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2-(4-N-Maleimidophenyl)-6-methoxybenzofuran: a superior derivatizing agent for fluorimetric determination of aliphatic thiols by high-performance liquid chromatography

Abdullah I. Haj-Yehia, Leslie Z. Benet*

Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA, 94143-0446, USA

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Abstract

The use of 2-(4-N-maleimidophenyl)-6-methoxybenzofuran as a fluorogenic pre-column derivatizing agent for sensitive high-performance liquid chromatographic (HPLC) determination of aliphatic thiols is described. The maleamic acid, its methyl ester and the N-maleimide derivatives of the common amine precursor were easily synthesized and characterized according to conventional methods. These derivatives lack fluorescent properties in their native form but display strong fluorescence upon reaction with aliphatic thiols. The reaction is rapid and highly selective for thiols over a wide pH range (7.1–8.8). Following derivatization, the thiol adducts were separated on a reversed-phase column (Ultrasphere-ODS) using 0.1% hexanesulfonic acid in 10 m*M* potassium hydrogen phosphate–acetonitrile (65:35, pH 4.5) and were detected fluorimetrically (excitation at 310 nm; emission at 390 nm). The method is highly sensitive (femtomole range) and is easily applied to the determination of SH-containing drugs and endogenous thiols in biological samples.

1. Introduction

The endogenous thiols, glutathione (GSH) and cysteine (Cys), are ubiquitous SH-containing compounds which play important metabolic, biochemical and pharmacological roles in the homeostatic mechanisms of the entire biological system.

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 the 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] and reactivity towards a conjugated double bond [6–10]. The resulting derivatives are monitored by ultraviolet or fluorescence spectrophotometry. Methods also exist involving enzymatic reactions [11], HPLC with electrochemical detection [12,13] and mass spectrometry [14] for the determination of thiols in biological samples. Of special interest is the addition reaction to a conjugated double bond, usually in the imide form, of chromophoric maleic acid derivatives. Recently, the modified substituted free acid [15,16] as well as its methyl ester [17] were reported as useful fluorogenic derivatizing reagents for thiols. In addition to chromophoric maleic (maleamic) acid deriva-

* Corresponding author.

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tives, bimanes [18,19], dansyl chloride [20] and 7-fluoro-2,1,3-benzoxadiazoles [21,22] have been reported to be useful reagents for derivatizing thiols in HPLC analyses with fluorimetric detection.

Other than being selectively attacked by the SH group (over a certain pH range), the advantage of utilizing the substituted maleic moiety results from the lower or absence of fluorescence of the native form of the derivatizing reagent versus the strong fluorescence displayed by the addition product upon reaction with SH-containing compounds. Unfortunately, without exception, all existing fluorogenic thiol reagents are unrealistically expensive and hence their use on a routine basis is limited. Moreover, in some cases, the derivatization and detection procedures are nonspecific, time-consuming and necessitate the use of complex elution conditions for the separation of the derivatives [20]. In other cases, limited sensitivity [12,13] or fluorescent impurities in the reagents, even after extensive purification procedures, interfere with the resolution and detection of certain thiols [8].

We recently explored the properties of substituted benzothiazoles for the fluorimetric determination of aliphatic thiols [23]. Here, we describe a facile method for the preparation of 2-(4-N-maleimidophenyl)-6-methoxybenzofuran, its acid and methyl ester derivatives from their easily synthesized amine precursor. All three derivatives were found to be useful for the rapid and sensitive determination of various thiols, including glutathione (GSH) and cysteine (Cys) in biological samples.

2. Experimental

2.1. Chemicals

2-(4-Aminophenyl)-6-methoxybenzofuran (I, Fig. 1) was synthesized in our laboratory and yields the following analytical data: m.p 207–208°C, IR (Nujol, cm^{-1}),–NH_2 3400, CH-aryl 1620, ^1H NMR ($CDCl_3$-TMS) δ (ppm) = 3.85 (3H, s,–OCH_3), 6.47–7.57 (8H, m, ArH), $UV_{ethanol}$ λ_{max} = 310 nm, (log ϵ = 4.14), MS (*m/z*): 239 (M^+). Analysis calculated (%) for $C_{15}H_{13}NO_2$: C 75.29, H 5.47, N 5.84; found, C 74.74, H 5.31, N 5.75 (a complete description of the synthesis of this and other thiol selective precursors to be reported elsewhere).

