCY FOR THE METABOLITES OF ASPIRIN IN URINE

Metabolite	*Loss by extraction (%)*	
	From alkaline media	*From acid media*
GU	12	9
SU	7	3
GA	14	15
SA	2	13

(GA), salicyluric acid (SU) and gentisuric acid (GU) (Fig. 2), as previously reported to occur in human urine after ingestion of aspirin[5,27,28,33–39]. Aspirin is usually quickly deacetylated to salicylic acid, thus no aspirin spot was found in the urine. After the addition of the standard compounds, and also after hydrolysis of the urine, the peaks corresponding to these metabolites were increased and no other TLC spots were found. The surface under the densitometric peaks (measured by weights of the excised peaks) increased proportionally to the amount of the standard added to the urine (Table II). The proportionality was linear up to 2000 μg/ml urine (Fig. 3). The control urine from a patient who did not take aspirin was devoid of TLC spots, which could interfere with the determination.

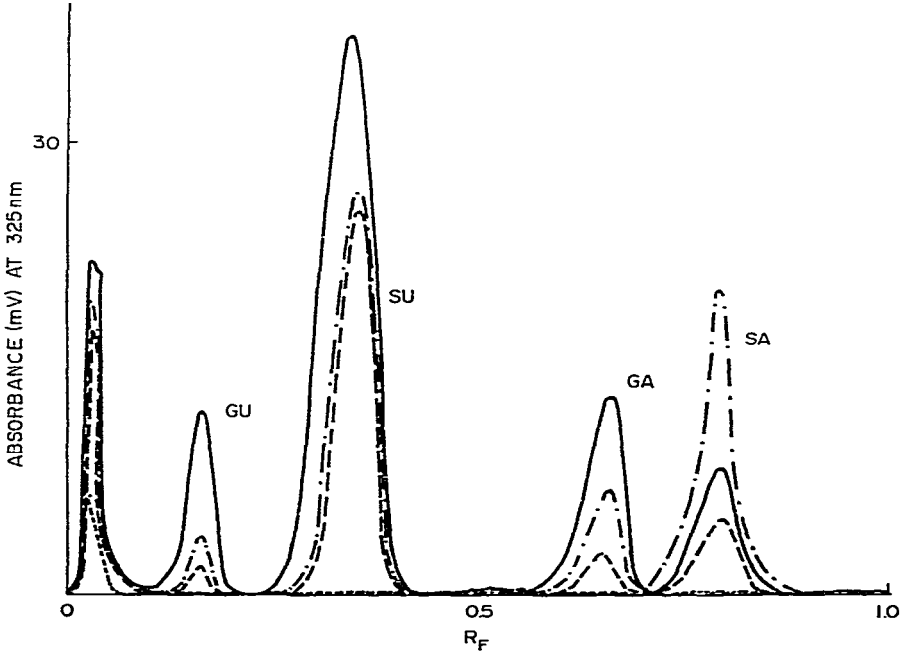

Fig. 2. TLC of urine in an aspirin-treated patient. The graph was obtained on the densitometer at 325 nm as described in methods and in Tables II and III. The urine was marked as described in Table II under D. If *a*, *b*, *c*, and *d* are the surfaces of the corresponding curves, [*a* = control urine (———); *b* = patient's urine (— — —); *c* = acid hydrolyzed patient's urine (— . —); and *d* = patient's urine marked with standards (———),] then the contents of free acids in the urine are $b \cdot y/(d-b)$, where y are the concentrations of the corresponding standards in the marked urine, and the contents of the corresponding conjugates are $(c-b) \cdot y/(d-b)$ (expressed in the same units as the standard).

