Simple Modified Colorimetric Method for Total Salicylate Assay in Urine after Salicylate Administration

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Abstract □ A simpler and yet sensitive colorimetric method, modified after Levy's method for the assay of total urinary salicylate, is presented. The values of salicylate recovery after oral administration in humans, based on the present method and Trinder's reagent method, are compared.

Keyphrases □ Salicylates, total—modified colorimetric method for assay in urine □ Colorimetry—analysis, total salicylates in urine

Measurements of urinary salicylate excretion have been used to study the bioavailability of salicylates such as aspirin and sodium salicylate from their dosage forms. Two analytical methods are most often employed: *(a)* Trinder's reagent method (1–4), and *(b)* Levy's modification (5–9) of the method of Smith *et al.* (10).

The first method is simpler than the second. It merely involves the measurement of absorbance at 540 nm of the mixture of 1 ml of urine sample and 5 ml of Trinder's reagent. The absorbance is then corrected for the value due to blank urine taken before drug administration. However, it suffers from two major drawbacks. First, the urinary blank contribution not due to absorbed salicylates may vary markedly during the day (11). As a result, this analytical method may underestimate or overestimate the salicylate excretion. For example, one subject was estimated to excrete 159 mg of sodium salicylate in 24 hr even if no salicylate was ingested. Second, Trinder's reagent does not react with the glucuronide metabolites of salicylic acid and underreacts with salicyluric acid, a major metabolite, when compared with salicylic acid on an equimolar basis (10). Consequently, this method tends to underestimate the total salicylate excretion if the blank contribution remains constant during the study. The method is further complicated by the fact that the relative composition of excreted aspirin metabolites may vary markedly with dose and time during the study (8).

In Levy's modified method, 3 ml of the urine sample and 3 ml of concentrated hydrochloric acid are heated in a sealed 50-ml (5) or 20-ml (6) glass ampul at 100° for 16 hr to hydrolyze completely all metabolites to salicylic acid. An aliquot of the hydrolyzed sample is then accurately removed, acidified, and extracted with 30 ml of ethylene chloride (5) or carbon tetrachloride (6) in a glass-stoppered bottle. The salicylic acid from an aliquot of the organic solvent phase was assayed colorimetrically after complexing with ferric ion in the acidic medium. In this method, the total salicylate, which includes aspirin, salicylic acid, and all other metabolites, is analyzed. Since essentially all absorbed aspirin or sodium salicylate can be recovered from the urine after oral administration (6, 8, 12), this method is obviously better than the Trinder's reagent method for studying the total bioavailability of salicylates from dosage forms.

This article reports a simpler and yet sensitive modified procedure for assaying the total salicylate in urine specimens. The values of urinary salicylate recovery after oral administration of aspirin based on the Trinder's reagent method and the presently proposed method also are compared. The reported studies are relevant, especially in light of the facts that the salicylates are the most widely used drugs today and that their adequate bioavailability from dosage forms is critical, particularly for patients on extensive and chronic medication (13).

EXPERIMENTAL

Chemicals—The following reagent grade chemicals were used: sodium salicylate[1], hydrochloric acid[2], chloroform[3], mercuric chloride[3], and ferric nitrate[4].

Analytical Procedure—Concentrated hydrochloric acid (2 ml) was added to 3 ml of urine samples in a screw-top Pyrex culture tube[5] or screw-top disposable culture tube[6]. After sealing the tubes with plastic caps[6], they were incubated in an oven at 100° for 17 hr or 65° for 1 week.

After cooling, 0.5 ml of approximately 6 *N* hydrochloric acid (prepared by dilution of concentrated hydrochloric acid with an equal volume of distilled water) and 6 ml of chloroform were added. The tubes were shaken[7] for 10 min. They were then centrifuged for 5 min in a regular clinical centrifuge[8], and the aqueous phase was aspirated. Three milliliters of the chloroform layer was then accurately transferred to another screw-top culture tube. Six milliliters of Trinder's reagent (1) without mercuric chloride (11) was added. The tubes were shaken for 10 min and centrifuged. The chloroform phase was aspirated, and the absorbance of the aqueous layer was measured[9] at 540 nm. Trinder's reagent without mercuric chloride was used for 100% transmittance adjustment. To calculate the concentration of total salicylate in urine specimens, a standard curve was constructed using known concentrations of sodium salicylate (0.125, 0.25, 0.5, 1.0, and 2 mg/ml) in distilled water and the same incubation and extraction procedure. All assays were run in duplicate. Reproducible results were obtained throughout the study.

