Determination of Aliphatic Thiols by Fluorometric High-Performance Liquid Chromatography After Precolumn Derivatization with 2-(4-N-Maleimidophenyl)-6-Methylbenzothiazole

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A sensitive, fluorometric high-performance liquid chromatographic method for the detection of aliphatic thiols, following pre-column derivatization with 2-(4-N-maleimidophenyl)-6-methylbenzothiazole, has been developed. The N-maleimide, the acid and the methyl ester derivatives of the commercially available 2-(4-aminophenyl)-6-methylbenzothiazole were synthesized and found to be equally effective for the precolumn derivatization procedure. The resulting fluorescentic derivatives of aliphatic thiols were separated on a reversed-phase column (Ultrasphere-ODS) using 0.1% hexane-sulfonic acid in 10 mM potassium hydrogen phosphate: acetonitrile (65:35) as a mobile phase and were detected fluorometrically (excitation 320 nm; emission 405 nm). The method is highly sensitive (femtomole range) and is easily applied for determination of SH-containing drugs and endogenous thiols in biological samples.

KEY WORDS: N-Maleimidophenylbenzothiazole; fluorometric detection; HPLC determination, thiols.

INTRODUCTION

Several methods are available for the selective determination of thiols. These are based on the unique reactivity of the SH group towards various chemical entities: an oxidation of S-S bond as in Ellmans' reagent (5,5'-dithiobis-2-nitrobenzoic acid) (1-3), a substitution and cleavage of an aziridine ring as in N-dansyl aziridine (4,5) or a reactivity towards a conjugated double bond (6-10). The resulting derivatives are monitored by ultraviolet or fluorescence spectrophotometry. Methods involving enzymatic reactions (11), HPLC with electrochemical detection (12,13) and mass spectrometry (14) for the determination of thiols in biological samples also exist.

Of special interest is the addition reaction to a conjugated double bond, usually in the imide form, of chromophoric maleic acid derivatives. Recently, however, the modified substituted free acid (15,16) as well as its methyl ester (17) were reported as useful fluorogenic derivatizing reagents for thiols. Besides chromophoric maleic (maleamic) acid derivatives, bimanes (18,19), dansyl chloride (20) and 7-fluoro-2,1,3-benzoxadiazoles (21,22), were reported to be useful reagents for derivatizing thiols in HPLC analyses with fluorometric detection.

Other than being selectively attacked by the SH group (in a certain pH range), the advantages of utilizing the substituted maleic moity are due to the lower or absence of fluorescence in the native form of the derivatizing reagent and the strong fluorescence displayed by the addition product upon reaction with SH containing compounds. Unfortunately, with no exception, all existing fluorogenic thiol reagents are unrealistically expensive and hence their use in a routine basis is limited. Moreover, in some cases, the derivatization and detection procedures are nonspecific, prolonged and necessitate the use of complex elution conditions for the separation of the derivatives (20). In other cases, limited sensitivity (12,13) or, even after extensive purification procedures, fluorescentic impurities contained in the reagent interfere with the resolution and detection of certain thiols (8).

The present work describes a facile method for the preparation of 2-(4-N-maleimidophenyl)-6-methylbenzothiazole, its acid and methyl ester derivatives from the commercially available amine precursor. All three derivatives were found to be useful for the fast and sensitive determination of various thiols including glutathione (GSH) and cysteine (Cys) in biological samples.

MATERIALS AND METHODS

Chemicals

2-(4-Aminophenyl)-6-methylbenzothiazole (I, Fig 1) (brown plates) was purchased from Aldrich (Milwaukee, WI) and was recrystallized from chloroform (white prisms) prior to use. Maleic anhydride, acetic anhydride, sodium acetate, hexanesulfonic acid, N-acetyl-*d,l*-penicillamine, penicillamine and captopril were purchased from Aldrich and used as received. Cysteine (Cys), N-acetylcysteine (NAC), cystamine and coenzyme A were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were HPLC grade obtained from Fischer (Santa Clara, CA). Deionized distilled water was used and the mobile phase filtered through a 4 micron filter (Rainin, Emeryville, CA, USA) and degassed under vacuum prior to use.

