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Abstract

An attempt was made to establish the method for the estimation of glucuronide formation in vivo using salicylamide and further to study the alteration in the glucuronide formation in liver disease. The results were as follows: 1. The method for the determination of free salicylamide separately from other conjugates of salicylamide in urine, without involving any hydrolysis of the other conjugates, was presented. When 1 g. of salicylamide was administered to the subjects with or without liver injuries, no free salicylamide was detected by the present method in the urine following the salicylamide administration. 2. The analytical method for the determination of salicylamide glucuronide was also devised by employing a hydrolysis with β-glucuronidase. The ratio of the salicylamide liberated by the enzymatic hydrolysis of the 10-hour urine following the administration of 1 g. of salicylamide to the total salicylamide excreted in the same urine was neither affected by the total recovery of the salicylamide nor by the urine volume. This ratio was thus used as a means of estimating the capacity of the glucuronide formation in vivo, although it was considered that the ratio might be affected to some extent by the competition between the glucuronide and other conjugate formations in vivo. 3. As a result of this salicylamide glucuronide excretion test, it was indicated that the in vivo formation of salicylamide glucuronide in the patients with postnecrotic cirrhosis was slightly decreased compared with that in normal controls.

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URINARY RECOVERY OF SALICYLAMIDE AS ITS GLUCURONIDE IN LIVER DISEASE

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The determination of the glucuronide formation *in vivo* has been based on the estimation of the urinary excreted glucuronic acid after administration of the substance to be conjugated with glucuronic acid, such as camphor, borneol, and menthol[1]. The method of SANPPER and SALTZMAN[2,3] using benzoic acid as a test substance for this purpose was not a suitable means of estimating mainly the glucuronide formation *in vivo* without reference to other conjugate formations, because the principle of their method was based on the competition between glycine and glucuronic acid conjugations for benzoic acid. BARNIVILLE and MISK[4], for the same purpose, used salicylamide which was known to be conjugated *in vivo* primarily with glucuronic acid. However, their means also depended on the determination of the glucuronic acid increased in the urine following salicylamide load. SCHMID and HAMMAKER[5], also for this purpose, administered N-acetyl-p-aminophenol and determined the corresponding glucuronide in the blood and urine specifically by employing a hydrolysis with β-glucuronidase. This method was considered, however, to be still imperfect in that N-acetyl-p-aminophenol appeared to be conjugated *in vivo* less favorably with glucuronic acid than with other substances, such as sulfuric acid.

In view of these unsatisfactory methods and the results obtained therefrom, present study was made attempting to establish a specific method of estimating the *in vivo* formation of glucuronide in human subject and to apply it to the patients with liver disease. For this purpose salicylamide was selected as a test substance, because it was known to be rapidly absorbed, excreted in urine mainly as its etherial glucuronide[6], and also non-toxic.

MATERIALS AND METHODS

Five normal subjects, 19 patients with viral hepatitis including 4 with acute hepatitis, 2 with acute exacerbation of chronic hepatitis, 7 with chronic hepatitis, and 6 with postnecrotic cirrhosis, and 2 patients with Dubin-Johnson syndrome, all hospitalized to Okayama University Hospital were subjected to a salicylamide glucuronide excretion test. All these clinical diagnoses were con-

firmed by peritoneoscopy, histological examination of the liver, and chemical tests for liver function. The procedures of the salicylamide glucuronide excretion test were as follows.

The subject was allowed to take dinner at 5:00 p. m. and fasted thereafter. The administrations of the drugs capable of being conjugated with glucuronic acid or of the drugs containing phenolic compounds were also withheld during the period of this test. A dose of 1 g. salicylamide was administered orally with approximately two hundred ml. of water at 10:00 p. m., and the following 10-hour urine was collected. This urine sample was immediately subjected to the following analyses after measurement of the urine volume. Total and free salicylamide and salicylamide glucuronide were analyzed according to the following modifications of the method described by BRODIE and others[7] for the determination of salicylic acid and of the method described by CRAMPTON and VOSS[8] for the determination of salicylamide.

