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Development and Application of Organic Reagents for Analysis. VIII.[1] Determination of Biological Thiols with a New Fluorogenic Thiol-Selective Reagent, *N*-{*p*-[2-(6-Dimethylamino)-benzofuranyl]phenyl}maleimide

KENICHIRO NAKASHIMA, KEN'ICHI NISHIDA, SHIN'ICHI NAKATSUJI, and SHUZO AKIYAMA*

Faculty of Pharmaceutical Sciences, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852, Japan

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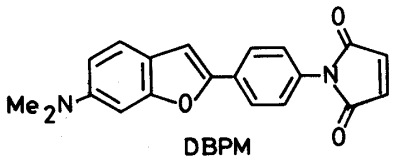
A method for the determination of thiol compounds is described. A new fluorogenic reagent, *N*-{*p*-[2-(6-dimethylamino)benzofuranyl]phenyl}maleimide, has been found to give fluorescent products when reacted with certain biological thiols, *e.g.*, reduced glutathione and cysteine. The reaction is very sensitive, and concentrations as low as 10^{-9} M can be detected. Disulfides such as oxidized glutathione and cystine can also be determined after reduction of the disulfide to thiol with KBH_4. The proposed method has been successfully applied to the determination of total thiol compounds in rat tissues and rat blood.

Keywords——*N*-{*p*-[2-(6-dimethylamino)benzofuranyl]phenyl}maleimide; fluorogenic reagent; disulfide; glutathione; oxidized glutathione; rat tissue; rat blood

Several thiol-selective reagents bearing the maleimide ring have recently been reported.[2–7] For the development of sensitive and useful fluorogenic thiol reagents, we investigated the synthesis of 2-phenylbenzofuran derivatives, which might be expected to be strongly fluorescent because of the presence of a latent *trans*-stilbene skeleton.

Recently, a new fluorogenic reagent, *N*-{*p*-[2-(6-dimethylamino)benzofuranyl]-phenyl}maleimide (DBPM, Chart 1), was synthesized and its application to the determination of reduced glutathione (GSH), as a representative thiol, was investigated.[8] Oxidized glutathione (GSSG) was also determined as GSH after reduction with KBH_4. In the present work, the proposed method was successfully applied to the determination of total thiol and disulfide contents in rat tissues and rat blood.



DBPM

Chart 1. Structural Formula of DBPM

Experimental

Apparatus——The fluorescence spectra and intensities were measured with a Shimadzu RF-540 fluorescence spectrophotometer in 10 × 10 mm quartz cells. A Toa HM-7B pH meter was used for pH measurements.

Reagents——Water was deionized and further distilled. All reagents except for DBPM were of analytical grade, and were not further purified. DBPM was synthesized in our laboratory,[9] and purified by thin-layer chromatography on silica gel plates (Kieselgel 60, Merck) using benzene–chloroform (1 : 1, v/v) as a developing solvent. The product was recrystallized from acetone. DBPM was used as acetonitrile solution.

Standard Procedure for the Determination of GSH, as a Representative Thiol——GSH Assay: A buffer solution (pH 8.5, 0.1 M borate–carbonate, 2.25 ml) and a solution of DBPM in CH_3CN (8×10^{-6} M, 2.5 ml) are added to a

solution (0.25 ml) of GSH ($\leqq 2 \times 10^{-5}$ M) in 20 mM ethylenediaminetetraacetic acid (EDTA). The well-mixed solution is heated for 30 min at 60 °C, then cooled to room temperature, and the relative fluorescence intensity (RFI) is measured at 457 nm with excitation at 355 nm.

GSSG Assay: *N*-Ethylmaleimide (NEM) (4 mM, 0.1 ml) is added to a solution (0.25 ml) of GSSG ($\leqq 1.2 \times 10^{-5}$ M) in 20 mM EDTA. After 10 min, KBH_4 (18%, 0.25 ml) is added to this solution. The solution is heated for 10 min at 60 °C and mixed with metaphosphoric acid (30%, 0.2 ml) for 30 s to decompose excess KBH_4. An aliquot (0.2 ml) of the mixture is subjected to the proposed GSH assay method.

