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122. Morizo Ishidate and Mitsuo Watanabe: Separatory Determination of Glucuronic Acid and Glucuronides in Biological Fluid by Ion-Exchange Resin.

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A method for the microdetermination of glucuronic acid and its conjugated derivatives in biological materials had been reported.[1] In this method, a weakly alkaline sample is passed through the anion exchange resin, Amberlite IRA-411, to allow adsorption of glucuronic acid and its conjugates, the resin is washed with water to desorb other sugars and impurities, and eluted first with hydrochloric acid to remove glucuronic acid, and then with methanolic hydrochloric acid to remove the stable conjugate, ether-type conjugated O-glucuronide, and the amount of each in the effluent is determined by color reaction with carbazole. In the living body, glucuronic acid is present as labile conjugates such as the ester-type O-glucuronides, and N-glucuronides, as well as the free glucuronic acid and stable ether-type conjugated O-glucuronides. In the foregoing method, these labile conjugates would be eluted with the free glucucronic acid by hydrochloric acid and the method would not be appropriate for separatory determination of free glucuronic acid and these labile conjugates.

The methods used in the past for separatory determination of free glucuronic acid and its conjugates were decomposing the free acid chemically, determining the conjugates alone, and calculating the amount of free glucuronic acid from the difference in the amount of total and conjugated glucuronic acids. For the decomposition of free glucuronic acid, there were various methods such as oxidation with iodine[2] or bromine,[3] reduction with sodium borohydride,[4] and decomposition with sodium hydroxide.[4,5] Oxidation with iodine and bromine, and decomposition with sodium hydroxide can be used for separatory determination of free acid and stable O-glucuronides but not for that of free acid from the ester-type and N-conjugated glucuronides. Reduction with sodium borohydride can be used under certain conditions for separatory determination of free acid from the ester-type and comparatively stable N-glucuronides, such as sulfapyridine N^4-glucosiduronate,[6] but not for labile N-glucuronides.

The present writers attempted separatory determination of free glucuronic acid, N-glucuronides, and O-glucuronides by improving the previous ion exchanger method.[1] As a sample, representative O-glucuronides and comparatively labile N-glucuronides were prepared and used.

The samples used were sodium aniline N-glucosiduronate[7] and sodium 2-naphthylamine N-glucosiduronate[8] as N-glucuronides, and phenyl O-glucosiduronic acid[9] and potassium menthyl O-glucosiduronate as O-glucuronides.

The fact that O-glucuronide is not desorbed from the resin by hydrochloric acid, thereby separated from free glucuronic acid, indicates that conjugates have stronger

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1) M. Ishidate, S. Takitani, T. Nakajima: Yakugaku Zasshi: **79**, 843(1959)
2) W. H. Fishman, S. Green: J. Biol. Chem., **215**, 527(1957).
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affinity to the resin than the free acid, while the reason why N-glucuronides are eluted with free glucuronic acid is because N-glucuronides are easily hydrolyzed in acid medium and elution with hydrochloric acid changes N-glucuronides to free acid, thereby eluted with free glucuronic acid. Therefore, attempt was made for elution with 0.25*N* ammonium chloride solution, in place of hydrochloric acid, adjusting its pH to 8 at which the N-glucuronides were considered to be most stable.

A column of anion exchange resin was first eluted with 20 cc. of 0.25*N* ammonium chloride solution (pH 8) to remove the free acid and the column was then eluted with 10 cc. of *N* hydrochloric acid to decompose and remove N-glucuronides. The column was further eluted with 20 cc. of *N* methanolic hydrochloric acid to remove O-glucuronide. By this means, as far as the present samples were concerned, separatory determination of free glucuronic acid, and N- and O-glucuronides was effected (Table I).