Analysis of Total Salicylamide in Urine: A one-thousandth part of the volume of the 10-hour urine sample was placed in a 50 ml. centrifuge tube with a well-fitting glass stopper and diluted to 1 ml. with distilled water. One ml. of concentrated hydrochloric acid was added to the diluted urine sample. The tube was closed with the glass stopper and placed in a boiling water bath for 15 minutes[1)], and then cooled in running tap water. To the hydrolyzed urine, 2 ml. of distilled water followed by 25 ml. of benzene[2)] was added and the tube was shaken vigorously for one minute. After centrifugation of the tube, 15 ml. of the organic phase was transferred to another similar centifuge tube receiving 2 ml. of 0.05 N sodium hydroxide and the tube was again shaken vigorously for one minute followed by centrifugation. One ml. of the aqueous phase was transferred to a test tube. To the tube, 1.5 ml. of 0.1 M glycine buffer, pH 2.6, 1.5 ml. of ferric nitrate solution one per cent in 0.07 N nitric acid, and 2 ml. of distilled water were added in this order. The optical density of this solution was read at a wave length of 535 mμ against the control solution which was similarly prepared from 1 ml. of 0.05 N sodium hydroxide by adding the buffer, the reagent, and water[3)]. Concentration of the salicylamide was determined by a

1) CRAMPTON and VOSS[8] hydrolyzed conjugated salicylamide in 1 N hydrochloric acid for six hours in a boiling water bath. In the present method, however, the time required for the hydrolysis was reduced to fifteen minutes by employing the hydrolysis in 6 N hydrochloric acid in a boiling water bath. Under this condition, the hydrolysis was complete and did not involve any degradation of salicylamide.

2) Benzene was substituted for ethylene dichloride which was generally used for the extraction of salicylic acid[7] and salicylamide[8], because benzene facilitated the extraction of salicylamide from a weak acid medium to a satisfactory degree.

3) Various substances contained in urine did not interfere with salicylamide in the processes of the extractions, the color developement, and the colorimetry.

comparison with the standard curve prepared from aqueous salicyamide solutions with varied concentrations. The amount of the salicylamide liberated by the acid hydrolysis was calculated and expressed in terms of total salicylamide.

Analysis of Salicylamide Glucuronide in Urine: A one-thousandth part of the volume of the 10-hour urine sample was placed in a similar centrifuge tube and diluted to 1 ml. with distilled water. Two ml. of 0.2 M acetate buffer, pH 6.0, containing 80 units of bacterial β-glucuronidase was added to the diluted urine sample, which was then incubated for 5 hours[4] at 37°C and then cooled in running tap water. To the enzymatically hydrolyzed urine, 1 ml. of 1 N hydrochloric acid was added, and the salicylamide being present in the hydrolyzed urine was extracted with 25 ml. of benzene. The amount of the extracted salicylamide was determined thereafter by the same procedure as described for the measurement of total salicylamide. From this amount of salicylamide, the amount of the free salicylamide existed in the original urine before the hydrolysis was subtracted in order to obtain a net amount of the salicylamide derived from salicylamide glucuronide following the hydrolysis. This amount of the salicylamide liberated by the enzymatic hydrolysis was expressed in terms of salicylamide glucuronide.

Analysis of Free Salicylamide in Urine: A one-two hundred and fiftieth part of the volume of the 10-hour urine sample was placed in a similar centrifuge tube and diluted to 3 ml. with distilled water. One ml. of 1 N hydrochloric acid[5] was added to the diluted urine sample, and free salicylamide was extracted with 25 ml. of benzene. The amount of the free salicylamide was determined thereafter by the same procedure as described for the measurement of total salicylamide.

RESULTS

Urinary excretion of salicyamide glucuronide in case of the administration of varied amounts of salicylamide to a normal subject was estimated and indicated in Table 1. With the increase in the amount of salicylamide administered, not only the amount of salicylamide glucuronide excreted in urine but also the

4) The liberation of salicylamide from the salicylamide glucuronide contained in the urine sample, following the hydrolysis with β-glucuronidase, was almost complete even in 2 hours of incubation under the present conditions. On the other hand, in the case of the incubation of a similar sample without the addition of β-glucuronidase, the liberation of salicylamide was not observed even in 5 hours of incubation. No sulfatase activity was demonstrated in the β-glucuronidase used in the present assay.

5) The amount of salicylamide extracted with benzene from the urine sample with the addition of 1 N hydrochloric acid was equal to that extracted from the urine sample in 3 N hydrochloric acid, while the hydrolysis of conjugated salicylamide was not involved at all in the process of extraction from the urine with the addition of 1 N hydrochloric acid.