Pretreatment of Biological Materials——Male Wistar rats (7—8 weeks old) were anesthetized by intraperitoneal injection of Nembutal^R (pentobarbital sodium solution, Dainippon Pharmaceutical Co., Ltd., Japan). Various organs (liver, kidney, and spleen) were removed and perfused with 0.9% (w/v) NaCl solution. The tissues were each homogenized in 20 mM EDTA solution in a Teflon-glass Potter-Elvehjem homogenizer. The homogenates were individually adjusted to 1% (w/v) for the determination of thiols. Whole blood was collected with a heparinized syringe from the cervical vein. The blood was used after dilution with 20 mM EDTA.

Measurement of Thiols and Disulfides in Biological Samples——An outline of the measurement procedures for both thiols and disulfides is shown in Chart 2.

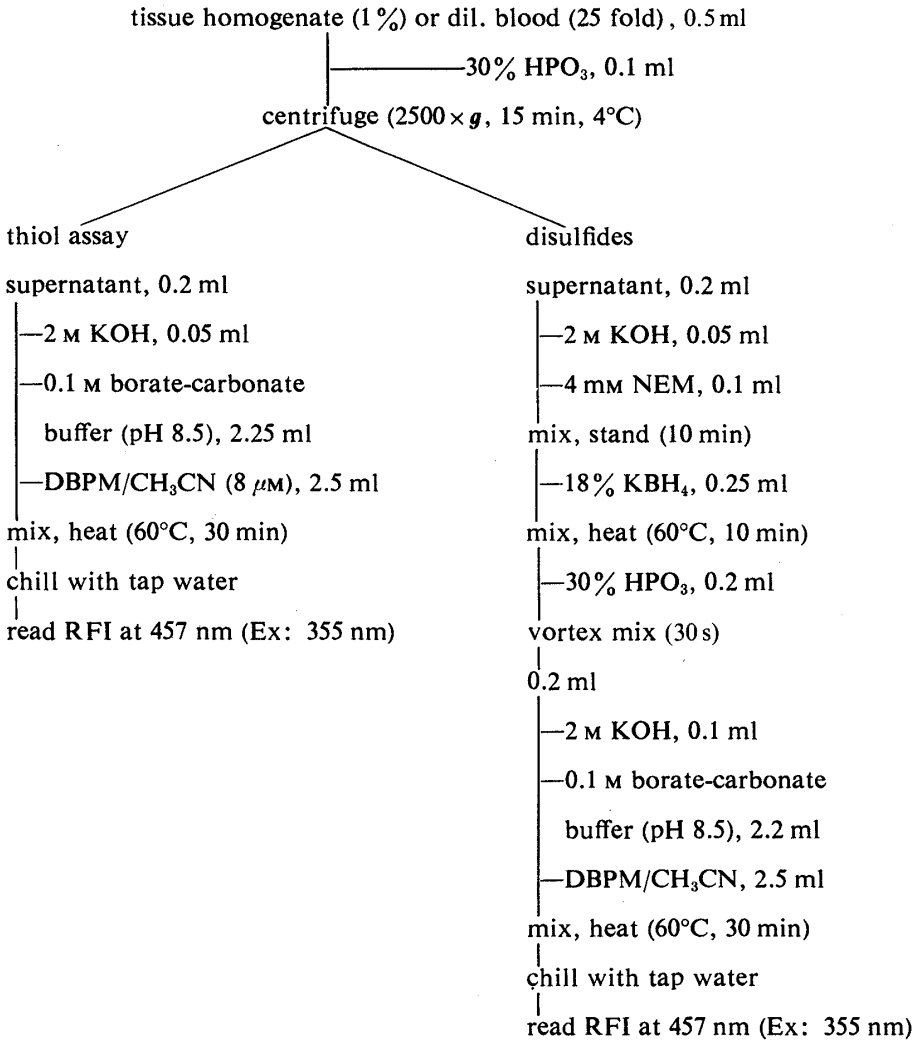


Chart 2. Assay Procedure of Biological Samples

Results and Discussion

Measurement of Thiols

Fluorescence spectra of the reaction mixture of GSH, as a representative thiol, and DBPM are shown in Fig. 1. The excitation maximum was a 355 nm and the emission maximum was at 457 nm (uncorrected). The fluorescence intensity of DBPM itself is quite