Instrumentation

The liquid chromatograph consisted of a Shimadzu LC 600 pump and a Shimadzu RF 535 Fluorescence detector (Shimadzu, Tokyo, Japan) operating at excitation and emission wavelengths of 320 and 405 nm, respectively. Samples were either injected using an autosampler (Millipore Corp., Marlborough, MA, USA) or manually using a Rheodyne 20 microliter sample loop (Rainin, Emeryville, CA). Ultra violet (UV) spectra were recorded with a Diode array Spectrophotometer 8452A (Hewlett-Packard, Palo Alto, CA). Fluorescence was recorded on a DM 3000 Fluorometer (SPEX Industries, NJ, USA); NMR spectra were recorded on QE-300 (GE-NMR Instruments, CA).

Synthesis of the Reagents

2-(4-N-Maleimidophenyl)-6-metylbenzothiazole IV, the

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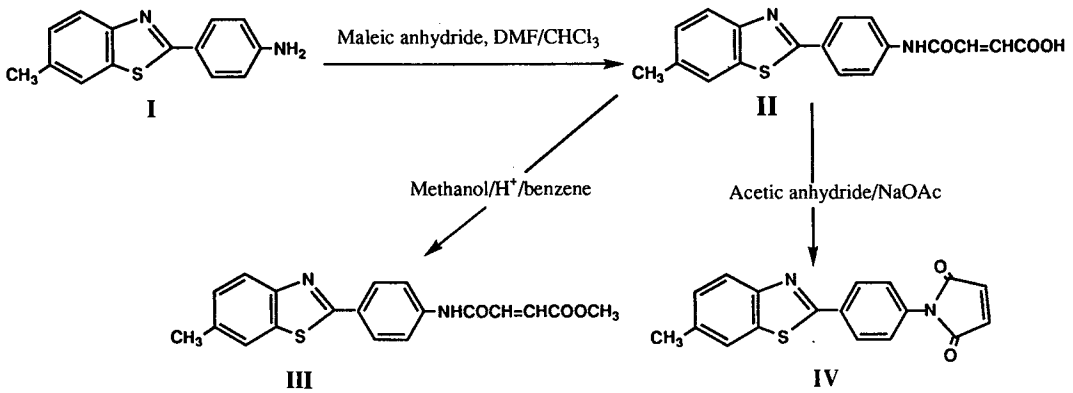

Fig. 1. Synthesis scheme for 2-(4-N-maleimidophenyl)-6-methylbenzothiazole (IV), the acid (II) and the methyl ester (III).

acid II and the methyl ester III, were synthesized following the scheme depicted in Fig. 1.

2-(4-N-Phenylmaleamic acid)-6-methylbenzothiazole (N-[4-(6-methyl-2-benzothiazyl) phenyl] maleamic acid)-II. Maleic anhydride (0.5 g, 5 mmol) in chloroform (2 ml) was added dropwise to a solution of I (1.2 g, 5 mmol) in 10 ml dimethylformamide. The mixture was stirred for 2 hr at room temperature. The deposited crystals were filtered off, washed with 30 ml of chloroform and recrystallized from dimethylformamide to yield II (1.4 g, 82%, yellow crystals). Melting point 242°C (dec.); IR (Nujol, cm^{-1}), C*O*OH 1709 C*O*NH 1610, ^1H NMR (DMF-d_7-TMS) δ (ppm) = 2.49 (3H, s, CH_3–), 6.46 and 6.65 (1H,1H, d, d, J = 12 Hz, –CH=CH–), 7.37–8.14 (7H, m, ArH), $UV_{ethanol}$ λ_{max} = 320 nm, (log ε = 4.04), MS(*m/z*) : 339 (MH^+). Analysis calculated (%) for $C_{18}H_{14}N_2O_3S$: C 63.88, H 4.17, N 8.28, S 9.47; found, C 63.99. H 4.34, N 8.18, S 9.47.

