in 1% AcOH (2.0 ml) was fractionated on a Sephadex G-15 column (2.6×65 cm) as described above. Fractions 40 to 55 (containing the octapeptide) were pooled, and evaporated to dryness *in vacuo*. Paper chromatography revealed the presence of two ninhydrin-positive spots with Rf^1 0.06 (major), 0.23 (minor) and Rf^2 0.04 (major), 0.29 (minor). A solution of the crude product in Waley's solvent was applied to a cellulose powder column (2.6×50 cm) and eluted with the same solvent system. Fractions of 4 ml were collected at a flow rate of 4 ml/15 min and the peptide was located by ninhydrin reaction. Fractions No. 39 to 46 (containing the octapeptide) were pooled, evaporated to dryness and lyophilized. Yield 36 mg (58%), mp 151—158°, $[\alpha]_D^{24}$ −42.3° (c=1.0, H_2O), Rf^1 0.06, Rf^2 0.04, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Glu 1.00, Asp 0.97, Gly 2.00, Ser 1.91, Lys 0.97, Ala 0.97. Amino acid ratios in the AP-M digest: Gln 1.00, Asn 0.90, Ala 1.02, Gly 2.00, Ser 1.96, Lys 0.99.

E-rosette Formation——Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient.[11] The lymphocytes were adjusted to a concentration of 5×10^5/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 5%. Sheep erythrocytes were washed with PBS and a suspension (1×10^6/ml) was prepared. Lymphocytes suspended in GVB^{2+} or FCS (1.0 ml) were incubated for 30 min at 37° with MMS (3 mg) and STF fragments. The lymphocytes were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in GVB^{2+} (1.0 ml). The suspension was mixed with sheep erythrocytes (0.5 ml) and then incubated for 18 hr at 4°. The mixture was centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding three more sheep erythrocytes was determined. Monocytes or polymorphonuclear cells forming rosettes were excluded (Table I).

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Fluorometric Determination of Acetaminophen and Its Conjugates in Whole Blood

Juichiro Shibasaki, Ryoji Konishi, and Keiji Yamada

Faculty of Pharmaceutical Sciences, Nagasaki University[1]

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A fluorometric procedure has been developed for the determination of acetaminophen and its conjugates, not only in blood plasma and serum, but also in whole blood. This method, in principle, is based on a method reported previously for the determination of acetaminophen in preparations, in which the drug is oxidized with potassium ferricyanide to give a fluorescent substance.

The present method has the advantage of being able to determine the drug without hydrolysis to *p*-aminophenol, in contrast to many of the published methods. The detection limit is about 4 μg/ml of acetaminophen in whole blood, which is comparable to that of spectrophotometric assays reported previously.

Keywords——fluorometric determination; acetaminophen; acetaminophen glucuronide; acetaminophen sulfate; *p*-aminophenol; potassium ferricyanide oxidation; determination in whole blood

Although many types of acetaminophen determination procedures in biological fluids are described in the literature, no fluorometric assay has been reported for this drug. We present here a fluorometric assay for acetaminophen and its conjugates in whole blood. This

1) Location: *1–14, Bunkyo-machi, Nagasaki 852, Japan.*

method, in principle, is based on the determination procedure for acetaminophen in preparations reported by Kaito *et al.*,[2] in which acetaminophen is oxidized in a slightly alkaline aqueous medium with potassium ferricyanide to give a fluorescent substance (2,2′-dihydroxy-5,5′-diacetylaminobiphenyl), and the fluorescence is enhanced by the addition of dimethylformamide. In this study, some modifications have been made in order to apply the method to biological samples. In addition, since the previous spectrophotometric determinations were exclusively concerned with blood plasma and serum samples, a further aim of the present study was to establish a method capable of determining the drug not only in plasma and serum, but also in whole blood.

Experimental

Materials——Acetaminophen was of J.P. grade. Acetaminophen glucuronide ($C_{14}H_{17}NO_8$)[3] and potassium acetaminophen sulfate ($C_8H_8KNO_5S$)[4] used as standards in preparing standard curves were synthesized. All other reagents were of reagent grade and were used without further purification.

Standard Solutions——One mg% acetaminophen, 2.2 mg% acetaminophen glucuronide, and 1.8 mg% potassium acetaminophen sulfate solutions were prepared by dissolving the materials in purified water. The concentrations of the latter two compounds are equivalent to 1 mg% acetaminophen.