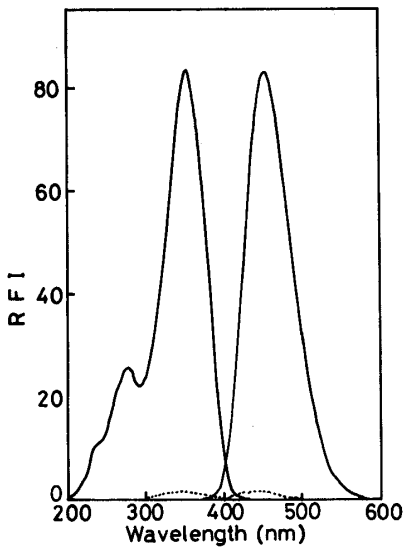


Fig. 1. Fluorescence Spectra of the Reaction Mixture of GSH and DBPM

------, blank. GSH, 2 μM; DBPM, 4 μM.

TABLE I. The Reactions of Thiols and Related Compounds with DBPM[a)]

Compound	RFI
GSH	100
GSSG	13.2
Cysteine	110
Cystine	0
N-Acetyl-L-cysteine	83.8
Methionine	0
Coenzyme A	74.2

a) The reactions and the measurements of the fluorescence intensities were performed according to the procedure described in the experimental section (GSH assay). The final concentrations of the test compounds were 2 μM.

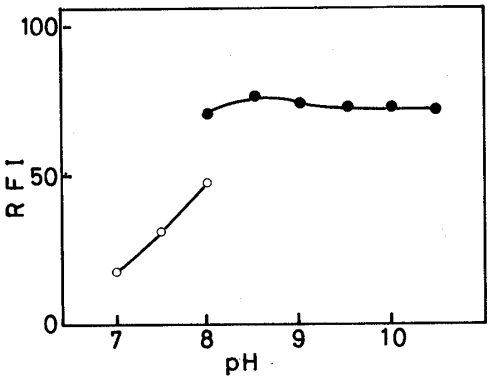


Fig. 2. Effect of pH

—●—, 0.1 M H_3BO_3–KCl–Na_2CO_3 buffer; —○—, 0.1 M KH_2PO_4–Na_2HPO_4 buffer. GSH, 2 μM; DBPM, 4 μM.

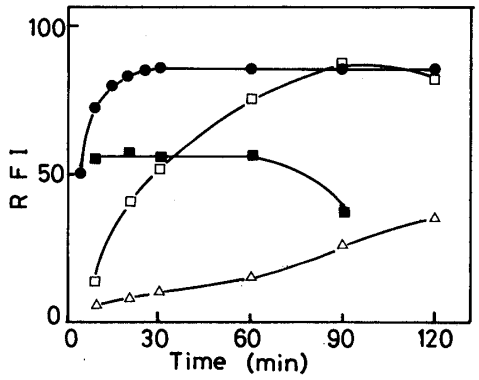


Fig. 3. Effect of the Reaction Temperature and Time

—●—, 60 °C; —□—, 37 °C; —■—, 80 °C; —△—, 20 °C. GSH, 2 μM; DBPM, 4 μM.

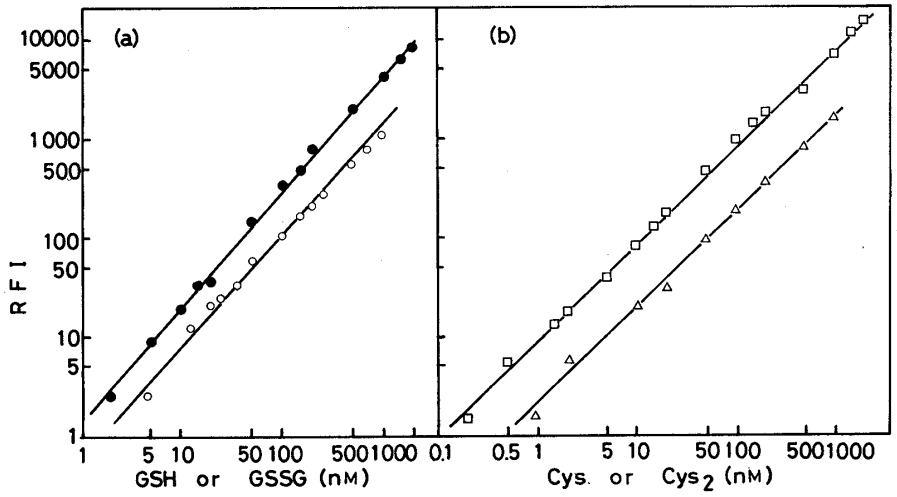


Fig. 4. Standard Curves

(a) GSH (—●—) and GSSG (—○—); (b) Cys (—□—) and Cys_2 (—△—).