Methyl-N-[4-(6-methyl-2-benzothiazyl)phenyl] maleate-III. A mixture of the acid II (2 g, 6 mmol), 10 ml of methanol and 100 ml of anhydrous benzene in the presence of 2 drops of concentrated sulfuric acid was refluxed for 2 hours. Upon cooling to room temperature, the reaction mixture was extracted with 100 ml of 10% aqueous sodium hydrogen carbonate solution and subsequently washed twice, each with 50 ml of saturated aqueous sodium chloride solution. The benzene layer was dried over anhydrous magnesium sulfate and evaporated *in vacuo* to dryness. The residual solid was crystallized from methanol to yield off white crystals of the methyl ester III, 1.8 g (86%) : m.p. 209–211° C, ^1H NMR ($CDCl_3$ – TMS), δ (ppm) = 2.49 (3H, s, CH_3—), 3.88 (3H, s, $-OCH_3$), 6.24 and 6.44 (1H,1H, d, d, j = 12 Hz, –CH=CH–), 7.24–8.07 (7H, ArH). IR (Nujol, cm^{-1}) C*O*OCH_3 1722, C*O*NH 1669, $UV_{ethanol}$ λ_{max} = 320 nm, (log ε = 4.07), MS(*m/z*) : 353 (MH^+). Analysis calculated (%) for $C_{19}H_{16}N_2O_3S$: C 64.75, H 4.58, N 7.95, S 9.09; found, C 66.71, H 4.70, N 7.77, S 9.05.

2-(4-N-maleimidophenyl)-6-methylbenzothiazole-IV. A mixture of the acid II (2 g, 6 mmol), anhydrous sodium acetate (100 mg, 1.2 mmol) and acetic anhydride, 25 ml, was refluxed for 2 hr. Upon cooling on ice, the deposited crystals were collected and washed with water. The filtrate was neutralized with cold 10% aqueous NaOH and extracted with chloroform which then was dried over anhydrous magnesium sulfate and evaporated *in vacuo* to dryness. The combined product was crystallized from isopropanol to yield yellow needles, 1.4 g (74%) of the maleimide IV: m.p 254–256° C, ^1H NMR ($CDCl_3$ – TMS), δ (ppm) = 2.50 (3H, s, CH_3—), 6.89 (2H, s, –CH=CH–), 7.3–8.18 (7H, m, ArH), IR (KBr, cm^{-1}) C*O*NH 1715, $UV_{ethanol}$ λ_{max} = 315 nm, (log ε = 4.05), MS (*m/z*) : 321 (MH^+). Analysis calculated (%) for $C_{18}H_{12}N_2O_3S$: C 67.47, H 3.78, N 8.74, S 9.90 found, C 67.28, H 3.93, N 8.37, S 9.72.

Chromatographic Conditions

The HPLC apparatus was connected to an Ultrasphere-ODS column (25 cm, 4.6 mm i.d, 5 μ, Beckman, Palo Alto, CA, USA). The samples were eluted isocratically at ambient temperature with 10 mM KH_2PO_4, 0.1% in hexanesulfonic acid (sodium salt): acetonitrile 65 : 35, pH 4.5 for the thiol adducts of reagents III and IV and 70 : 30, pH 5.6 for the adducts of reagent II at flow rates of 1.5 and 1 ml/min, respectively.

Preparation of Standards

Fresh standard solutions of thiols containing 10 nmol/ml of either cysteine (Cys), N-acetyl-*l*-cysteine (NAC), glutathione (GSH), homocysteine, *d,l*-penicillamine, N-acetyl-*d,l*-penicillamine and coenzyme A were prepared by dilution with distilled water of 1 mM solutions of each thiol in a 20 mM EDTA (disodium salt) solution. For determination of GSH and Cys in biological samples, 100 μl of NAC solution (0.05 mM) were added to the sample as an internal standard prior to derivatization. Solutions of each of reagents II, III and IV were prepared by dissolving 0.05 mmol of the reagent in 10 ml dimethylformamide; 1 ml of this stock solution was diluted 25 fold with acetonitrile and 100 μl of the resulting solution was used for the derivatization procedure.

