Fluorometric Determination of Glutathione by N-(9-Acridinyl)-maleimide and Its Application to Biological Materials

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A fluorescent reagent, N-(9-acridinyl)maleimide, was studied for its application to the determination of GSH and the total thiol content (crude GSH) in mammalian tissues. The fluorescence was reproducible and stable at pH 8.8. The fluorescence intensity was linear in the range of the GSH concentration from 0.001 nmole/ml to 100 nmole/ml. The total thiol content of the mammalian tissue determined by this method agreed well with that obtained by the DTNB colorimetric method. The sensitivity of the present method was more than fifty times higher than the DTNB method; determination with 0.03~15 mg of tissue was adequate.

Thiol compounds such as GSH, cysteine and ergothioneine are widely distributed among living tissues and involved in the important biological functions. Various analytical methods are available such as the ultraviolet absorption methods with PCMB¹⁾ and NEM,²⁾ the colorimetric method with DTNB³⁾ and the enzymatic method⁴⁾ in addition to the classical methods.⁵⁾ Recently, Hissin *et al.* reported a specific and sensitive fluorometric method for GSH and GSSG assay, which depended on the reaction with *o*-phthalaldehyde at pH 8.0 for GSH or pH 12.0 for GSSG.⁶⁾ The fluorometric method is one of the most sensitive analyses and seems useful in the field of biological and medical sciences.

Since Friedman *et al.*⁷⁾ reported the quantitative reaction of NEM with thiol compounds under a mild condition, several N-substituted maleimide derivatives have been synthesized for the colorimetric purpose⁸⁾ and applied for the modification of proteins.⁹⁾ Recently, Kanaoka *et al.* developed several N-substituted maleimide reagents^{10~12)} which had no fluorescence but revealed strong fluorescence by

the coupling with thiol compounds. They applied these reagents for the modification of proteins.^{13,14)} They also suggested a possibility of the application of the reagents to the micro-determination of thiol compounds in biological materials.¹⁵⁾

In this paper we describe the application of N-(9-acridinyl)maleimide (NAM) to the micro-determination of GSH and related substances in biological materials. NAM is a N-substituted maleimide-type reagent^{16,17)} which has negligible fluorescence, but shows strong blue fluorescence by coupling with thiol compounds. Application of NAM has been reported¹⁸⁾ as a spray reagent for the detection of GSH, cysteine and related thiol compounds on thin-layer chromatography.

MATERIALS AND METHODS

Reagents and equipments. NAM was synthesized in our laboratory.¹⁷⁾ NAM was used as freshly prepared acetone solution. GSH and other thiol compounds were commercial reagents of high purity and were used without further purification or drying. Unless otherwise noted, the 0.5 M Na₂CO₃-H₃BO₃-KCl, pH 8.8 was used as buffer solution.

Fluorescence spectra and fluorescence intensities were measured with a Hitachi 204 Fluorescence Spectrophotometer (slit width 10 nm) equipped with a Hitachi Recorder 056. All the fluorescence spectra were uncorrected. The volume of the quartz cells used was

Abbreviations: NEM, N-ethylmaleimide; NAM, N-(9-acridinyl)maleimide; PCMB, *p*-chloromercuribenzoate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GSH, glutathione; GSSG, glutathione oxidized; EDTA, disodium ethylenediaminetetraacetate; TCA, trichloroacetic acid.

10×10×44 mm. Fluorescence measurement was carried out at 435 nm excited at 360 nm.

Preparation of biological materials. Rats (6~7 months of age) and mice (2~3 months of age) were anesthetized by ether. Blood was collected from the heart with a heparinized syringe. Livers and spleens were blotted and weighed. The perfusion with saline of the liver was not carried out. They were frozen and stored at -20°C until used for the assay.

Just before assay, 500 mg portion of the tissue was weighed and homogenized in the presence of 0.02 M EDTA with a glass homogenizer cooled in ice. The homogenate was made up to 5.0 ml, to which 1.0 ml of 0.02 M EDTA and 1.5 ml of 25% TCA were added. The homogenate was transferred to a centrifuge tube, and was centrifuged at 4000 rpm for 15 min at 4°C to precipitate proteins. Six ml of the supernatant was neutralized with 1 N NaOH. This solution was diluted to appropriate concentrations for assay.