Table 1. Total salicylamide, free salicylamide, and salicylamide glucuronide excreted in urine following each administration of varied amounts of salicylamide to a normanl subject

Salicylamide Administered (mg.)	100	300	1000	2000
Total salicylamide excreted (mg.)	85	259	831	1724
Free salicylamide excreted (mg.)	0	0	0	0
Salicylamide glucuronide excreted (mg.)	22	98	527	1202
G/T* (%)	25.9	37.9	63.4	69.7

$$* \ G/T = \frac{\text{Salicylamide glucuronide excreted}}{\text{Total salicylamide excreted}} \times 100$$

The amounts of total salicylamide and salicylamide glucuronide are each expressed as mg. of the salicylamide liberated by each hydrolysis.

ratio of the salicylamide glucuronide to the total salicylamide was increased. The results of a preliminary experiment indicated that following the administration of 1 g. of salicylamide a normal subject excreted urinary salicylamide more than sixty per cent of the administered salicylamide in 5 hours, more than eighty per cent in 10 hours, and a far less amount thereafter. As shown in Table 2, the patients with liver disease also excreted more than eighty per cent of the administered salicylamide into the following 10-hour urine. No free salicylamide was detected in the urine in all of these cases.

The amount of the salicylamide liberated following the hydrolysis of the conjugated salicylamide in the urine sample with β-glucuronidase was almost constant even when more than 80 units of β-glucuronidase were added to the sample and the mixture was incubated more than 5 hours at 37°C. The total recovery of the administered salicylamide from the 10-hour urine was not affected by the volume of the urine. The ratio of the salicylamide glucuronide in the 10-hour urine to the total salicylamide in the same urine, (G/T), was not also affected by the total recovery and the urine volume.

The G/T ratios in 5 normal controls were 54 to 64 per cent. Those in postnecrotic cirrhosis were slightly lower, while those in Dubin-Johnson syndrome were slightly higher. To some of these patients, 5 per cent glucose solution was intravenoulsy administered as a curative drug immediately before the salicyamide glucuronide cxcretion test. The G/T ratios in the patients treated with the glucose all remained above the lower range of the normal and appeared to be slightly higher than those in the patients who were not treated with the glucose. On the other hand, the administration of glucuronic acid preparations failed to cause such an increased excretion of the glucuronide. There was not an apparent relationship between the G/T ratio and the result of routine tests for liver function.

Table 2. Urine volume, total salicylamide, free salicylamide, and salicylamide glucuronide of 10-hour urine following the administration of 1 g. of salicylamide in normal subjects and the patients with liver disease

Clinical Diagnosis	Patient	Urine Volume	Salicylamide Excreted			
			total	free	glucuronide	G/T*
		ml.	mg.	mg.	mg.	%
Normal control	K. T.	380	831	0	527	63.4
	S. H.	360	862	0	541	62.8
	Y. N.	156	840	0	527	62.7
	K. K.	126	860	0	461	53.6
	T. H. †	390	873	0	491	56.2
Acute viral hepatitis	· H. T. §	255	886	0	479	54.1
	· H. N. †	226	887	0	521	58.7
	S. N. §	395	911	0	582	63.9
	· S. N. †	220	888	0	591	66.6
Acute exacerbation of chronic viral hepatitis	· A. I.	430	814	0	495	60.8
	S. Y. †	240	862	0	352	40.8
Chronic viral hepatitis	K. I.	490	836	0	511	61.1
	S. H.	250	897	0	512	57.1
	M.T.	210	862	0	515	59.7
	D. S. §	490	928	0	549	59.2
	T. H. †	270	851	0	542	63.7
	Y. S.	290	889	0	478	53.8
	· K. Y.	240	888	0	540	60.8
Postnecrotic cirrhosis	Y. I.	360	912	0	463	50.8
	K. T.	430	851	0	358	42.1
	T. K.	650	850	0	358	42.1
	· G. T.	490	907	0	531	58.5
	· U. K. †	740	888	0	511	57.5
	T. Y.	218	937	0	571	60.9
Dubin-Jonson syndrome	· S. M.	223	888	0	622	70.0
	H. M.	345	899	0	647	72.0

$$* \ G/T = \frac{\text{Salicylamide glucuronide excreted}}{\text{Total salicylamide excreted}} \times 100$$

The amounts of total salicylamide and salicylamide glucuronide are each expressed as mg. of the salicylamide liberated by each hydrolysis. ·, case with an intravenous infusion of 500 to 1000 ml. of 5 per cent glucose solution immediately before this test; §, case with daily oral administration of 2 g. of sodium glucuronate as a curative drug; †, case with daily oral administration of 2 g. of glucuronamide as a curative drug.