Maleic anhydride, acetic anhydride, sodium acetate, hexanesulfonic acid, N-acetyl-L-penicillamine and penicillamine were purchased from Aldrich (St. Louis, MO, USA) 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, USA). Deionized distilled water was used and the mobile phase filtered through a 4-μm filter (Rainin, Emeryville, CA, USA) and degassed under vacuum prior to use.

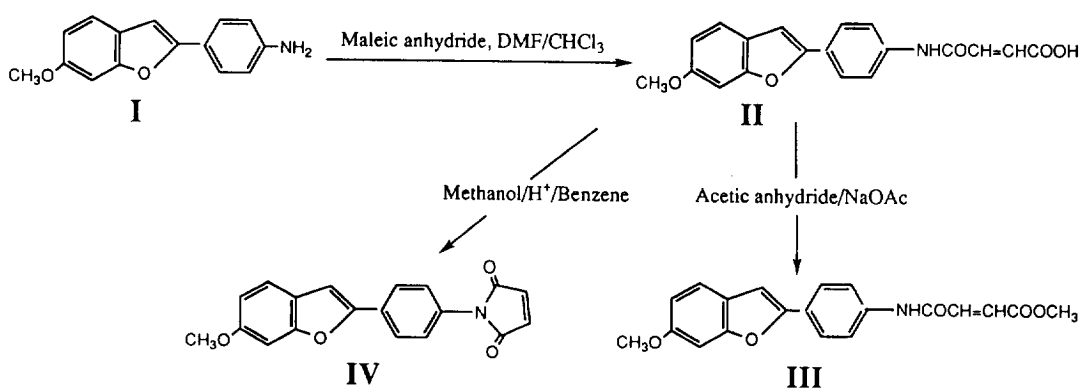

Fig. 1. Synthesis scheme for 2-(4-N-maleimidophenyl)-6-methoxybenzofuran (IV), the acid (II) and the methyl ester (III).

2.2. 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 310 and 390 nm, respectively. Samples were either injected using an autosampler (Millipore Corp., Marlborough, MA, USA) or manually using a Rheodyne 20-μl sample loop (Rainin, Emeryville, CA, USA). Ultraviolet (UV) spectra were recorded with a diode-array spectrophotometer 8452A (Hewlett-Packard, Palo Alto, CA, USA). Fluorescence was recorded on a DM 3000 fluorometer (SPEX Industries, Edison, NJ, USA); NMR spectra were recorded on QE-300 (GE-NMR Instruments, Fremont, CA, USA).

2.3. Synthesis of the reagents

2-(4-Maleimidophenyl)-6-methoxybenzofuran (IV), the acid (II) and the methyl ester (III), were synthesized from the amine (I) following the scheme depicted in Fig. 1.

2-(4-N-Phenylmaleamic acid)-6-methoxylbenzofuran (N-[4-(6-methyl-2-benzofuranyl)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 of dimethylformamide. The mixture was stirred for 2 h at room temperature. The deposited crystals were filtered off, washed with 30 ml of chloroform and recrystallized from chloroform–DMF (3:1) to yield compound II (1.54 g, 91%, reddish crystals). Melting point 237°C (dec.) ; IR (Nujol, cm^{-1}), C*O*OH 1711 C*O*NH 1620, ^1H NMR (DMF-d_7-TMS) δ (ppm) = 3.89 (3H, s,–OCH_3), 6.41 and 6.63 (1H,1H,d,d, J = 12 Hz,–CH = CH–), 7.34–8.21 (8H, m, ArH), $UV_{ethanol}$ λ_{max} = 310 nm, (log ϵ = 3.90), MS (*m/z*): 337 (M^+). Analysis calculated (%) for $C_{19}H_{15}NO_5$: C 67.64, H 4.48, N 4.15; found, C 66.97, H 4.50, N 4.17.

