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Review

The determination of thiols and related compounds using high-performance liquid chromatography

DAVID PERRETT* and SUSAN R. RUDGE[2]

[1] *Department of Medicine, St Bartholomew's Hospital Medical College, West Smithfield, London EC1A 7BE, UK*
[2] *Department of Rheumatology, City Hospital, Nottingham, UK*

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Introduction

To many workers in biochemistry, medicine and pharmacology, thiols (also referred to as sulphydryl, mercapto- and -SH compounds), and especially thiol-containing drugs, hold a particular fascination. They form an important class of compounds with an extensive and interesting chemistry [1, 2], their reactions including disulphide bond formation, metal chelation and thiazolidine formation. Such varied chemistry leads in turn to a complex biochemistry [3, 4] for both free thiols and -SH groups of proteins. Of the naturally occurring thiols, cysteine and reduced glutathione are best known since they play important roles in cellular metabolism and protein structure. The ability of the -SH group to undergo so many types of chemical reaction has led to its incorporation into many drugs.

There has thus arisen a need both to quantify naturally occurring thiols and to measure therapeutic concentrations of thiol-containing drugs. Unfortunately the very properties of the -SH group which make it an important biochemical moiety also cause great difficulties in its reliable estimation. Historically many methods have proved non-specific, difficult and inaccurate. D-Penicillamine, for example, had been in clinical use for nearly 25 years before it could be reliably assayed in body fluids. A number of approaches to the assay of thiols now exist: most rely upon the recent developments in the field of high-performance liquid chromatography (HPLC) that form the subject of this review.

* To whom correspondence should be addressed.

Biologically Important Thiols

The most abundant simple thiol is the amino acid cysteine: along with its disulphide cystine it occurs in body fluids and cells as well as in most proteins. Homocysteine is found as the free thiol, its homodisulphide and mixed disulphides in man. Methionine, an *S*-methyl amino acid, is intimately linked with the metabolism of the thiol amino acids. These amino acids are associated with various metabolic disorders [4] such as homocystinuria and cystinuria where quantitative diagnostic data are required. The cysteine-containing tripeptide reduced glutathione (GSH) is found in high concentration in most mammalian tissues where it fulfils many roles including maintainence of the oxidation status of the cell [5].

Probably the oldest thiol-containing drug is BAL (British anti-lewisite, 2,3-dimercaptopropanol) which was introduced during World War II as an anti-arsenical agent. In 1956 Walshe introduced D-penicillamine, a chemical degradation product of penicillin, as a metal chelator in the treatment of Wilson's disease [6]. The same compound has since become the drug of choice in the treatment of cystinuria and is also widely used as an anti-rheumatic agent. A number of compounds related to cysteine are employed as mucolytic agents. Recently captopril (D-3-mercapto-2-methyl propanoyl-L-proline) has been successfully introduced as a converting enzyme inhibitor in the treatment of hypertension. Mercapto-derivatives of the purine bases, such as 6-mercaptopurine and thioguanine, are used in the treatment of various cancers. Thiols have been extensively studied as radioprotective agents. To prevent excessive oxidation of the thiol group in some drugs, e.g. azathioprine, a labile protective group is attached to the thiol and the free thiol is only liberated following absorption.

A more comprehensive listing of natural thiols and thiol-containing drugs is given in Tables 1 and 2.

Table 1
Some natural sulphur-containing compounds

Free thiols (−SH)	Disulphides
Cysteine	Cystine
Homocysteine	Homocystine
Glutathione (Red.)	Glutathione (Ox.)
Co-enzyme A	Lipoic acid
Propane thiol	
Ethane thiol	
Ergothione	

Metabolism of Thiols

The metabolic pathways of the sulphur amino acids are well documented [3, 4] but values given for their relative concentrations in tissues and physiological fluids are often inaccurate. The reactivity of the -SH group, particularly in the presence of oxygen and/or catalytic quantities of heavy metals, means that thiol drugs are readily transformed in physiological systems. Since the concentrations of the sulphur amino acids in body fluids are high compared with therapeutic levels of thiol drugs, the latter tend to form disulphides, particularly with cysteine in plasma and urine and glutathione in tissues. Disulphides with homocysteine and thiolactate are also possible. With an