Preparation of Biological Samples

Male Sprague-Dawley rats (300–400 g) were sacrificed by decapitation. The organs (aorta, heart, lung, liver, kidney, testes, spleen, brain and small intestine) were immediately removed and perfused with normal saline. The metabolic reaction in tissue material was quenched by means of the freeze-stop technique (liquid nitrogen, –180°C), and the frozen samples were either stored (–70°C) or pulverized (Tis-

sue Pulverizer, Fisher Scientific, Santa Clara, CA, USA) and processed immediately. To an aliquot of powered tissue, (0.1–0.5 g), four volumes of ice-cold 2 M $HClO_4$, 25 mM in EDTA, were added, vortex mixed quickly and homogenized (Brinkman, NY). Blood samples were collected into pre-cooled EDTA tubes and plasma was separated by centrifugation (4°C) at 4000 g for 5 min. The plasma (1 ml) was treated by addition of an equal volume of ice-cold 2 M $HClO_4$, 4 mM in EDTA, vortex mixed and centrifuged (4°C) at 4000 g for 10 min to remove protein. An aliquot of the deproteinized tissue extract or plasma was neutralized with a predetermined volume of cold 2 M LiOH solution and was individually adjusted with borate buffer pH 8.4 to 1% w/v (brain, lung, liver, kidney, testes, small intestine), 2.5% (aorta, heart and spleen) or 10% v/v (plasma). 100 µl of the resulting solutions were used as the biological samples.

Derivatization Procedure

The following were successively added to screw capped tubes each containing 0.1 ml of a sample (standard or biological), prepared as described above: 0.1 ml of the internal standard solution, 0.1 ml of the reagent solution and 0.7 ml of borate buffer pH 8.4. The tubes were vortex mixed for a few seconds. The mixture was incubated for 60 min after which it was mixed with an equal volume of the mobile phase. 10 µl of the resulting solution was injected onto the chromatograph. In the case of reagent III, after dilution with the mobile phase, the samples were centrifuged at 4000 g for 5 min to precipitate the insoluble unreacted ester and 10 µl of the supernatant was injected.

For the determination of GSH and Cys in biological samples, a calibration curve was constructed by following the same procedure, except that the tissue extract sample was replaced by standard solution containing 0.5-5 nmol each of GSH and Cys.

Recovery Study

Table I shows the summary of the recovery studies performed with reagent IV. Tissue (1 g) were extracted as described above and their GSH and cysteine concentrations were determined following derivatization according to the above procedures before and after the addition of known amounts of standard GSH and Cys (standard thiols were added to tissue homogenate). Freshly separated plasma was diluted 10-fold with reaction buffer and immediately derivatized before and after the addition of known amounts of Cys and GSH. No other suitable blanks for the determination of thiols in biological samples are available since thiols (GSH or Cys or both) are present at different concentrations in all biological material including plasma and urine.

Table I. Recoveries of the Amounts of Glutathione (GSH) and Cysteine (Cys) Added to the Biological Sample Extracts

Sample	Thiol	Amount added (nmol)	Amount (nmol) recovered (±S.D.)	C.V.% (n = 4)
Liver	GSH	5	4.51 ± 0.27	5.9
	Cys	7	7.04 ± 0.30	4.3
Kidney	GSH	7	8.02 ± 0.60	7.5
	Cys	5	5.17 ± 0.51	10.0
Spleen	GSH	5	4.70 ± 0.20	4.2
	Cys	7	6.35 ± 0.26	4.1
Heart	GSH	5	5.12 ± 0.28	5.4
	Cys	5	4.10 ± 0.39	9.5
Plasma	GSH	5	5.21 ± 0.31	6.0
	Cys	7	6.69 ± 0.44	6.6

RESULTS AND DISCUSSION

All three reagents can be utilized to yield sensitive and selective measurements of thiols. We have chosen to describe results preferentially with reagent IV, commenting on differences between this reagent and reagents II and III where appropriate, because most of the existing thiol reagents are commercially available in the imide form resembling the structure of reagent IV. This makes comparison of the current method with other existing HPLC determinations of thiols with fluorometric detection (most of which also involve an imide reactant) more appropriate.

Figure 2 shows a typical chromatogram of the adducts of seven different thiols after derivatization with reagent IV. The conditions of the derivatization reaction were examined using 0.1-1 µM solutions of GSH, NAC and Cys. No pH dependence of the yield of the derivatization reaction for these thiols was observed over pH range 7.8–9.6. Constant peak heights were obtained using a molar ratio as low as 1.2 for the reagent to the total thiols present. The effect of temperature on the yield and duration of the derivatization reaction was also studied. Figure 3 shows the time course for the completion of the derivatization at 80°, 65°C and room temperature at pH 8.4. Increased reaction rates were observed at these temperatures with higher pH values.