small, which is convenient for the measurement of GSH. The effect of pH on the fluorescence reaction was examined and maximal RFI was obtained over the pH range of 8—10.5 (Fig. 2). Next, the effects of the reagent (DBPM) concentration and the solvent (CH_3CN) were examined. Final concentrations of 4 μM DBPM and 50—60% (v/v) CH_3CN gave maximal RFI. The effect of reaction temperature and time were checked, and it became apparent that fluorescence development of GSH with DBPM was almost completed within 20—30 min at 60 °C (Fig. 3). The influence of EDTA concentration was checked in the final concentration range of 1—20 mM. The maximal RFI was obtained with 1—4 mM EDTA. DBPM reacted with various other thiols, as shown in Table I. The fluorescence spectra of these thiols were almost identical with that of GSH. Although most disulfides and sulfides gave no fluorescence upon reacting with DBPM, GSSG exhibited weak fluorescence, due probably to the partial conversion of GSSG into GSH during incubation in the alkaline medium. As shown in Fig. 4, linear relationships were observed between thiol [GSH and cysteine (Cys)] concentration and RFI over the indicated ranges: GSH, 2—2000 nM [relative standard deviation (RSD), 2.4% at the 200 nM level, $n=10$; 0.52%, 25 nM, $n=5$]; Cys, 0.2—2000 nM [2.27%, 200 nM, $n=5$; 2.47%, 2 nM, $n=5$].

Measurement of Disulfides

For the determination of disulfides in biological samples, coexistent thiols should be masked with NEM to prevent their interference with the disulfide assay. GSSG, as a representative disulfide, was used for the examination of the determination conditions. To begin with, the final concentration of NEM was checked in the range of $(7.5—50)\times10^{-6}$ M in the presence of GSH (10×10^{-6} M) and GSSG (1×10^{-6} M). Since GSH was trapped completely at pH 7 by more than 15×10^{-6} M NEM within 10 min at room temperature, 20×10^{-6} M was selected as the optimum. Conditions for reduction of GSSG to GSH were examined, and it was found that GSSG (1×10^{-6} M) could be quite easily reduced to GSH by means of KBH_4 [7.5% (w/v)] within 10 min at 60 °C. The excess of KBH_4 was easily decomposed with metaphosphoric acid [7.5% (w/v)]. The relationships between disulfide

Table II. Recovery Assay

Sample	Amount of sample	Original (nmol)	Added[a] (nmol)	Found (nmol)	Recovery (%)	RSD[b] (%)
Thiols						
Liver	2 mg	14.0	7.5	21.8	104.0	0.4
			15.0	28.8	98.8	1.3
Kidney	2 mg	4.9	7.5	12.6	102.4	2.5
			15.0	18.9	93.5	1.7
Spleen	2 mg	8.2	7.5	15.9	104.3	1.1
			15.0	22.8	97.4	3.8
Blood	10 μl	10.3	7.5	18.1	104.5	0.1
			15.0	24.7	95.6	2.8
Disulfides						
Liver	15 mg	6.5	4.0	10.7	105.6	1.0
			12.0	18.5	100.3	1.0
Kidney	2 mg	1.2	4.0	5.4	103.5	2.5
			12.0	13.6	103.2	1.2
Spleen	2 mg	0.8	4.0	5.2	118.8	4.1
			12.0	12.7	99.4	2.7
Blood	10 μl	1.1	4.0	4.7	91.5	3.8
			12.0	13.1	98.5	3.3

a) Cys or Cys_2 was added to kidney; GSH or GSSG was added to other samples. b) $n=5$.