Methyl-N-[4-(6-methoxy-2-benzofuranyl)phenyl]maleate (III)

A mixture of the acid II (2.02 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 h. 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 yellow crystals of the methyl ester (III), 1.86 g (88%): m.p. 219–221°C, ^1H NMR ($CDCl_3$-TMS), δ (ppm) = 3.91 (3H, s, aryl-*O*CH_3), 3.85 (3H, s, ester-OCH_3), 6.29 and 6.47 (1H,1H,d,d, J = 12 Hz,–CH = CH–), 7.32–8.17 (8H, ArH). IR (Nujol, cm^{-1}) C*O*OCH_3 1712, C*O*NH 1674, $UV_{ethanol}$ λ_{max} = 310 nm, (log ϵ = 4.03), MS (*m/z*): 351 (M^+). Analysis calculated (%) for $C_{20}H_{17}NO_5$: C 68.36, H 4.88, N 3.99; found, C 68.62, H 4.93, N 4.07.

2-(4-N-maleimidophenyl)-6-methoxybenzofuran (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 h. 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 dissolved in a minimal volume of DMF and crystallized from methanol to yield deep purple needles, 1.4 g (74%) of the maleimide (IV): m.p. 249–251°C, ^1H NMR ($CDCL_3$-TMS), δ (ppm) = 3.90 (3H, s,–OCH_3), 6.83 (2H, s,–CH = CH–), 7.39–8.38 (8H, m, ArH), IR (KBr, cm^{-1}) C*O*NH 1726, $UV_{ethanol}$ λ_{max} = 310 nm, (log ϵ = 3.95), MS (*m/z*): 319 (M^+). Analysis calculated (%) for $C_{19}H_{13}NO_4$: C 71.46, H 4.10, N 4.39; found: C 70.98, H 3.98, N 4.40.

2.4. Chromatographic conditions

The HPLC apparatus was connected to an Ultrasphere-ODS column (250 × 4.6 mm I.D., 5 μm Beckman, Palo Alto, CA, USA). The sam-

ples were eluted isocratically at ambient temperature with 10 m*M* KH_2PO_4 [supplemented with 0.1% hexanesulfonic acid (sodium salt)]–acetonitrile (65:35, v/v), adjusted to pH 4.5 (after degassing) 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 1.0 ml/min, respectively.

2.5. Preparation of standards

Fresh standard solutions of thiols containing 10 nmol/ml of either cysteine (Cys), N-acetylcysteine (NAC), glutathione (GSH), homocysteine, L-penicillamine, N-acetyl-L-penicillamine and coenzyme A were prepared by dilution with distilled water of 1 m*M* solutions of each thiol in a 20 m*M* EDTA (disodium salt) solution. For determination of GSH and Cys in biological samples, 100 μl of NAC solution (0.05 m*M*) 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 of dimethylformamide; a 1-ml volume of this stock solution was diluted 20 fold with acetonitrile and 100 μl of the resulting solution was used for the derivatization procedure.

2.6. Preparation of biological samples

Male Sprague–Dawley rats (300–400 g) were sacrificed by decapitation. The organs (heart, lung, liver, kidney, testes, and spleen) were immediately removed and perfused with normal saline. The metabolic reaction in tissue material was quenched by means of the freeze-stop technique with liquid nitrogen, and the frozen samples were either stored or pulverized (Tissue Pulverizer, Fisher Scientific, Santa Clara, CA, USA) and processed immediately. Portions (50–100 mg) of tissue were homogenized in 1 ml of ice-cold 20 m*M* EDTA aqueous mixture containing 30% acetonitrile. After centrifugation (4°C) at 4000 *g* for 5 min to remove protein, each supernatant was transferred into another precooled test tube and individually adjusted to 1–2.5% w/v with borate buffer pH 8.4. The resulting solutions were kept on ice and immediately used as the biological samples for the derivatization procedure described below.