DISCUSSION

In the analysis of salicylamide glucuronide, the degree of the enzymatic hydrolysis was a problem. Since in the present study the hydrolysis of salicylamide glucuronide with β-glucuronidase was constant and reproducible, the amount of salicylamide glucuronide measured by the enzymatic hydrolysis was considered as equal to the amount of the whole salicylamide glucuronide actually contained in the urine sample, or even if not, as proportional to the whole salicylamde glucuronide. Hence, at any rate, the amount of salicylamide glucuronide determined by the enzymatic hydrolysis could be used as a measure of

glucuronide formation *in vivo*.

The amount of total salicylamide excreted in the 10-hour urine sample was almost constant, ranging from 800 to 950 mg., without reference to the volume of the urine sample. The G/T ratio was also neither affected by the urine volume nor by the amount of the total salicylamide excreted. Accordingly, the possible effect of renal funtion on the G/T ratio was considered not to be involved to an appreciable extent. In this connection, the G/T ratio was considered to serve as representing the capacity of glucuronide formation *in vivo* without reference to renal function.

The evidence in the present experiment that no free salicylamide was detected in the urine following the administration of salicylamide disagreed with the results obtained by other workers[6,9]. Since, however, free salicylamide was extracted with benzene from a weak acid medium to the same degree as from the strong acid medium, it was rather convincing that in the human subjects tested no free salicylamide was excreted in the urine following the administration of 1 g. of salicylamide.

MANDEL and others[6] reported that when 300 mg. of C^{14} salicylamide was administered to the patient with advanced carcinoma of the cervix and with normal liver function, most of the urinary metabolites was accounted for as the etherial glucuronide and no other metabolites, except the etherial sulfate in a negligible amount, were excreted. In the present study, however, the glucuronide portion of the total salicylamide excreted in the urine was only 25.9 per cent when 300 mg. of salicylamide was administered to a normal subject, and it rather increased in accordance with the increase in the amount of salicylamide administered. This result indicated that when a smaller amount of salicylamide was administered, the salicylamide preferred to form the other conjugate than the glucuronide, possibly the sulfate, rather than to form the glucuronide, and when a larger amount of salicylamide was administered, the glucuronide conjugation was increased compensatory. Accordingly, it was considered that it was necessary to administer more than 1 g. of salicylamide in order to estimate the capacity of glucuronide formation. This was further supported by the result in a previous report[10] that the glucuronyl transferase activity of liver tissue correlated significantly with the G/T ratio obtained following the administration of 1 g. of salicylamide.

The result that the patients with postnecrotic cirrhosis indicated lower G/T ratio than that in normal subjects was pertinent with the result of BARNIVILLE and MISK[4]. The other patients with viral hepatitis indicated the G/T ratio within the normal range, and thus this salicylamide glucuronide excretion test was not so sensitive as to be utilized as a liver function test.

SUMMARY

An attempt was made to establish the method for the estimation of glucuronide formation *in vivo* using salicylamide and further to study the alteration in the glucuronide formation in liver disease. The results were as follows:

1. The method for the determination of free salicylamide separately from other conjugates of salicylamide in urine, without involving any hydrolysis of the other conjugates, was presented. When 1 g. of salicylamide was administered to the subjects with or without liver injuries, no free salicylamide was detected by the present method in the urine following the salicylamide administration.

2. The analytical method for the determination of salicylamide glucuronide was also devised by employing a hydrolysis with β-glucuronidase. The ratio of the salicylamide liberated by the enzymatic hydrolysis of the 10-hour urine following the administration of 1 g. of salicylamide to the total salicylamide excreted in the same urine was neither affected by the total recovery of the salicylamide nor by the urine volume. This ratio was thus used as a means of estimating the capacity of the glucuronide formation *in vivo*, although it was considered that the ratio might be affected to some extent by the competition between the glucuronide and other conjugate formations *in vivo*.

3. As a result of this salicylamide glucuronide excretion test, it was indicated that the *in vivo* formation of salicylamide glucuronide in the patients with postnecrotic cirrhosis was slightly decreased compared with that in normal controls.

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