For determination of thiols in biological samples, the optimal derivatization conditions were found as incubation at room temperature of the biological sample extract with the reagent at pH 8.4 for 1 hr. Under these conditions, maximal peak heights for both GSH and Cys occurred with no interfering peaks present. When heat was applied, particularly at high pH (*i.e.*>9), the reaction was completed within *ca.* 20 min but was accompanied by unidentified peaks, especially

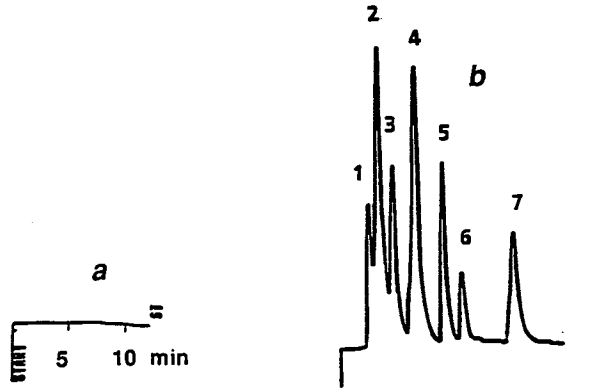

Fig. 2. Chromatograms of reagent IV-thiol derivatives (b) and the regent blank (a). A mixture consisting of 100 µl of each of the standard solutions (10 nmol/ml) of the thiols coenzyme A,1; homocysteine, 2; GSH, 3; NAC, 4; Cys, 5; N-acetyl-*d,l*-penicillamine, 6 and *l*-penicillamine, 7, was treated with reagent IV according to the procedures described in the text.

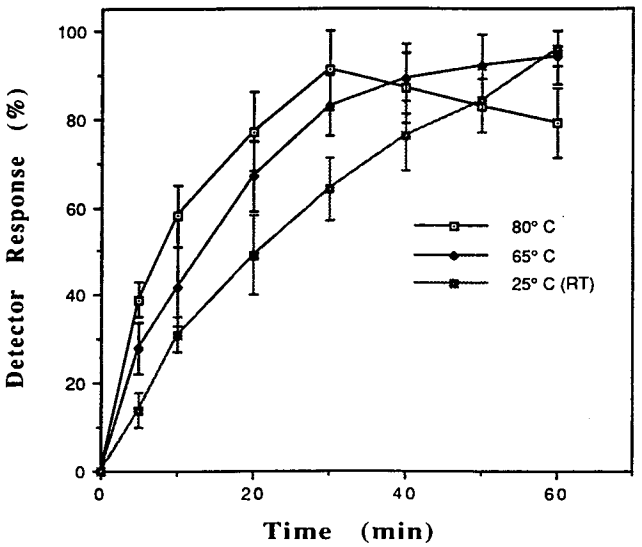

Fig. 3. Dependence of the detector response and the time of completion of the derivatization reaction upon temperature. Reactions were carried out at pH 8.4 with reagent IV in the presence of GSH (0.1-1 μmol) at the specified temperatures. Aliquots of the reaction mixture were taken at different time points and injected directly onto the chromatograph. Each time point represents the mean ± S.D. of 6 different experiments.

when reagent IV was utilized for the derivatization. In the later case, hydrolysis of the imide might have occurred under these conditions, and/or products of the reaction of the reagent with the amino group of other amino acids present in the biological sample may have been formed.

To preclude peak splitting, the mobile phase is maintained at pH 4.5. The effect of the pH of the mobile phase on the separation of the adducts of reagent IV was studied. Figure 4 shows splitting of NAC and Cys adducts peaks at pH>4.8. The peak of GSH adduct shows complete splitting at pH>8.6 when eluted with the same mobile phase at a slower rate (~0.5 ml/min). Other thiols, (*i.e.*, penicillamine