Table 2
Thiol-containing drugs

Clinical use	Non-proprietary name	Proprietary names
Metal chelation	Dimercaprol	BAL
	D-Penicillamine	Distamine
		Pendramine
Mucolytic agents	*N*-Acetylcysteine	Airbron
		Fabrol
	Carbocysteine	Mucodyne
		Mucolex
	Methylcysteine	Visclair
Anti-thyroid drugs	Carbimazole	Neo-mercazole
	Propylthiouracil	
Anti-hypertensives	Captopril	Capoten
Anti-helminthics	Levamisole	
	Thiobendazole	Mintezol
Immunosuppressants	Azathioprine	Imuran
	6-Mercaptopurine	Puri-nethol
	Thioguanine	Lanvis
Anti-rheumatic drugs	D-Penicillamine	Distamine
	5-Thiopyridoxine	
	Pyrithioxine	
	Thiopronine	
	Mercaptopropylglycine(Thiola)	
	Sodium aurothiomalate	Myocrisin
	Aurothioglucose	Solganol
	Aurothiopropanol sulphonate	Allochrysine
Radioprotective agents	Cysteamine	

excess of the free thiol the homodisulphide is also found in quantity. The ability of various thiols to form disulphides or undergo thiol–disulphide exchange is dependent on their relative concentrations, equilibrium constants and redox potentials. True metabolism of the -SH group in drugs is rare. For example, less than 7% of a dose of D-penicillamine is found in its *S*-methylated form in urine whilst 40% occurs as disulphides [7]. The metabolism of the -SH group of thiopurines is similar to that of simpler thiols although the rates of reaction are slower. The purine ring continues to be metabolized by both degradative and salvage pathways and compounds such as thio-uric acid and thio-nucleotides can occur. Little is known of possible desulphurization pathways that may exist particularly in the gut. It is possible that some thiols occur in body fluids as metal chelates. In plasma free thiols as well as disulphides can be detected but a large proportion of most thiol drugs is usually protein-bound.

Sample Preparation

Thiols and thiol-containing drugs are notoriously unstable both in neutral buffer solutions and in physiological fluids. Careful sample preparation is thus essential to ensure that assay techniques accurately reflect *in vivo* thiol concentrations.

Standard solutions of free thiols, even if prepared in deionized water, are readily oxidized to homodisulphides. The rate of oxidation is dependent on the structure of the thiol, an example being the steric hindrance of D-penicillamine oxidation by its two methyl groups [8]. Thiols may be stabilized by lowering the pH ≤ 2 or by adding disodium EDTA which may act by complexing the trace metals catalysing thiol oxidation.

Physiological thiols such as cysteine and thiol drugs such as D-penicillamine, captopril and thiomalate are readily lost from plasma *in vitro* (Fig. 1). This loss is probably due to a combination of protein binding (for example to the cysteine residues of serum albumin) and disulphide formation with endogenous thiols. This loss may be minimized by the collection of whole blood into disodium EDTA. The blood should be rapidly centrifuged at 4°C using pre-cooled buckets, followed by immediate acid precipitation of plasma proteins. Trichloroacetic acid, perchloric acid and metaphosphoric acid have been satisfactorily employed as the protein precipitant. The authors have used sulphosalicylic acid (final concentration in plasma, 0.2 mol/l containing 1 mmol/l EDTA), since the supernatant can be injected directly on to HPLC columns. Provided the above steps are completed within 3–5 min recoveries of ca. 95% for free thiomalate [9] and 104% for D-penicillamine [10] have been reported. Despite flash-freezing and storage at −20°C a slow further loss of thiol can occur [11]. It is therefore recommended that plasma samples are assayed as soon as possible after collection. Similar data on the extraction and storage of the thiopurines do not appear to be available and many authors have stored plasma prior to assay without any precautions. Since thiopurinol and to a lesser extent 6-mercaptopurine bind to serum proteins [12] some losses of thiopurines may occur between collection of the plasma and assay, unless suitable precautions are taken.

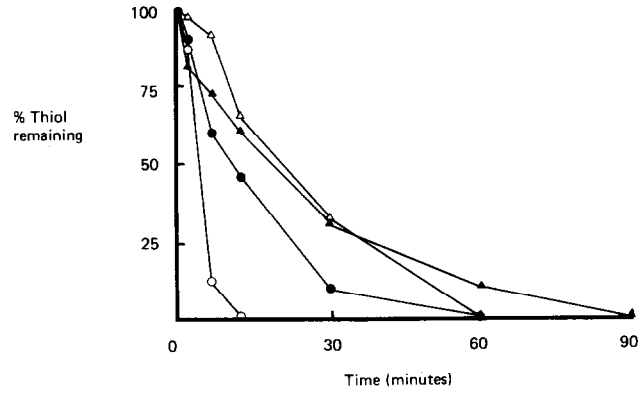


Figure 1
Relative rate of loss of physiological concentrations of free thiols from plasma at 37°C. ○—○ cysteine, ●—● captopril, △—△ thiomalate and ▲—▲ D-penicillamine.

