[Chem. Pharm. Bull. 30(1) 358—361 (1982)]

Improved Fluorometric Determination of Acetaminophen and Its Conjugates with 1-Nitroso-2-naphthol in Whole Blood and Urine

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(Received June 27, 1981)

An improved fluorometric determination procedure has been established for the determination of acetaminophen and its conjugates, not only in whole blood but also in urine, by means of the reaction with 1-nitroso-2-naphthol to give a fluorescent substance.

Keywords——fluorometric determination; acetaminophen; acetaminophen glucuronide; acetaminophen sulfate; 1-nitroso-2-naphthol; *p*-aminophenol; whole blood; urine

In the previous paper,[1] we presented a fluorometric assay method for acetaminophen and its conjugates in whole blood by means of an oxidation procedure with potassium ferricyanide. Though the method was successfully applied to whole blood samples, its application to urine samples was not successful because of the high blank values of the fluorescence.

In 1964, Umeda[2] reported that acetaminophen react with 1-nitroso-2-naphthol in the presence of nitric acid and nitrite to form a colored product, 10-acetamido-5*H*-benzo[*a*]phenoxazine-5-one. Based on this reaction, Kaito *et al.*[3] developed a fluorometric determination method for the drug in preparations and Sugiyama *et al.*[4] applied the method of Kaito *et al.* to the determination of the drug in biological samples. The method of Sugiyama *et al.*, however, requires a time-consuming extraction procedure and the details of the procedure have not been reported. In the present study, an improved fluorometric assay which permits the determination of the drug and its conjugates in both whole blood and urine was established by making several modifications to the procedure published by the previous investigators.

Experimental

Materials——Acetaminophen was of J.P. grade. All other reagents were of reagent grade and were used without further purification.

Acetate Buffer (pH 5.0)——A solution of 0.1 M acetic acid was adjusted to pH 5.0 with 0.1 M sodium acetate solution.

Borate Buffer (pH 9.0)——A solution containing equal volumes of 0.1 M boric acid and 0.1 M KCl was adjusted to pH 9.0 with 0.2 N NaOH.

Standard Solution of Fluorescein (0.01 and 0.03 μg/ml)——Fluorescein was dissolved in borate buffer quantitatively in a stepwise manner to obtain solutions having concentrations of 0.01 and 0.03 μg/ml.

1-Nitroso-2-naphthol Solution (0.01%)——1-Nitroso-2-naphthol was dissolved in ethanol.

Sodium Nitrite Solution (0.1%)——One hundred mg of sodium nitrite was dissolved in 10 ml of purified water and diluted with ethanol to adjust the total volume to 100 ml.

β-Glucuronidase Solution——A commercial preparation (Tokyo Zoki Co., Tokyo, Japan, from calf liver, approximately 13000 Fishman U/ml)[5] was diluted 6 times with acetate buffer.

β-Glucuronidase/Arylsulfatase Solution——A commercial preparation [Boehringer Mannheim GmbH, West Germany, from *Helix pomatia*, approximately 5.2 U/ml (with phenolphthalein monoglucuronide as a substrate) of β-glucuronidase and 2.6 U/ml (with phenolphthalein disulfate as a substrate) of arylsulfatase] was diluted 40 times with acetate buffer.

Drug-free Whole Blood and Urine——Drug-free whole blood and urine for the preparation of standard curves were taken from rabbits that had received no drug. Blood was withdrawn from an ear vein with a syringe containing 3.8% sodium citrate solution (one-ninth of the volume of blood collected). Urine was taken by bladder catheterization.

Instrumentation——Fluorescence was determined with a Hitachi 512 spectrophotofluorometer.

Assay Procedure

Determination of Free Acetaminophen in Whole Blood and Urine (Method 1)——Purified water was added to biological samples (0.1—0.5 ml of whole blood or 1.0 ml of appropriately diluted urine containing 1—10 μg of acetaminophen) to adjust the total volume to 4.0 ml. After the addition of 2.5 g of NaCl, the mixture was shaken with 10 ml of ether for 10 min. Eight ml of the organic layer was transferred to a glass-stoppered test tube containing 1.5 ml of 0.01 N NaOH solution and the mixture was shaken for 10 min. One ml of the alkaline layer was then transferred to another glass-stoppered tube and 1.0 ml of 1-nitroso-2-naphthol solution, 1.0 ml of $NaNO_2$ solution, and 1.0 ml of 1 N HNO_3 were added successively. The mixture was heated at 60°C for 40 min and cooled to room temperature. After the addition of 10 ml of purified water and 5 ml of $CHCl_3$, the mixture was shaken for 10 min, then centrifuged at 2000 rpm for 5 min. The lower organic layer was transferred to a test tube and dried over 1 g of anhydrous Na_2SO_4 for 20 min, then the fluorescence intensities were measured at the maximum activation and emission wavelengths (467 and 552 nm), taking the fluorescence of 0.01 μg/ml standard solution of fluorescein as 70.