Table I. Results with Samples

	No. 1. Free glucuronic acid			No. 2. N-Glucuronide (glucuronic acid equivalent)			No. 3. O-Glucuronide (glucuronic acid equivalent)		
	Calcd.	Found (γ)	(%)	Calcd.	Found (γ)	(%)	Calcd.	Found (γ)	(%)
S. 1 Sodium glucuronate	320	320	(100.0)	0	18		0	12	
	666	638	(95.7)	0	20		0	10	
S. 2 Sodium aniline N-glucosiduronate	0	16		243	235	(96.7)	0	0	
	0	4		382	376	(98.4)	0	0	
	0	0		382	376	(98.4)	0	12	
	0	20		370	361	(97.5)	0	15	
S. 3 Sodium 2-naphthylamine N-glucosiduronate	0	16		478	478	(100.0)	0	10	
	0	16		478	473	(98.9)	0	12	
S. 4 Phenyl O-glucosiduronic acid	0	0		0	14		391	371	(94.8)
	0	8		0	15		431	420	(97.4)
S. 5 Potassium menthyl O-glucosiduronate	0	0		0	25		403	400	(99.2)
	0	38		0	0		403	388	(96.2)
Mixture: S.1+S.2+S.4	638	644	(100.9)	271	271	(100.0)	431	422	(97.9)

For the determination of glucuronic acid in each of the eluted fraction, a modified method of Dische's colorimetry using carbazole[10] was employed. In the original method of Dische's, the sample is heated with sulfuric acid, then carbazole is added to this mixture, and this is allowed to stand for gradual coloration. In the present experiments, carbazole was added with sulfuric acid to the sample and the whole was heated to effect coloration, by which the period required for the whole procedure was shortened. The original method required about 3 hours from the beginning of heating to measurement but the present improved method can be completed within 30 minutes. Although there is a slightly more interference from other sugars in the present method, the sugars would have been removed in the preliminary procedure and their interference is considered to be negligible. The absorption curve of the colored solution has a maximum at 520 mμ (Fig. 1) and the wave length of 520 mμ was used for colorimetry. The optimal concentration range of glucuronic acid for the measurement is 10～60 γ/cc. (Fig. 2).

An aliquot of 1 cc. was taken from each of effluents obtained by elution with ammonium chloride and hydrochloric acid, while 2 cc. of methanolic hydrochloric acid effluent was evaporated to dryness under a reduced pressure and the residue was dissolved in 1 cc. of *N* hydrochloric acid to make the sample solution.

Experiments were then made using human urine which was basified by addition of sodium hydroxide solution and 1 cc. of this solution was added with sodium glucuronate

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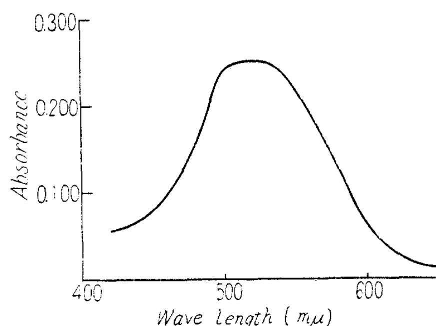

Fig. 1. Absorption Spectrum of the Colored Solution

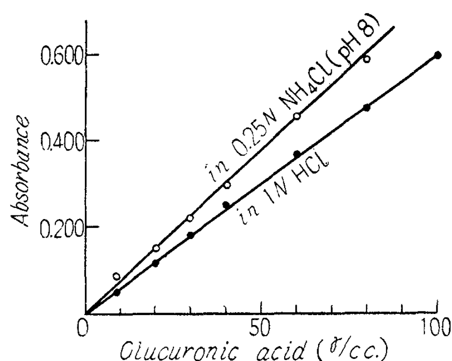

Fig. 2. Standard Curve (Wave length: 520 mμ)

From carbazole and 40 γ of glucuronic acid in 1 cc. of 1*N* HCl by the modified procedure. A mixure of 1 cc. of sample solution, 5 cc. of conc. H_2SO_4, and 0.2 cc. of 0.2% dehyd. EtOH solution of carbazole, mixed with ice cooling, was heated for 15 min. at 100°, cooled in water for 5~10 min., and absorbancy measured with Beckmann spectrophotometer.

as free glucuronic acid, sodium aniline N-glucosiduronate as N-glucuronide, or potassium menthyl O-glucosiduronate as O-glucuronide to examine the amount of the substances recovered. In any of these cases, recovery was quantitative (Table II). Determination procedure with the urine effluent without addition of carbazole was found to effect coloration only with the effluent of methanolic hydrochloric acid and, therefore, a control solution of the same effluent without addition of carbazole was used.