Borate Buffer (pH 7.5, 0.4 M)——About 1 part of 0.4 M Na_2CO_3 solution was added to 10 parts of 0.4 M boric acid solution containing equimolar KCl.

Acetate Buffer (pH 5.0, 0.1 M)——About 1 part of 0.1 M acetic acid solution was added to 2 parts of 0.1 M sodium acetate solution. The final pH of each buffer was checked with a pH meter.

β-Glucuronidase Solution——A commercial preparation (Tokyo Zoki Co., Tokyo, Japan, from calf liver, approximately 13000 Fishman U/ml) 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.

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

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 means of bladder catheterization.

Assay Procedures

Method 1 (Determination of Free Acetaminophen)

Purified water was added to 0.1—0.5 ml of whole blood sample to adjust the 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 shaken for 10 min. The alkaline layer was then transferred to an evaporating tube as completely as possible, and the remaining ether was removed on a rotary vacuum evaporator for 10 min at room temperature.[5] One ml of the ether-free alkaline solution and 0.5 ml of borate buffer[6] were pippeted into a test tube. After allowing the mixture to stand at 0° for 5 min, 0.5 ml of 0.05% $K_3Fe(CN)_6$ solution was added. After standing at 0° for 5 min again, 1.0 ml of 0.25% ascorbic acid solution and 2.5 ml of dimethylformamide were added. The fluorescence was measured at the maximum activation and emission wavelengths (337 and 425 nm), taking the fluorescence of 0.02 mg% quinine sulfate solution as 100.

2) T. Kaito, K. Sagara, T. Yoshida, and Y. Ito, *Yakugaku Zasshi* **94**, 633 (1974).
3) J. Shibasaki, E. Sadakane, R. Konishi, and T. Koizumi, *Chem. Pharm. Bull.*, **18**, 2340 (1970).
4) A.J. Cummings, M.L. King, and B.K. Martin, *Brit. J. Pharmacol. Chemother.*, **29**, 150 (1967).
5) Random levels of fluorescence intensity were observed when the alkaline layer was subjected to oxidation before the removal of remaining ether.
6) The optimal pH for the oxidation was ascertained to be 8.3—8.5, in accord with the result of Kaito *et al.* In order to bring the pH to the optimum, borate buffer (pH 7.5, 0.4 M) was added.

Method 2 (Determination of the Sum of Free Acetaminophen and Acetaminophen Glucuronide)

A sample of 0.1—0.5 ml of whole blood was treated with 0.5 ml of β-glucuronidase solution and an appropriate volume of acetate buffer to adjust the volume to 3.0 ml. The mixture was incubated at 37° for 24 hr and then 1.0 ml of 5% $NaHCO_3$ solution was added to adjust the pH to about 7.0.[7] 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 test tube containing 1.5 ml of 0.05 N NaOH solution[8] and the mixture was shaken for 10 min. Subsequent procedures were the same as for method 1.

Method 3 (Determination of the Sum of Free Acetaminophen, Acetaminophen Glucuronide and Acetaminophen Sulfate)

β-Glucuronidase/arylsulfatase solution (0.5 ml) was used for hydrolysis. In other respects, the procedure was the same as for method 2.

The concentration of the conjugates was calculated from the results of methods 1, 2, and 3.

Preparation of Standard Curves

Working standard solutions (1.0 ml) containing 0, 2, 4, 6, 8, and 10 μg of acetaminophen or equivalent amounts of the conjugates were prepared by aqueous dilution of standard solutions and run through the procedures described above in the presence of 0.1, 0.3, and 0.5 ml of drug-free whole blood. Standard intensities obtained by methods 1, 2, and 3 for 0.3 ml of drug-free whole blood spiked with 10 μg of acetaminophen or equivalent amounts of

Table I. Fluorescence Intensities[a] determined by the Present Methods for 0.3 ml of Drug-free Whole Blood spiked with 10 μg of Acetaminophen or Equivalent Amounts of the Conjugates[b]