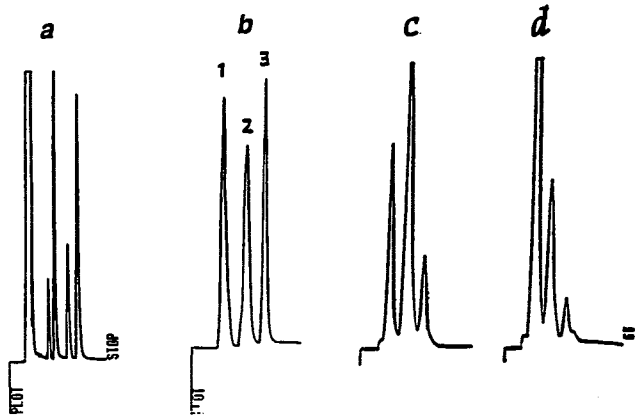

Fig. 4. Chromatograms of the adducts of GSH, 1; NAC, 2; and Cys, 3, with reagent IV when eluted with the same mobile phase at pH 7.4 (a) and pH 4.5 (b) demonstrating splitting of the adducts at basic pH (see text for details). 4c and 4d represent chromatograms of the endogenous thiols GSH, 1; and Cys, 3, following derivatization with reagent IV of rat lung and liver extracts, respectively, using NAC, 2, as internal standard.

and its N-acetyl derivative) also showed splitting at pH 4.8–6.9.

Similar results were obtained when the ester III was used, whereas no splitting occurred under these conditions with reagent II. Best separation of Cys, GSH, NAC, and other thiols adducts within 10 min were obtained at pH 4.5 for reagents III and IV and at pH 5.6 for reagent II. Lowering the pH of the mobile phase to below 4.5 resulted in increased retention times for all of the thiols tested. A similar trend was obtained upon increasing the concentration of the ion-pairing agent, hexanesulfonic acid. Splitting of the derivatization products of thiols with thiol reagents, although not documented previously, is a known phenomenon (14) and is probably due to the separation of the two diastereoisomer adducts obtained upon addition of thiols to the double bond of the reagent.

Amongst various buffering, ion-pairing and organic modifying agents examined, isocratic elution at 1.5 ml/min with 10 mM KH_2PO_4, 0.1% in hexanesulfonic acid: acetonitrile, 65 : 35, at pH 4.5 when derivatized with reagents III and IV (70 : 30 at 1 ml/min and pH 5.6 for reagent II) gave the best separation within 10 min for most thiols tested. Acetate buffers, triethylammonium (14), tetrabutylammonium salts (11) and methanol, ethanol or tetrahydrofuran alone or in combination with acetonitrile as the organic modifier promoted splitting of the adducts peaks even at acidic pH.

Native fluorescentic compounds such as sulfonated aminonaphthalene (11) and saturated naphthoylacrylic acids (14) could be used as internal standards and exhibit linearity over a wide range of thiol concentrations. However, in the current work we utilized nonfluorescentic thiols (NAC or penicillamine) which, upon undergoing the same treatment (reaction with the reagent), yield a fluorescentic product with the same absorption characteristics (excitation and emission), and hence behave as 'true' internal standards. Under the derivatization conditions described, a typical calibration graph of the peak-height ratio of derivatized GSH to derivatized NAC (y) versus GSH concentration (x) was, $y = 0.289x + 0.00274$ ($r = 0.998$, $n = 7$) and was more precisely reproducible than when utilizing non-thiol native fluoroscentic compounds of different chemical and spectral characteristics as internal standards. For the determination of GSH and Cys in biological samples, adding the postderivatized adducts of NAC or penicillamine as internal standards at the end of the reaction of the reagent with the endogenous thiols offered no advantage over utilizing the thiols themselves prior to the addition of the reagent to the sample.

The lower limit of detection at a signal-to-noise ratio of 2 varied from 2 fmole (GSH and coenzyme A) to 20 fmole (penicillamine) with all other thiols tested exhibiting values within this range. The stability of GSH-adducts with the reagents was studied. The results showed increased stability with reagent III (>2 weeks at room temperature) and 3 and 5 days at room temperature with reagents IV and II, respectively. Although not significantly different, the derivatization reaction rate was fastest with reagent IV and slowest with II.