Table II. Results of Recovery Test in Human Urine

	No. 1. Free glucuronic acid			No. 2. N-Glucuronide (glucuronic acid equivalent)			No. 3. O-Glucuronide (glucuronic acid equivalent)		
	Calcd. (γ)	Found	(%)	Calcd. (γ)	Found	(%)	Calcd. (γ)	Found	(%)
Human urine 1 cc. (A drop of *N* NaOH added)	—	50		—	7		—	120	
S. 1 added (400 γ)	450	458	(102.0)	7	7		120	140	
S. 2 added (333 γ)	50	60		340	332	(97.5)	120	135	
S. 5 added (342 γ)	50	60		7	18		462	440	(93.5)

It is considered that the present method will not be suitable for samples containing a large amount of ester-type conjugated O-glucuronides since they will not be separated from other conjugates and they might undergo decomposition when the sample solution is basified.

Experimental

1) **Separation**—Anion Exchange Resin: About 100 cc. of Amberlite IRA-411 (20~50 mesh, DVB 4%) was extracted with methanolic HCl for ca. 1 hr. in a Soxhlet extractor to remove colored material, the resin was packed in a column, and washed thoroughly with water. The column was washed with ca. 500 cc. of 4*N* NaOH to change the resin into OH-type and again washed with water. The column was then washed with 500 cc. of 4*N* HCl to change the resin back to its Cl-type and the resin was washed thoroughly with water until Cl^- was no longer detected in the effluent.

0.25*N* NH_4Cl solution (pH 8): One cc. of 28% NH_4OH was added to a solution of 13.3 g. of NH_4Cl (special grade) dissolved in distilled water and the whole was diluted to 1000 cc. with distilled water.

Methanolic HCl: A 44-cc. portion of conc. HCl (special grade) was diluted to 500 cc. with MeOH.

Procedure: About 3.5 cc. of Amberlite IRA-411 was packed in a column (7 mm. internal dia-

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meter, 150 mm. long) provided with a stop cock, and 1~2 cc. of the sample solution basified to weak alkalinity with 1 drop of *N* NaOH was passed through the column. The column was washed with 50~60 cc. of water and eluted with 0.25*N* NH_4Cl solution (pH 8) at the rate of 0.5~1 cc./min., and exactly 20 cc. of the effluent (Test solution No. 1) was collected. The column was again washed with 20~30 cc. of water and eluted with *N* HCl at the rate of ca. 1 cc./min., and 10 cc. of the effluent (Test solution No. 2) was collected exactly. The column was then eluted with *N* methanolic HCl solution warmed to ca. 50°, at the rate of 0.5 cc./min., and exactly 20 cc. of the effluent was collected (Test solution No. 3).

2) **Determination**—Carbazole : Commercial carbazole was dissolved in conc. H_2SO_4 with cooling, the solution was poured into water, and the solid was collected. After removal of colored substances, it was recrystallized from dehyd. EtOH or toluene.

Carbazole Solution : The purified carbazole was dissolved in dehyd. EtOH to make a 0.2% solution.

Procedure : One cc. each of the test solution No. 1 and No. 2 was placed in separate test tubes. Test solution No. 3 (2 cc.) was evaporated to dryness under a reduced pressure by heating on a boiling water bath and the residue was dissolved in 1 cc. of *N* HCl.

To each of these test solutions, under ice cooling, 5 cc. of conc. H_2SO_4 and 0.2 cc. of the carbazole solution were added, shaken thoroughly, and tubes were stoppered tightly. The tubes were heated in a boiling water bath for 15 min., cooled in water for 5~10 min., and absorbance of each solution was measured at 520 mμ with a Beckman spectrophotometer. The values so obtained represent the amount of free glucuronic acid from test solution No. 1, that of N-glucuronide from test solution No. 2, and that of O-glucuronide from test solution No. 3.

One cc. of 0.25*N* NH_4Cl solution (pH 8) for No. 1, and 1 cc. of *N* HCl for No. 2 and No. 3, were treated as for the sample and used as the references.