To an 1.0 ml aliquot of heparinized whole blood, 11.0 ml of 0.02 M EDTA and 3.0 ml of 25% TCA were added. It was centrifuged at 4000 rpm for 15 min at 4°C. Eleven ml of this supernatant was neutralized with 1 N NaOH. For NAM assay, 0.1 ml of this supernatant solution was sufficient, whereas 1.0 ml was needed for the colorimetric method with DTNB.

Human urine was collected freshly for every determination. Two ml of the urine was diluted with 8.0 ml of 0.02 M EDTA. A 0.5 ml aliquot of the solution was used for assay by the NAM method. For the DTNB method, to 40.0 ml of the urine, 6.0 ml of 0.02 M EDTA and 14.0 ml of 0.1 M phosphate buffer, pH 8.0 were added. A 3.0 ml aliquot was used for assay.

Unless otherwise noted the procedures were carried out at room temperature.

Analytical procedure for thiol content. A 0.2 ml aliquot of the prepared solution from the tissue homogenate was added to 3.3 ml of the buffer solution. As an internal standard, 0.1 ml of a GSH solution containing 0.02 M EDTA was added. The amount of GSH was about half to two fold of the expected amount of thiols in the sample solution. Then, NAM was added as 0.4 ml of acetone solution. After 1 hr at room temperature, the fluorescence intensity was measured.

RESULTS AND DISCUSSION

Reaction of thiol compounds with NAM

As shown in Table I, NAM reacted with various thiol compounds except certain forms of thiol compounds such as 2-thiouracil and 2-benzimidazolethiol which were slightly soluble in water and existed in the thione form in

TABLE I. THE REACTION OF THIOL COMPOUNDS WITH NAM

To 3.5 ml of buffer, 0.1 ml of each sample solution and 0.4 ml of NAM (1.0×10^{-6} mole) were added. After 1 hr, the fluorescence intensity was measured.

Compound	R.F.I. ^a	Concentration
Glutathione	100.0	5.0×10^{-9} mole/ml
Cysteine	114.1	"
N-Acetylcysteine	80.2	"
Thiomalic acid	29.3	"
Penicillamine	93.0	"
Homocysteine	66.9	"
2-Benzimidazolethiol	0.4	1.3×10^{-7}
2-Thiouracil	7.4	1.0×10^{-7}
Glutathione oxidized	4.5	1.0×10^{-8}
Cystine	0.1	saturated concentration
Methionine	0.7	1.3×10^{-7}
Thiourea	0.0	"

^a Relative fluorescence intensity of the reaction mixture.

aqueous solution.¹⁹⁾ NAM reacted scarcely with another type of sulfur-containing compounds such as GSSG, cystine, methionine and thiourea. Lysine and histidine did not react with NAM under the present analytical condition. Although NEM has been reported to react with the ε-amino group of lysine and the imidazole group of histidine under certain conditions.²⁰⁾

The relative fluorescence yields of water-soluble thiol compounds by the NAM method are shown in Table I.

The rate of the fluorescence development by NAM with thiol compounds was rapid in alkaline solutions and slow in acid solutions. The fluorescence intensity was pH-dependent and stronger at higher pHs than at lower pHs.

The hydrolytic opening of the succinimide ring has been reported to occur in alkaline solutions in the case of the coupling product of thiol with N-[p-(2-benzimidazolyl)phenyl]maleimide.¹⁰⁾ In alkaline solutions, presumably the same phenomenon took place in the coupling products of thiols with NAM. In the case of cysteine, the formation of a thiazane ring compound was observed,¹⁷⁾ although it might have occurred after the hydrolytic opening of the succinimide. Thiazane ring formation is

impossible from the coupling products of GSH or homocysteine, because they do not have a primary amino group at β -position from the sulfur atom.

Determination of GSH by NAM

Fluorescence spectra of the reaction mixture of GSH and NAM are illustrated in Fig. 1. The excitation maximum was at 358 nm and the fluorescence emission maximum was at 438 nm at pH 8.8. These spectra were identical with those of other thiol compounds.

Fluorescence development by GSH with NAM almost completed within 30~40 min at pH 7~9 (Fig. 2). The fluorescence was most stable at pH 8.8 and was constant for more than 24 hr. Therefore, in the determination of biological thiols, the measurement was carried out after the reaction of 1 hr at pH 8.8. The presence of 10% acetone in the reaction mixture did not affect the fluorescence characteristics.

The calibration curve is shown in Fig. 3. Straight linear relationship was obtained in the

FIG. 1. Fluorescence Spectra of NAM-GSH and NAM-Blood.