2.7. Derivatization procedure

The following solutions 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 diluted with an equal volume of the mobile phase. A 10-μl volume of the resulting solution was injected onto the chromatographic system. 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 samples were replaced by standard solutions containing 0.5–5 nmol each of GSH and Cys.

2.8. Recovery study

Table 1 summarizes the recovery studies performed with reagent IV. Tissues (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. 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.

3. Results and discussion

All three reagents can be utilized to yield sensitive and selective measurements of thiols.

Table 1
Recoveries of the amounts of glutathione (GSH) and cysteine (Cys) added to biological sample extracts

Sample	Thiol	Amount added (nmol)	Amount recovered (mean ± S.D., $n = 5$) (nmol)	C.V. (%)
Heart	GSH	15	15.44 ± 0.98	6.3
	Cys	25	22.49 ± 2.09	9.3
Liver	GSH	15	14.51 ± 0.81	5.6
	Cys	25	26.05 ± 1.90	7.3
Kidney	GSH	25	24.22 ± 1.96	8.1
	Cys	15	15.23 ± 0.59	3.9
Spleen	GSH	15	14.61 ± 0.87	5.9
	Cys	25	26.65 ± 2.16	8.1

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 fluorimetric detection (most of which also involve an imide reactant) more appropriate.

Fig. 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 m*M* solutions of GSH, NAC and Cys. No pH dependence of the yield of the derivatization reaction for these thiols was observed over the pH range 7.1–8.8 when the reaction was carried out for 1 h at room temperature. 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. Fig. 3 shows the time course for the completion of the derivatization at 80°, 65°C and room temperature at pH 8.4. In each case the reactions appear to be complete by 1 h. Increased reaction rates were observed at these temperatures with higher pH values (data not shown).

For determination of thiols in biological samples, the optimal derivatization conditions were found to be incubation of the biological sample extract with the reagent at pH 8.4 for 1 h at room temperature. 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

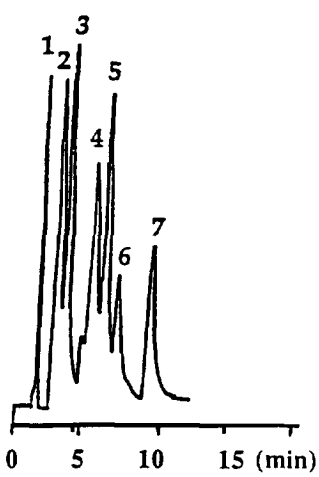

Fig. 2. Chromatogram of reagent IV-thiol derivatives. A mixture consisting of 100 μl of each of the standard solutions (10 nmol/ml) of the thiols was treated with reagent IV according to the procedures described in the text. Peaks: 1 = coenzyme A, 2 = homocysteine, 3 = GSH, 4 = NAC, 5 = Cys, 6 = N-acetyl-L-penicillamine, 7 = L-penicillamine.

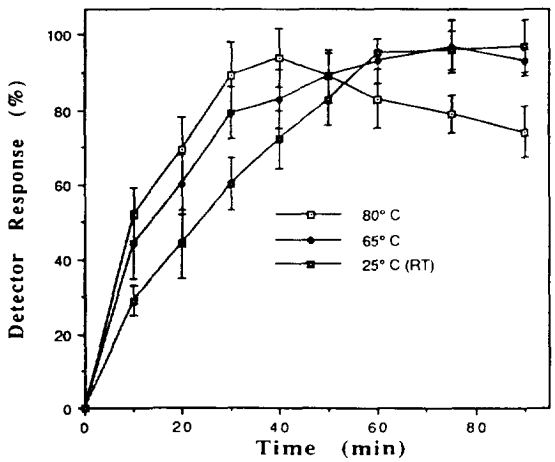

Fig. 3. Dependence of the detector response and the time of completion of the derivatization reaction upon temperature. Reactions were carried out at pH 8.4 with reagent IV in the presence of GSH (0.1–1 μmol) at the specified temperatures. Aliquots of the reaction mixture were taken at different time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