Determination of the Sum of Free and Glucuronidated Acetaminophen (Method 2)——Biological samples as for Method 1 were treated with 0.5 ml of β-glucuronidase solution and an appropriate volume of acetate buffer to adjust the total volume to 3.0 ml. The mixture was incubated at 37°C for 24 h[6] and then 1.0 ml of 5% $NaHCO_3$ solution was added to adjust the pH to about 7.0. After the addition of 2.5 g of NaCl the mixture was shaken with 10 ml of ether for 10 min. Eight ml of the ether layer was transferred to a glass-stoppered tube containing 1.5 ml of 0.05 N NaOH solution and the mixture was shaken for 10 min. The subsequent procedure was the same as for Method 1.

Determination of the Sum of Free, Glucuronidated, and Sulfated Acetaminophen in Whole Blood and Urine (Method 3)——β-Glucuronidase/arylsulfatase solution (0.5 ml) was used for hydrolysis.[6] In other respects, the procedure was the same as for Method 2.

The concentrations of the glucuronide and sulfate were calculated from the results of Methods 1, 2, and 3.

Preparation of Standard Curves——Method 1: One ml aliquots of working standard solutions containing 0, 1, 2, 3, 4, and 5 μg/ml of acetaminophen were run through the procedure of Method 1 in the presence of 0.1, 0.2, 0.3, and 0.5 ml of drug-free whole blood. Blank intensities were low enough to permit the determination of acetaminophen without difficulty and the net fluorescence intensities were practically the same regardless of the volume of drug-free whole blood examined, though a small increase of blank intensities was noted on increasing the volume of drug-free whole blood. Data for the standard curve with 0.3 ml of drug-free whole blood are given in Table I.

Standard curves were also examined in the presence of 1.0 ml of diluted drug-free urine. On diluting drug-free urine over 10 times, the blank fluorescence intensities became low enough for accurate determination; the data for the standard curve are shown in Table II.

Method 2 and 3: Since blank intensities of drug-free whole blood were hardly influenced by enzyme treatment, the standard curve for the conjugates in whole blood agreed well with that for free acetaminophen

Table I. Standard Curve Data for Acetaminophen in Whole Blood

	Acetaminophen added (μg)	Fluorescence mean ± S.D.[a]	Fluorescence/ acetaminophen (μg)
Method 1[b]	1	16.7±2.6	16.7
	2	34.4±0.8	17.2
	3	49.8±6.6	16.6
	4	66.0±4.6	16.5
	5	84.2±5.8	16.8
Method 2[b]	1	17.5±1.2	17.5
	2	33.8±3.0	16.9
	3	50.1±3.1	16.7
	4	65.4±5.6	16.4
	5	86.8±8.2	17.4
Method 3[b]	1	16.5±1.1	16.5
	2	33.2±2.0	16.6
	3	48.4±3.8	16.1
	4	66.1±6.3	16.5
	5	86.3±8.0	17.3

a) Based on 3—5 determinations.
b) Determination was done in the presence of 0.3 ml of drug-free whole blood, taking the fluorescence of 0.01 μg/ml standard solution of fluorescein as 70.