— NAM-GSH; to 3.5 ml of buffer, 0.1 ml of GSH (5.0×10^{-8} mole) and 0.4 ml of NAM (1.0×10^{-7} mole) were added.

--- NAM-blood; instead of GSH, 0.1 ml of a prepared sample solution of rat blood was added.

..... NAM; GSH or a sample solution was replaced by 0.1 ml of EDTA.

After 1 hr, the fluorescence spectra were measured. The left and right curves express the excitation and emission spectra, respectively.

FIG. 2. Fluorescence Development of the Reaction Mixture of GSH and NAM at Various pHs.

To 7.8 ml of buffer, 0.3 ml of GSH (6.2×10^{-8} mole) and 0.9 ml acetone solution of NAM (6.0×10^{-7} mole) were added. The following buffer systems were used: pH 3.5 (○—○), pH 5.0 (×—×); 1 M HCl-CH₃COONa pH 7.0 (△—△), pH 8.0 (□—□); 0.1 M NaH₂PO₄-Na₂HPO₄, pH 8.8 (●—●), pH 9.2 (■—■); 0.1 M Na₂CO₃-H₃BO₃·KCl.

FIG. 3. Calibration Curves for GSH.

To 4.0 ml of buffer, 0.5 ml of GSH and 0.5 ml of NAM were added. The fluorescence intensity was measured after 1 hr. The final concentrations of NAM were 1.1×10^{-7} , 5.2×10^{-9} and 1.0×10^{-10} mole/ml according to the concentration of GSH. The concentration of GSH: $(1.0 \sim 100) \times 10^{-8}$ mole/ml, +—+; $(1.0 \sim 100) \times 10^{-11}$, □—□; $(1.0 \sim 100) \times 10^{-12}$, ○—○.

range of GSH concentration from 0.001 nmole/ml to 100 nmole/ml.

The blank fluorescence of NAM was small and it was compensated on the measurement. NAM itself was not stable in the aqueous solution. Below pH 7 the fluorescence increased gradually, presumably because of the

protonation of acridine ring as has been discussed for the acridine compounds.²¹⁾ Above pH 8 the fluorescence did not vary, therefore there was no trouble with the measurement due to the variation of blank fluorescence. However the hydrolysis of the maleimide moiety of NAM might have occurred (Fig. 4).

FIG. 4. Variation of Fluorescence of NAM at Various pHs.

To 8.1 ml of buffer, 0.9 ml of NAM (6.0×10^{-7} mole) was added. The buffer systems were identical with those in Fig. 2. The Fluorescence intensity was illustrated about four times sensitive relative to Fig. 2.

Application to biological materials

Since NAM reacts with various thiol compounds, the measurement indicates the total amount of the thiol compounds; the fluorescence intensity somewhat varies with the compounds, and therefore exact measurement cannot be expected. However, the main thiol compound of mammalian tissues and blood is GSH. The content of cysteine, ergothioneine and coenzyme A is less than one tenth.²²⁾ Therefore, the total thiol content can be regarded as crude GSH content. In this study, the total fluorescence was estimated as crude GSH in comparison with standard GSH solutions. The internal standard technique was preferred because the fluorescence intensity might be influenced by various factors.

The total thiol content (C) was calculated as follows:

$$C = C_{st} \times \frac{F_{s\text{amp}} - F_{\text{blank}}}{F_{\text{inst}} - F_{s\text{amp}}} \times D_f$$

C_{st} : the amount of GSH added as the internal standard.

$F_{s\text{amp}}$: the fluorescence intensity of the

sample.

F_{inst} : the fluorescence intensity of the sample added with GSH as the internal standard.

F_{blank} : $F_a + F_b - F_c$, where F_a is the fluorescence intensity of NAM without sample, F_b is the fluorescence intensity of the sample containing acetone without NAM, F_c is the fluorescence intensity of acetone, the solvent of NAM. Blank fluorescence was below 10% of the total fluorescence in this experiment.

D_f : the reciprocal of dilution factor.

The recovery value of GSH added as the internal standard was calculated as follows:

$$\text{Recovery (\%)} = \frac{F_{\text{inst}} - F_{s\text{amp}}}{F_{st} - F_a} \times 100$$

Where, F_{st} is the fluorescence intensity corresponding to C_{st} .

Colorimetric determination was made by the DTNB method³⁾ for comparison. The internal standard technique was also applied.