In case of urine samples, the following three blanks were prepared for test solution No. 2 and No. 3.

(A) Real blank : 1 cc. of *N* HCl treated as for the test solution without addition of the carbazole solution.

(B) Unknown blank : 1 cc. of *N* HCl treated as for the test solution.

(C) Carbazole blank : Test solution No. 3 treated as usual, without addition of carbazole solution.

Absorbance was measured using (A) as the reference. Readings of (B), (C), test solution No. 2, and No. 3 are represented by *b*, *c*, *x*, and *y*, respectively.

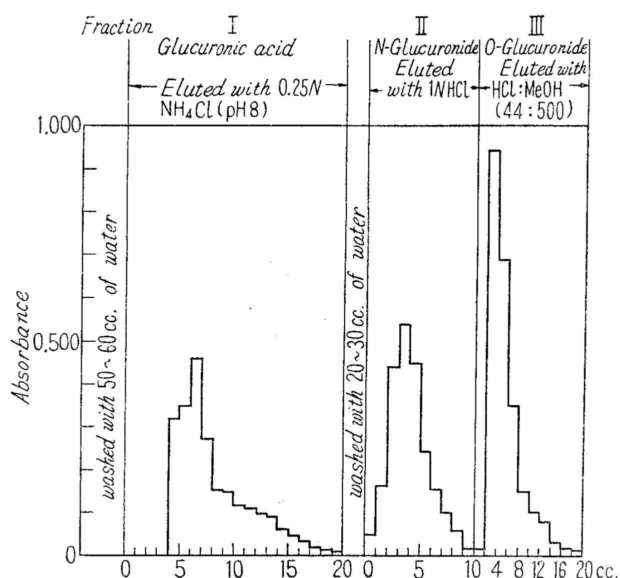

Fig. 3

Chromatogram of Glucuronic Acid and its Conjugates by Anion Exchange Resin

Sample : Sodium glucuronate (as free glucuronic acid)
Sodium aniline N-glucosiduronate (as N-glucuronide)
Potassium menthyl O-glucosiduronate (as O-glucuronide)
Each 400 γ (glucuronic acid equivalent)

Resin : Amberlite IRA-411 3.5 cc.

Fractions I and II : 1 cc. used.

Fraction III : 2 cc. each is evaporated in vaccum and 1 cc. of HCl added.

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Value of N-glucuronide corresponds to $x-b$.
Value of O-glucuronide corresponds to $y-b-c$.

3) **Elution Curve**—Sodium glucuronate as free glucuronic acid, sodium aniline N-glucosiduronate as N-glucuronide, and potassium menthyl O-glucosiduronate as O-glucuronide, at the rate of 400 γ each calculated as glucuronic acid, were adsorbed on the anion exchange resin and the separation procedures were carried out. The effluents were collected in 1-cc. fractions for test solution No. 1 and No. 2, and in 2-cc. fractions for test solution No. 3, and submitted to colorimetric determination. The elution curves thereby obtained are indicated in Fig. 3.

Summary

For the separatory determination of free glucuronic acid and conjugated glucuronic acids of various types, such as N-glucuronides and ester-type or ether-type O-glucuronides in urine, a revised method using a column of anion-exchange resin was investigated. A column of Amberlite IRA-411, after passing a mixed solution of sodium glucuronate, sodium aniline or 2-naphthylamine glucosiduronate, and phenyl glucosiduronic acid or potassium menthyl O-glucosiduronate, was first eluted with ammonium chloride solution at pH 8 to separate the free glucuronic acid, and then eluted with hydrochloric acid to hydrolyse and remove the N-glucuronide. The column was further eluted with methanolic hydrochloric acid to remove the stable O-glucuronide.

For the colorimetric measurement of glucuronic acid in each of eluted fractions, a modified procedure of Dische's method using carbazole was employed, in which carbazole was added with sulfuric acid to the sample in order to save the time required for the whole procedure. By this improved method, so far as the present samples were concerned, the separatory and recovery determination of glucuronic acid, N-glucuronide, and O-glucuronide, excluding ester-type O-glucuronide, in urine, was quite satisfactory.

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