Table II. Standard Curve Data for Acetaminophen in Diluted Urine

	Acetaminophen added (μg)	Fluorescence mean±S.D.[a]	Fluorescence/ acetaminophen (μg)
Method 1[b]	1	17.4±1.2	17.4
	2	34.9±2.5	17.5
	3	48.9±4.3	16.3
	4	70.0±3.7	17.5
	5	87.3±8.0	17.5
Method 2[c]	2	11.0±0.0	5.5
	4	21.2±2.6	5.3
	6	31.0±1.6	5.2
	8	40.8±2.4	5.1
	10	55.6±4.5	5.6
Method 3[c]	2	10.7±0.2	5.4
	4	21.3±4.5	5.3
	6	31.5±1.5	5.3
	8	42.0±4.1	5.3
	10	55.4±4.0	5.5

a) Based on 3—5 determinations.
b) Determination was done in the presence of 1.0 ml of 10 times diluted drug-free urine, taking the fluorescence of 0.01 μg/ml standard solution of fluorescein as 70.
c) Determination was done in the presence of 1.0 ml of 50 times diluted drug-free urine, taking the fluorescence of 0.03 μg/ml standard solution of fluorescein as 70.

in whole blood, as listed in Table I. On the other hand, the blank intensities of drug-free urine after enzyme treatment became so large that the determination of the conjugates in urine was disturbed at the dilution used for the determination of free acetaminophen, suggesting that some glucuronide and/or sulfates in drug-free urine are responsible for the blank fluorescence. By diluting the urine sample over 50 times, the blank value was reduced to a permissible level and the data for the standard curve are shown in Table II.

Results and Discussion

Sugiyama *et al.*, in their attempt to estimate acetaminophen in biological fluids, extracted the drug with ethyl acetate and then evaporated off the solvent. The drug thus isolated was, after dissolving it in ethanol, subjected to the reaction. These procedures are time-consuming and the solid extract is probably contaminated with substances other than acetaminophen. Hence in the present study, acetaminophen was extracted with ether, reextracted with dilute alkali, and then subjected to the reaction. Kaito *et al.* used excess 1-nitroso-2-naphthol for

Table III. Influence of Coexisting *p*-Aminophenol on the Fluorescence of 5 μg of Acetaminophen observed according to Method 1 in the Present Fluorometric Assay[a]

Coexisting *p*-aminophenol (μg)	Fluorescence intensity[b]
0	89.0
0.1	87.3
0.2	88.7
0.5	91.2
1.0	89.2
2.0	88.4
5.0	88.2

a) Determination was done in the presence of 0.3 ml of drug-free whole blood.
b) The fluorescence intensity of 0.01 μg/ml fluorescein in pH 9.0 buffer solution was taken as 70.

the reaction, and consequently removal of the remaining reagent was necessary. We could omit the removal procedure since we used a smaller amount of the reagent. The effect of coexisting *p*-aminophenol (a known metabolite of acetaminophen) on the determination was examined. The results, shown in Table III, indicate no interference with the determination.

The detection limits of acetaminophen and its conjugates in whole blood and urine are summarized in Table IV. The detection limit of acetaminophen and its conjugates in whole blood is as low as 2.0 μg/ml, so that this procedure is a little more sensitive than our previous method. The sensitivity in urine, especially for the conjugates is inferior to that in whole blood, but the present assay method may be of practical value for most cases of determination following administration of acetaminophen to man and animals, because the excretion of the conjugates is generally quite rapid and this results in high urinary concentrations.

Table IV. Detection Limits of Acetaminophen in Whole Blood and Urine by the Present Fluorometric Assay Method

Sample	Method	Maximum volume of sample (A)[a] (ml)	Detection limit	
			Amount (B) (μg)	Concentration (B/A) (μg/ml)
Whole blood	1	0.5	1	2
	2, 3	0.5	1	2
Urine	1	0.1[b]	1	10
	2, 3	0.02[c]	2	100

a) Volume that can be analyzed without disturbance from blank fluorescence intensities.
b) In practice, 1.0 ml of 10 times diluted urine was used.
c) In practice, 1.0 ml of 50 times diluted urine was used.

References and Notes

1) J. Shibasaki, R. Konishi, and K. Yamada, *Chem. Pharm. Bull.*, **28**, 669 (1980).
2) M. Umeda, *Yakugaku Zasshi*, **84**, 839 (1964).
3) T. Kaito, K. Sagara, and Y. Ito, *Bunseki Kagaku*, **25**, 776 (1976).
4) S. Sugiyama, T. Oikawa, N. Chiba, T. Kuwamura, and R. Ito, *J. Med. Soc. Toho, Japan*, **25**, 476 (1978).
5) This enzyme preparation was confirmed to be practically free from sulfatase activity in the previous paper.[1]
6) In the previous study using authentic acetaminophen glucuronide and sulfate as substrates,[1] complete hydrolysis to free acetaminophen was confirmed to occur under the conditions described here.