Blank Urine Analysis—The intrasubject variation of the urine blank value was studied in six healthy subjects between 22 and 34 years old. No drugs were taken for at least 1 week before the

[1] Fisher.
[2] J. T. Baker.
[3] Mallinckrodt.
[4] Matheson, Coleman and Bell.
[5] Fisher No. 14-932A, 125 × 16 mm, 16-ml size.
[6] Fisher No. 14-959-36A.
[7] Using an Eberbach shaker.
[8] Centricone, G. C. A. Corp., Chicago, Ill.
[9] Using a Beckman DBGT spectrophotometer.

Table I—Absorbances of Blank Urine Samples at 540 nm

Sample	Subject 1	2	3	4	5	6
			Day 1			
Before breakfast	0.015	0.000	0.006	0.000	0.000	0.019
Breakfast–lunch	0.010	0.005	0.005	0.000	0.006	0.017
Lunch–supper	0.008	0.010	0.005	0.000	0.004	0.015
Supper–bedtime	0.010	0.005	0.005	0.000	0.002	0.015
			Day 2			
Before breakfast	0.010	0.004	0.005	0.000	0.000	0.015
Supper–bedtime	0.010	0.010	0.01	0.025	0.004	0.005
			Day 3			
Before breakfast	0.009	0.001	0.005	0.01	0.000	0.01
Supper–bedtime	0.008	0.008	0.004	0.01	0.01	0.015
Mean	0.010	0.005	0.006	0.01	0.003	0.014
$\pm SEM$	0.001	0.001	0.001	0.003	0.001	0.002
Mean as milligrams equivalent aspirin per milligram urine sample	0.009	0.005	0.006	0.009	0.003	0.014

study and during the study. On the 1st day, urine samples were collected before breakfast, between breakfast and lunch, between lunch and supper, and between supper and bedtime. On the 2nd and 3rd days, only the urine samples before breakfast and between supper and bedtime were collected for analysis. The subjects were instructed to have normal food and fluid intake. The urine specimens were kept at about 5° until analyzed (within 1 week).

Aspirin Absorption Study in Humans—Two commercial 300-mg (5-gr) buffered aspirin tablets were administered before breakfast with 200 ml of lukewarm water to two healthy subjects (ages 22 and 34). Urine samples were collected at 3, 6, 12, and 24 hr after administration. The volumes were recorded and portions of samples were stored in culture tubes. The samples were analyzed for salicylate content by both the proposed method and the Trinder's reagent method. In both methods, correction was made for the contribution due to blank urine collected before drug administration.

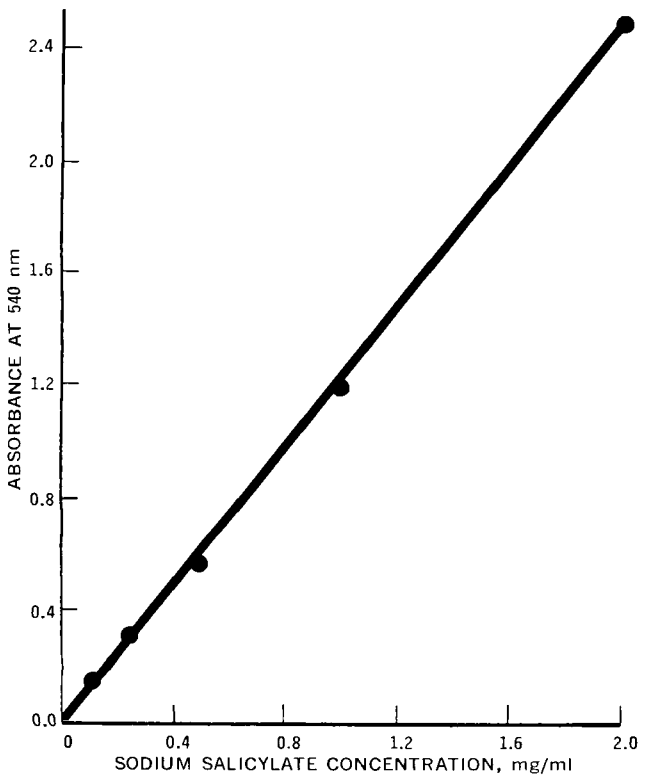

Figure 1—*Standard curve of sodium salicylate solutions.*

RESULTS AND DISCUSSION

The standard curve using the proposed procedure is shown in Fig. 1. The absorbances of the final solutions were proportional to the original concentrations of sodium salicylate. It can be calculated that 1 unit of absorbance corresponds to 0.84 mg/ml of sodium salicylate or 0.945 mg/ml of aspirin in the original sample. Based on the salicylate dose usually administered, this assay sensitivity is believed adequate to quantitate accurately the total amount of salicylate excreted in the urine. Although Levy's modified method has been used successfully in many studies (5–9), its sensitivity does not appear to have been reported. It must be emphasized that this investigation was not intended to challenge its accuracy or sensitivity but merely to explore another simpler and yet sensitive procedure for the assay of total salicylate in urine.