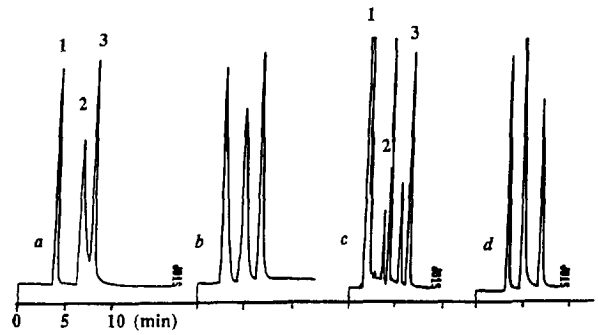

Fig. 4. Chromatograms of the adducts of GSH, NAC, and Cys, with reagent II when eluted with the same mobile phase at pH 4.5 (a) and pH 5.6 (b) and of the same thiols with reagent IV at pH 8.4 (c) and pH 4.5 (d) demonstrating the effect of pH on the splitting and retention time (see text for details). Peaks: 1 = GSH, 2 = NAC, 3 = Cys.

peaks, especially when reagent IV was utilized for the derivatization. In the latter case, hydrolysis of the imide might have occurred under these conditions, and/or the reagent may have reacted with the amino group of other amino acids present in the biological sample.

The effect of the pH of the mobile phase on the separation of the adducts of reagents II and IV was studied. Fig. 4 shows complete splitting of NAC and Cys adduct peaks at pH 8.4, versus that at pH 4.5 (Fig. 4c vs. 4d). In fact, the peaks of the IV-adducts of these thiols (NAC and Cys) exhibit splitting at pH values higher than 4.9, whereas those of the GSH-adduct show complete splitting at pH > 8.6 when eluted with the same mobile phase at a slower rate (~0.5 ml/min). Similar results were obtained when the ester III was used, whereas no splitting occurred under these conditions with reagent II (Fig. 4a,b). Best separation of Cys, GSH, NAC, and other thiol adducts within 10 min was obtained at pH 4.5 for reagents III and IV (Fig. 2) and at pH 5.6 for reagent II (Fig. 4). Therefore, to preclude peak splitting, when using reagents III and IV for derivatization, the endogenous thiols were determined with the mobile phase maintained at pH 4.5. Lowering the pH of the mobile phase below 4.5 yielded increased retention times for all of the thiols tested without significantly affecting the resolution. 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, has been alluded to [14] and is most likely due to the separation of the two diastereoisomer adducts obtained upon addition of thiols to the double bond of the reagent.

Amongst the various buffering, ion-pairing and organic modifying agents examined, isocratic elution at 1.5 ml/min with 10 m*M* KH_2PO_4 (supplemented with 0.1% hexanesulfonic acid)–acetonitrile, 65:35, at pH 4.5 (70:30, v/v at 1.0 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 tetrahydrofurane alone or in combination with acetonitrile as the organic modifier promoted splitting of the adduct peaks even at acidic pH.

Native fluorescent 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 non-fluorescent thiols (NAC or penicillamine) which, upon undergoing the same treatment (reaction with the reagent), yield a fluorescent 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: $y = 0.160 \times + 0.0036$ ($r^2 = 0.994$, $n = 6$). The results were more reproducible (precision $< 5\%$, accuracy $> 96\%$) than when utilizing non-thiol native fluorescent compounds of different chemical and spectral characteristics as internal standards. For the determination of GSH and Cys in biological samples, poorer results were obtained when the preformed adducts of NAC or penicillamine were added as internal standards at the end of the reaction with the reagent as compared to addition of NAC and penicillamine themselves as internal standards 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 8 fmol (GSH and coenzyme A) to 75 fmol (penicillamine) with the 4 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 (> 1 week 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 present method utilizing compound I, a benzofuran, as the precursor for the reagents (Fig. 1) has an advantage over our previously described method [23], since compared to benzothiazole, chromophoric benzofuranes are significantly more stable. Due to degradation products, further recrystallization of the benzothiazole derivatives is needed following storage for 1 month at −20°C, while under the same conditions, we find that the benzofuran derivatives described here are stable for more than one year.