Samples determined	Determination method		
	Method 1 without enzyme treatment	Method 2 β-glucuronidase treatment	Method 3 β-glucuronidase/arylsulfatase treatment
Acetaminophen	104.5	105.5	97.3
	104.8	106.6	97.4
	106.4	112.5	98.9
	mean 105.2	mean 108.2	mean 97.9
Acetaminophen glucuronide	0	103.8	96.5
		104.2	98.3
		106.0	99.5
		mean 104.7	mean 98.1
Acetaminophen sulfate	0	0	94.2
			98.0
			98.7
			mean 97.0

a) The fluorescence intensity of 0.02 mg% quinine sulfate solution was taken as 100.
b) Standard containing 10 μg of acetaminophen should give as much as 5.3 μg/ml of dilute NaOH solution through the double extraction procedure if each extraction is complete ((10 μg × 8/10)/1.5 ml). Thus, 5.3 μg/ml of acetaminophen solution was directly oxidized in the manner described in the text, affording a fluorescence intensity of about 150. Comparing this value with those listed in this table, the recoveries in methods 1 and 2 can be roughly estimated as 70% if the possible quenching effect of some components in whole blood is not taken into consideration.

7) When the incubation mixture was shaken with ether before neutralization, colored materials were also extracted, and interfered with the determination.
8) When 0.01 N NaOH solution was used, as in method 1, the pH of the aqueous layer was shifted so much to the acidic side by the effect of the buffer components incorporated into the ether that the reextraction of acetaminophen became inadequate.

the conjugates are listed in Table I. On increasing of the volume of drug-free whole blood, a slight decrease of net intensities was noted with a small increase of blank intensities. However, linearity was observed over the range of 2—10 μg of acetaminophen or equivalent amounts of the conjugates at a constant volume of added drug-free whole blood.[9] As indicated in Table I, the net intensities of the standards obtained by method 3 were about 10% less than those obtained by Methods 1 and 2,[10] which may be a result of some materials present in the enzyme preparation; however, this was not further investigated. It should be noted that the sulfate is reactive only in method 3, while the glucuronide is equally reactive in both methods 2 and 3, suggesting that the β-glucuronidase preparation used is practically free from arylsulfatase activity.

Results and Discussion

The present fluorometric method permits quantitative determination of 2—10 μg of acetaminophen in whole blood ranging in volume from 0.1 to 0.5 ml. Accordingly, the detection limit is as low as 4.0 μg/ml, which is comparable to the spectrophotometric assays reported previously.[11] Acetaminophen conjugates can be determined in the same manner after enzymatic hydrolysis. In addition, the present methods for whole blood are readily applicable to blood plasma and serum without modification, since they naturally contain less interfering material. Another feature of the present methods is that they do not require hydrolysis of the drug to *p*-aminophenol, in contrast to many of the published methods.

Table II. Effects of Various Amounts of Coexisting *p*-Aminophenol on the Fluorescence of 10 μg of Acetaminophen determined by Method 1 described in the Present Paper[a]

p-Aminophenol added (μg)	Fluorescence intensities[b]
0	105.2[c]
0.1	105.5, 108.3
0.2	102.6, 105.3
0.5	101.4, 102.4
1.0	99.7, 99.9
2.0	96.5, 98.6
5.0	74.8, 76.1

a) The determination was run with addition of 0.3 ml of drug-free whole blood.
b) The fluorescence intensity of 0.02 mg% quinine sulfate solution was taken as 100.
c) From Table I.

Kaito *et al.* have stated that coexisting *p*-aminophenol inhibits the fluorescence of acetaminophen. Since no more details were presented, the inhibitory effect of *p*-aminophenol was reinvestigated and found to be rather large at higher levels, but negligibly small at levels below one-twentieth of the level of acetaminophen, as shown in Table II. It is well-known that the major biotransformation of acetaminophen in man and most animals is conjugation with glucuronic acid and sulfuric acid, while deacetylation is relatively minor. Thus, we consider that the present methods allow the determination of acetaminophen in biological fluids of man and most animals without interference by *p*-aminophenol.

9) Net intensities of standards with 0.5 ml of drug-free whole blood were about 7% less than those with 0.1 ml. It is, therefore, necessary to prepare a standard curve for the particular volume of blood sample determined.
10) In order to determine the sulfate, a standard curve for method 3 should be prepared in addition to the curve for methods 1 and 2 (these give essentially the same standard curve).
11) For example, J.I. Routh, N.A. Shane, E.G. Arrendondo, and W.D. Paul, *Clin. Chem.*, 14, 882 (1968).