As reported previously (1), the absorbance of the salicylic acid–ferric-ion complex resulting from the reaction of salicylic acid with Trinder's reagent could vary markedly with the pH of the solution. Therefore, some precaution should be exercised in preparing Trinder's reagent (without mercuric chloride) and a standard curve should be established for each lot of the reagent prepared. (The prepared reagent was found to be stable for several months when stored at room temperature in a light-resistant, tight container.) Although significantly less of the organic solvent, chloroform, was used in the present method as compared to the ethylene chloride or carbon tetrachloride used in the previous methods (5, 6), the extraction efficiency of salicylic acid from the aqueous phase to the organic phase was quite high (91%). Moreover, chloroform is more commonly available in most laboratories and is probably less toxic. Therefore, it appears that this solvent should be preferred. The extraction of salicylic acid from the chloroform to the Trinder's reagent phase was found to be 65% complete, which is reasonably satisfactory in view of its high solubility in the organic solvent.

In developing an analytical method, the blank contribution should be of major concern. The results of the blank urine study on six subjects are shown in Table I. It is evident that the intra- and intersubject variation in blank readings was consistently low

Table II—Urinary Salicylate Excretion (Milligrams) in Terms of Equivalent Aspirin Analyzed by Two Methods

Time Interval, hr	Trinder's Reagent Method		Proposed Method	
	Subject 1	Subject 2	Subject 1	Subject 2
0–3	74.6	156.0	129.4	178.7
3–6	100.0	145.7	135.3	194.4
6–12	115.8	152.2	182.9	216.9
12–24	74.6	0	113.1	13.2
Total	365.0	453.9	560.7	603.2

as compared to the results obtained from the Trinder's reagent method (11). One can estimate that the average blank contribution from the subjects is equivalent to 7.7 mg aspirin/day, assuming 1 liter of daily urine output. This value is extremely low when compared to the usual dose of salicylates ingested. The individual blank values using Levy's modified method were reported to be less than 24 mg salicylic acid/day (5). The inter- and intrasubject variation in blank contribution, however, was not reported previously.

The screw-top culture tubes used in the present method are obviously more convenient and easier to use than the ampuls used in the previous methods. The sealing of the ampuls requires extra work, facility, and skill. Since the filled ampuls have to resist pressure when heated at 100° for 17 hr, incomplete sealing results in the loss of sample and makes the study invalid. On the other hand, tightening of a screw-cap on a culture tube can be accomplished in seconds without failure and requires no training and extra facility. Furthermore, the culture tubes and caps can be reused. Although there was some darkening of the rubber liner in the screw-cap after repeated use, it did not interfere with the assay sensitivity. The sorption, if any occurred, of aspirin or salicylic acid onto the screw-cap was found to be negligible. The evaporation of samples from the sealed tubes was also negligible, even after several days of incubation at 100°. Identical results were obtained with screw-caps with liners coated with Teflon, which were much more expensive than the regular caps. The presently proposed method also eliminates one step of the transfer of an aliquot of the hydrolyzed sample. Moreover, the sample in the ampul would presumably have to be poured into another container before the quantitative transferring of the hydrolyzed sample, and this would require additional work.

In the previous methods, an extraction process involving 30 ml of organic solvent and subsequent centrifugation for phase separation in a large enough glass-stopper bottle requires more careful manipulation than the extraction process performed in a small culture tube with a screw cap. Furthermore, the centrifugation in the present method can be carried out by the more common and less expensive clinical centrifuge, making the study possible in a laboratory with only a small budget. Due to its simplicity and sensitivity and the need for less facility, the present method was adopted in the undergraduate biopharmacy laboratory experiment for the bioavailability evaluation of commercial aspirin dosage forms. The preliminary results of bioavailability study on five commercial buffered aspirin dosage forms in human subjects employing the proposed assay method were published previously (12).

The urinary salicylate excretion in terms of equivalent aspirin after dosing of two 300-mg (5-gr) aspirin tablets in two subjects is shown in Table II. The ratios of values obtained from the Trinder's reagent method and proposed method were found to vary with time and subjects. In Subject 1, the value of estimated salicylate recovery in 24 hr based on the Trinder's reagent method was only 65% of that based on this method. In Subject 2 the relative recovery was 75.2%. These data indicate that the Trinder's reagent method, while simple, may significantly underestimate the extent of salicylate absorption from dosage forms. They also confirmed the pitfalls of using this method as already discussed.

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