By using TLC densitometry at 325 nm, we have calculated the content of the metabolites of salicylic acid and their conjugates in urine (Table III). The standard error of the mean between the duplicates was 0–6%, with an average calculated from 10 samples 1.7%. SU was the predominate metabolite in the urine of a patient and amounts of GU were similar to those of GA. Only a small amount of SU was present in conjugated form as estimated from acid hydrolysis, but much larger amounts of conjugates were found for salicylic, gentisic and gentisuric acids. The plasma from another patient was analyzed as described in Methods. Three metabolites were found

TABLE II

STANDARD CURVE FOR ASPIRIN METABOLITES IN URINE OF MAN

The surface of densitometric curves (in mg of the recording paper): A = patient's urine (1.3 g aspirin, 710 ml, collected 8 h); B = patient's urine + 200 μg GU + 2000 μg SU + 200 μg GA + 200 μg SA per 10 ml urine; C = patient's urine + 500 μg GU + 5000 μg SU + 500 μg GA + 500 μg SA per 10 ml urine; D = patient's urine + 1000 μg GU + 10,000 μg SU + 1000 μg GA + 1000 μg SA per 10 ml of urine; E = patient's urine + 2000 μg GU + 20,000 μg SU + 2000 μg GA + 2000 μg SA per 10 ml of urine; F = patient's urine hydrolyzed as described in Materials and methods (compare amount in F to A to estimate conjugates).

Urine sample	*SA*	*Mean*	*GA*	*Mean*	*SU*	*Mean*	*GU*	*Mean*
A 1	17.85		8.33		97.53		3.20	
		17.97		8.22		96.87		3.15
2	18.10		8.11		96.22		3.10	
B 1	22.01		17.06		113.12		5.38	
		22.08		16.99		113.56		5.39
2	22.15		16.91		114.00		5.40	
C 1	28.19		30.04		135.21		8.63	
		28.34		29.78		134.00		8.62
2	28.48		29.53		132.80		8.60	
D 1	38.10		51.46		175.32		14.24	
		38.39		51.25		174.74		14.33
2	38.67		51.03		174.16		14.41	
E 1	58.27		94.71		252.36		25.41	
		58.59		94.50		251.18		24.35
2	58.91		94.20		250.00		25.28	
F 1	63.23		22.40		99.40		6.12	
		63.80		22.09		99.48		5.61
	64.37		21.78		99.56		5.10	

as in the urine of the first patient, but no GU was found in plasma. These examples do not express the average values, because large individual and dose related differences in the amount of metabolites in urine or plasma from patients treated with aspirin have been described[5,37,38,40].

The identity of the estimated compounds was verified by the extraction of the spots from preparative TLC plate with acetone and by use of other solvent mixtures: benzene–methanol–acetic acid (45:8:4), benzene–dioxane–acetic acid (75:25:4) and toluene–ethyl formate–formic acid (5:4:1). The R_F values of the aspirin metabolites in urine corresponded to the standard compounds in all cases. GA and glycine were found after the hydrolysis (conc. HCl, 8 h) of the GU spot extracted by acetone from the preparative TLC plate. Maximum sensitivity of the method was 0.05–0.2 ng/ml urine, depending on the compound. Practical sensitivity which could be attained was 1 ng/ml urine or plasma (SA), 2.5 ng/ml (SU), 0.5 ng/ml (GA), and 2 ng/ml GU). The method is accurate, specific, and required only 3 h for the complete quantitative estimation of all metabolites including the hydrolyzed conjugates.

The control urine (20 ml) was at first extracted by ethyl acetate and diethyl ether (28+2 ml) at pH 8.5 to remove interfering substances. The ethyl acetate layer was then discarded and the urine layer was divided in two samples. The second sample was acidified by 1 ml conc. HCl. The standard mixture (containing 1 mg GU, 10 mg SU, 1 mg GA, and 1 mg SA in 100 μl ethanol) was added to both samples.