Fluorescence spectra of the reaction mixtures of NAM and mammalian tissues or blood preparations were identical with those with GSH as shown in Fig. 1. With 0.03~15 mg of liver (Fig. 5), linear relationship was obtained between the fluorescence intensity and the amount of the sample preparation. Fluorescence development by liver homogenate with NAM became constant after 40 min as well as GSH (Fig. 6).

The results of the measurement are listed in Table II. The values coincided with those found by the DTNB method. The reagent addition of 100 fold amount against total thiol concentration didn't disturb the measurement. The recovery of the internal standard was approximately 100%, suggesting the possibility of the application of the calibration curve technique without internal standard. In the case of human urine, higher values were obtained by the NAM method compared with the DTNB method. Since cysteine content is higher than GSH one in urine, GSH internal standard is not suitable. But, the values found

FIG. 5. Linearity of Fluorescence and the Amount of Liver.

An aliquot of a prepared sample solution of mouse liver was added to an appropriate volume of buffer. The total volume of this mixture was 3.6 ml. Then, 0.4 ml of NAM (2.0×10^{-7} mole or 2.0×10^{-8} mole) was added to the homogenates of 1~15 mg of liver or those of 0.03~0.6 mg liver, respectively. The fluorescence intensity was measured after 1 hr.

TABLE II. TOTAL THIOL CONTENT OF BIOLOGICAL MATERIALS

Material and method	Thiol content found ^a	Recovery ^b	Amount of sample provided
Mouse liver	$\mu\text{mole/g}$		
NAM	4.11	100.2%	1.00 mg
DTNB	4.23	99.4	10.0
Mouse spleen			
NAM	1.37	103.3	1.98
DTNB	1.33	101.5	19.8
Mouse blood	mmole/liter		
NAM	0.97	102.8	2.60 μl
DTNB	0.93	106.7	52.0
Rat blood			
NAM	1.11	100.8	5.05
DTNB	1.11	95.0	50.5
Human urine	mmole/liter		
NAM	0.082	98.1	0.06 ml
	0.066 ^c	96.0	
DTNB	0.057	84.7	1.00
	0.056 ^c	87.2	

^a As GSH, on fresh weight basis.

^b Recovery of fluorescence intensity or light absorption of the added internal standard.

^c As cysteine which was used as an internal standard.

FIG. 6. Development of Fluorescence by Liver Preparation and GSH.

x—x, to 6.2 ml of buffer, 1.0 ml of a prepared sample solution of mouse liver and 0.8 ml of NAM (1.1×10^{-7} mole) were added; O—O, to 7.0 ml of buffer, 0.2 ml of GSH (1.2×10^{-8} mole) and 0.8 ml of NAM (1.1×10^{-7} mole) were added.

by the DTNB method were not necessarily true, because the recovery values were somewhat low.

Table III shows the amount of each sample required for the present method. The mini-

TABLE III. AMOUNT OF SAMPLE REQUIRED

Material	Amount of sample ^a	Thiol content found ^b	Recovery ^c
Mouse liver	mg	$\mu\text{mole/g}$	%
	14.3	3.33	99.1
	7.1	3.36	99.7
	1.4	3.33	98.6
	0.7	3.24	99.3
	0.14	3.37	96.0
	0.07	3.26	99.7
	0.01	3.14	100.9
Rat blood	μl	mmole/liter	%
	29.9	1.18	99.8
	16.6	1.13	101.5
	3.3	1.16	102.8
	1.7	1.15	101.2
	0.3	1.08	105.8
Human urine	ml	mmole/liter	%
	0.3	0.042	96.0
	0.1	0.042	99.5
	0.05	0.040	98.1
	0.01	0.041	98.0

^a Fresh weight of the sample corresponding to final fluorescence measurement.

^b As GSH, on fresh weight basis.

^c Recovery of fluorescence intensity of the added internal standard.

mum amount of a sample required for the analysis by NAM method was 0.07 mg for fresh liver and 1.7 μ l for rat blood. On the other hand, the minimum amount of a sample required in the DTNB method was 5 mg for liver. Namely, the sensitivity of the NAM method was seventy times as high as that of the DTNB method. The standard deviation of the mean for these fluorometric measurements (12 samples) was $\pm 1.5 \sim 3.5\%$.

In conclusion, the method presented here is a sensitive and simple method of quantitative analysis of GSH in mammalian tissues. The method is reliable and requires less material than the conventional methods.

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