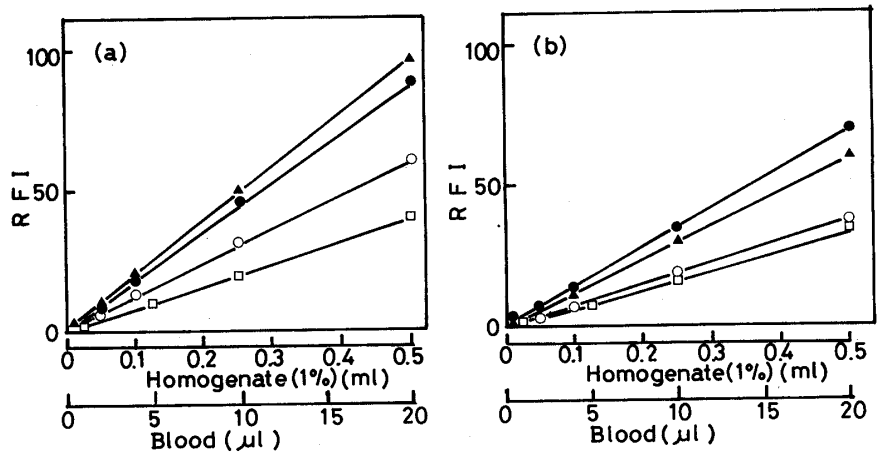


Fig. 5. Relationships between Sample Size and RFI

(a) thiols; (b) disulfides. —●—, liver; —▲—, spleen; —○—, kidney; —□—, blood. Sensitivity of the fluorometer: thiols, liver and kidney (Low × 32), spleen (Low × 64), blood, kidney, and spleen (High × 2).

TABLE III. Concentration of Thiols and Disulfides in Biological Materials

Sample	Thiols[a] (Mean ± S.D.)	Disulfides[b] (Mean ± S.D.)	n
Liver	6.30 ± 1.18 μmol/wet g	0.54 ± 0.18 μmol/wet g	5
Kidney	3.65 ± 0.79 μmol/wet g	0.30 ± 0.15 μmol/wet g	5
Spleen	3.64 ± 0.63 μmol/wet g	0.61 ± 0.08 μmol/wet g	4
Blood	0.95 ± 0.13 μmol/ml	0.19 ± 0.01 μmol/ml	3

a) Standard: Cys (kidney); GSH (other samples). *b*) Standard: Cys_2 (kidney); GSSG (other samples).

[GSSG and cystine (Cys_2)] concentration and RFI were linear over the indicated ranges: GSSG, 5—1000 nM (RSD, 2.37% at the 200 nM level, $n=5$; 2.50%, 20 nM, $n=5$); Cys_2, 1—1000 nM (2.38%, 200 nM, $n=5$; 2.31%, 10 nM, $n=5$) (Fig. 4).

Measurement of Total Thiols in Rat Tissues and Blood

The proposed method has been successfully applied to the determination of total thiol content in rat tissues and rat blood. The optimum conditions for the assay of biological samples were investigated as follows. Various deproteinizing agents (EtOH, CH_3CN, trichloroacetic acid, and metaphosphoric acid) were examined with rat liver homogenate [1% (w/v)] and rat blood. Metaphosphoric acid was selected as a superior agent and the final concentration of 5% metaphosphoric acid was used as the optimum. Next, recovery yields were estimated. As an index, a known amount of a specific thiol or disulfide abundantly found in each tissue was added to the respective assay samples: Cys or Cys_2 for kidney and GSH or GSSG for other tissues and blood. Good recovery yields were obtained in all cases as shown in Table II. The relationships between sample size and RFI for thiols and disulfides were linear over 0—0.5 ml for 1% homogenate and 0—20 μl for blood (Fig. 5).

On the basis of these experiments the amounts of thiols or disulfides (as total thiols or total disulfides) in tissues and blood of rat were estimated by the standard addition method (Table III). The values obtained are in good agreement with the reported values.[6,10]

Conclusion

As a result of examination of the fluorometric determination of thiols by means of

DBPM, we have developed a sensitive method for the assay of GSH, GSSG, Cys, and Cys_2. The sensitivity of the method is superior (*ca.* 100 times) to those of the conventional colorimetric methods,[11] and is comparable to that of the fluorimetric method using *N*-(9-acridinyl)maleimide.[6b,12] Our simple and sensitive method was successfully applied to the determination of micro amounts of glutathione peroxidase activity in rat liver, as will be reported in a subsequent paper.[13] Although the present assay does not permit the selective determination of individual thiols, high-performance liquid chromatography of DBPM-thiol derivatives is being explored to provide a means for the determination of each thiol in biological samples.

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