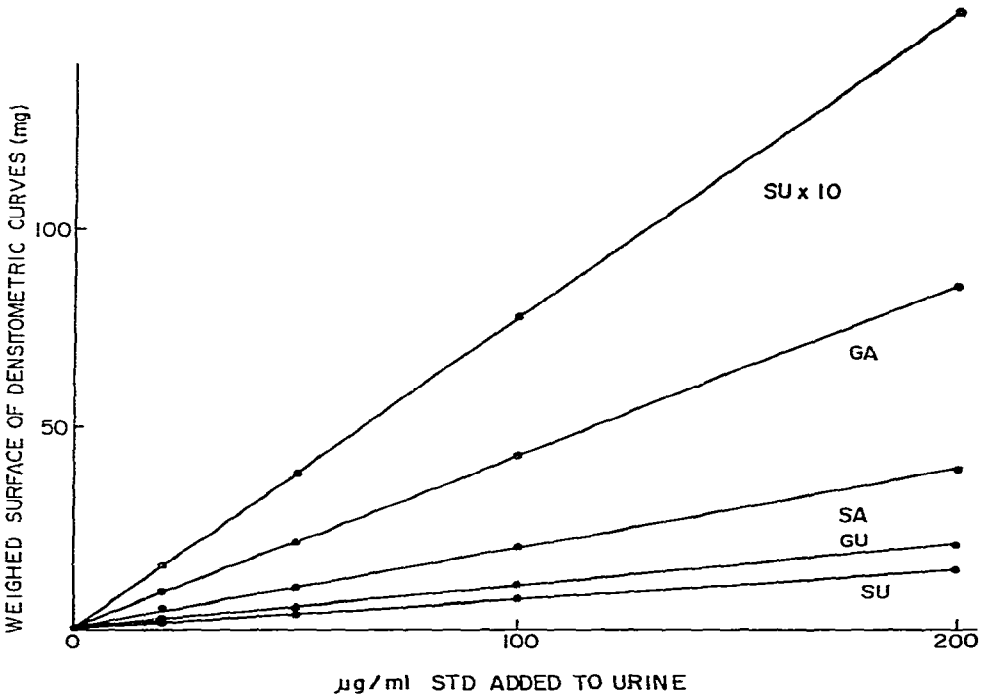

Fig. 3. Standard curve for the metabolites of aspirin in urine. The values in Table II are expressed in the form of standard curves. The surface of densitometric curves of the unmarked urine (A) was subtracted from the surface of densitometric curves of the marked urine (B to E) and the difference was plotted *versus* the amounts of standards added to the urine. The blank urine from the patient who was not treated with aspirin had no interfering peaks on TLC.

The first alkaline sample was extracted by ethyl acetate and ether (14+1 ml), and the upper layer was discarded. Then it was acidified by adding 1 ml conc. HCl. Both acid samples were then extracted by ethyl acetate and ether (14+1 ml) and 10 µl of the clear upper layers were used for TLC analysis. The 10 µl of standard solution

TABLE III

METABOLITES OF ASPIRIN IN URINE AND PLASMA OF MAN

The metabolites were estimated from the surface of the densitometric curves as described in Materials and methods. The urine from the first patient (83 kg body weight) was collected 8 h after a single dose of aspirin (1.3 g). The volume of urine was 710 ml. The plasma was taken from a second patient who was treated chronically with aspirin (1.5–2 g per day) for arthritis.

Sample	*Free acids (mg/l)*				*Conjugated acids (mg/l)*			
	SA	*GA*	*SU*	*GU*	*SA*	*GA*	*SU*	*GU*
Urine from patient 1 (duplicates)	88	19	1254	29	222	36	34	24
	88	19	1235	27	226	35	34	20
Mean	88	19	1245	28	224	36	34	22
Content (% of the dose)	4.8	1.0	68.0	1.5	12.2	1.9	1.9	1.2
Plasma from patient 2	126	5	190	0	5	1	6	0

added to ethyl acetate was used as the 100% control. By the comparison with this control the extraction efficiency was calculated for each extraction procedure. Higher loss by extraction was observed with cloudy, old urine.

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