The applicability of the method was tested for the determination of thiols in biological samples. When derivatized according to the procedure described, typical chromatograms are obtained (Fig. 4c and 4d). Table I shows results obtained from recovery studies carried out with this method

Table II. Concentrations of Glutathione (GSH) and Cysteine (Cys) in Various Rat Tissue Extracts and Rat Plasma

Sample	Dilution	Concentration	
		GSH	Cys
Heart	1:40	1.00 ± 0.11 μmol/gram	0.06 ± 0.004 μmol/gram
Liver	1:100	3.65 ± 0.27 μmol/gram	0.33 ± 0.06 μmol/gram
Aorta	1:40	0.38 ± 0.06 μmol/gram	0.17 ± 0.07 μmol/gram
Lung	1:100	1.23 ± 0.11 μmol/gram	0.80 ± 0.03 μmol/gram
Kidney	1:100	N.D**	3.13 ± 0.37 μmol/gram
Intestine	1:100	1.09 ± 0.09 μmol/gram	1.56 ± 0.34 μmol/gram
Testes	1:100	1.37 ± 0.14 μmol/gram	0.29 ± 0.04 μmol/gram
Spleen	1:40	0.69 ± 0.08 μmol/gram	0.73 ± 0.09 μmol/gram
Brain	1:100	1.36 ± 0.12 μmol/gram	0.29 ± 0.05 μmol/gram
Plasma	1:10	11.7 ± 1.1 nmol/ml	19.4 ± 4.6 nmol/ml

* Values indicate the mean ± S.D. (n = 6), **Non-detectable.

using reagent IV. The tissues used for the recovery study were chosen to reflect the diversity of thiol content in biological samples (table II). As shown in table I, when GSH (5 nmol) was added to liver extracts after homogenization, the amount added was accounted for in the recovery study even though the starting liver GSH concentrations (i.e. the subtracted blank) are approximately 700 fold greater (e.g. liver GSH in table II is 3.65±0.27 μmol/gram versus the 5 nmol added to the one gram of liver tissue extract in table I). Similarly, small amounts of GSH and Cys were recovered when added to kidney tissue extracts with no GSH present, but with high Cys content. Similar results were obtained using reagents II and III.

Table II shows GSH and Cys concentrations in various organs of fasted (12 hr) Sprague-Dawley rats as determined by this method. Compared to results in the literature and those obtained in our laboratory for the determination of thiols, especially endogenous thiols, on a routine basis, using a wide variety of commercially available imide chromophores, derivatization of thiols with 2-(4-N-maleimidophenyl)-6-methylbenzothiazol (reagent IV) according to the procedures described here offers the following advantages:

1. The regent is easily prepared from inexpensive, commercially available precursors.
2. The synthetic method is general and can be utilized for preparation of reagents II and III which were found to be as effective and sensitive as reagent IV.
3. None of these reagents require special equipment, neither for synthesis nor for purification. In fact, once separated from the crude reaction in the crystalline form, no further purification steps are necessary.
4. The derivatization procedure is simple, reproducible and easily applied for the determination of thiols in biological samples. Thiols of similar structures can be used as internal standards.
5. The chromatographic detection is selective, sensitive (fmole range), fast (less than 10 min per sample) and inexpensive, particularly when utilized for determination of thiols in a large number of samples.
6. Both the native reagent and its thiol adducts are stable for long periods of time and hence may be utilized with automated systems on a routine basis.
7. The chromatographic conditions are simple (no gradient elution is necessary) and enable a rapid simultaneous separation and detection of numerous thiols with complete resolution.
8. The method can be easily applied for the determination of SH-containing drugs (N-acetylcysteine and penicillamine) without the need for special separation conditions.

CONCLUSION

This work describes a sensitive and selective HPLC method for the determination of aliphatic thiols. The method is simple, inexpensive, rapid, reproducible and can simultaneously separate seven different thiols within 10 minutes. Once formed, the adducts are fairly stable for long periods of time even at room temperature (reagent III). The method is easily applied for the determination of endogenous thiols in biological samples. Furthermore, it can be routinely used for the determination of SH-containing drugs (NAC and penicillamine). Application of the method for the determination of the SH content of proteins, titration of an SH-containing catalytic site of various enzymes (pepsin, guanylyl cyclase) and determination of the activity of SH-producing or utilizing enzymes (GSH synthase and peroxidase and GSSG reductase) are under active investigation.

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