The applicability of the method was tested for the determination of thiols in biological tissue samples. When derivatized according to the procedure described, chromatograms are obtained as depicted in Fig. 5. Table 1 shows results obtained from recovery studies carried out with this method using reagent IV. The tissues used for the recovery study were chosen to reflect the diversity of thiol content in biological samples (Table 2). As shown in Table 1, when GSH (15 nmol) was added to liver extracts, the amount added was accounted for in the recovery study even though endogenous liver GSH concentrations (i.e. the subtracted blank) are approximately 250 fold greater (e.g. liver GSH in Table 2 is 3.78 ± 0.36 μmol/g versus the 15 nmol added to the one gram of liver tissue extract in Table 1). Similarly, small amounts of GSH and Cys were recovered with good accuracy 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 2 shows GSH and Cys concentrations in various organs of fasted (12 h) Sprague–Dawley

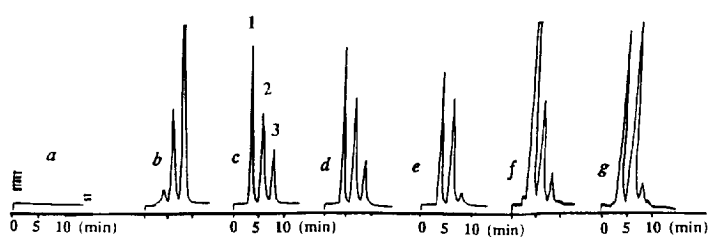

Fig. 5. Chromatograms of the endogenous thiols GSH (peak 1) and Cys (peak 3) following derivatization with reagent IV of rat tissue extracts using NAC (peak 2) as internal standard. A portion (100 μl) of each sample was treated according to the procedure. (a) Blank (no reagent added); (b) kidney; (c) lung; (d) spleen; (e) testes; (f) liver; (g) heart.

Table 2
Concentrations of glutathione (GSH) and cysteine (Cys) in various tissue extracts from fasted rats

Sample	Dilution	Concentration (μmol/g)	
		GSH	Cys
Heart	1:40	1.11 ± 0.13	0.05 ± 0.005
Lung	1:100	1.32 ± 0.14	0.69 ± 0.04
Liver	1:100	3.78 ± 0.36	0.25 ± 0.05
Kidney	1:100	N.D.[a]	2.93 ± 0.28
Spleen	1:40	0.63 ± 0.07	0.64 ± 0.07
Testes	1:100	1.49 ± 0.12	0.36 ± 0.06

[a] Non-detectable.

rats as determined by this method. When fasted, most rats exhibited similar thiol content in their organs (low S.D.), whereas fed animals exhibited a wide range of thiol concentrations (data not shown).

Compared to other thiol derivatizing reagents, reagent IV, for example, is easily prepared from inexpensive, commercially available precursors using general synthetic methods that neither require special equipment for synthesis nor for purification. The derivatization procedure is simple, reproducible and easily applied for selective and sensitive (femtomole range) determination of thiols in biological samples. Thiols of similar structures can be used as internal standards. Since both the native reagent and its thiol adducts are stable for long periods of time the method may be utilized with automated systems on a routine basis utilizing simple chromatographic conditions. In fact, SH-containing drugs (N-acetylcysteine and penicillamine) can be easily measured without the need for special procedures to separate these drugs from the endogenous thiols.

4. Conclusions

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 min. Once formed, the adducts are 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). Applications of the method for the determination of the SH content of proteins, titration of an SH-containing catalytic site of various enzymes (pepsin, guanylate 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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