Roston [13] has shown that small amounts of cysteine disappear rapidly from non-acidified urine in contact with air and more recently similar losses have been reported for both D-penicillamine [8] and thiomalate [9]. It is therefore essential to acidify the urine immediately after delivery. Collection of urine into HCl or immediate addition of HCl inhibits free thiol oxidation for at least 12 h at room temperature. Although freezing the urine inhibits further degradation it does not prevent additional loss of free thiols. Provided suitable precautions are taken to prevent disulphide formation from thiols,

disulphides in plasma or urine can be quantitated with adequate precision after acidification alone.

Dithiothreitol (DTT) has been used [14] to maintain thiols in the reduced form prior to analysis. This procedure could give erroneous results since DTT might release thiols from protein binding sites or from mixed disulphides. DTT can also react with many of the thiol-specific reagents used in the assays.

Several pre-column derivatization procedures are available all requiring distinct sample preparation methods. The derivatization can be performed directly on the biological fluid, or the latter may be pre-treated, e.g. plasma proteins precipitation. In either case the presence of many endogenous thiols means that a considerable excess of the derivatizing reagent may be required. The relatively long derivatization times of some reagents raise the possibility of disulphide formation occurring, with erroneous results.

When difficult extraction procedures are needed prior to HPLC an internal standard is often employed to improve quantitation. Internal standards are normally of a similar chemical composition to the unknown so that they will chromatograph and be detected in the same manner. They normally only correct for absolute errors such as volume changes and not for chemical or biological degradation of the sample. For thiols the only suitable compounds would be other thiols; the latter might also be unstable and their half lives would not necessarily parallel that of the analyte. In assays without derivatization mixed disulphides might be formed. With derivatization assays another thiol or a purified thiol-adduct would be more successful.

Early Methods

Ashworth [2] lists nearly 200 "specific" reagents and reactions for the thiol group. Many assays depend on the ability of the -SH group to form coloured complexes with heavy metals. The intense purple colour formed between thiols and alkaline nitroprusside is a commonly used screening procedure for both cystinuria and homocystinuria. The introduction of bis (5-carboxy-4-nitrophenyl)disulphide (DTNB) by Ellman in 1959 led to a simple and popular assay for thiols [15]. The formation of the mixed disulphide of DTNB and the thiol yields an intense yellow colour. Cleland [16] introduced DTT as an efficient reducing agent, able to maintain thiols in the reduced form and to reduce many disulphides. Since excess DTT can be complexed with arsenite it is possible to assay the liberated thiol using Ellman's reagent [17]. For many thiols spectrophotometric methods are available, e.g. cysteine gives a specific reaction with ninhydrin in concentrated acid [18] and glutathione reacts with *o*-phthalaldehyde giving a fluorescent product [19]. More details of the many non-chromatographic assays for thiols are given by Ashworth [2] and Jocelyn [3].

Early chromatographic methods

The occurrence of many -SH-containing compounds in physiological fluids prevents many of the simple methods mentioned above being applied to the analysis of individual thiols. This is particularly true in the quantitation of thiol-containing drugs at therapeutic levels substantially lower than endogenous thiol concentrations. Additionally the clinically important disulphides cannot be measured directly using thiol-specific reagents. The sulphur-containing amino acids, cystine, methionine and homocystine, are readily separated by both paper and thin-layer chromatography. Many earlier studies on their

metabolism employed these techniques, using ninhydrin to detect the amino groups or sulphur-specific reagents. The prolonged analysis time meant that the free thiols chromatographed poorly, often being oxidized during analysis.

The advent of the amino acid analyser improved both the specificity and accuracy of the determination of the disulphide amino acids and methionine. Notable studies on the separation of sulphur amino acids on ion-exchange columns were performed by Purdie *et al.* whilst working on the radio-protective effects of thiols [20, 21]. Most studies using amino acid analysers use ninhydrin, but some authors [22, 23] have commented on the unusually low colour response of sulphur-containing amino acids with this reagent.

Amino acid analysers were less unsuccessful in the separation of thiol-amino acids since the elevated temperatures at which the analytical columns were maintained led to rapid oxidation. There was one early but important attempt to circumvent this problem. In 1960, Stein and Moore, the inventors of the amino acid analyser, recognised that oxidation could be prevented by derivatizing the -SH group. They employed iodoacetate to convert cysteine quantitatively into a stable *S*-carboxymethyl derivative, which was readily separated from other amino acids [24]. Using this technique they were able, for the first time, to provide accurate data on the ratio of cystine to cysteine in plasma, i.e. 2.5:1. *N*-Ethylmaleimide (NEM), iodoacetamide and iodoacetic acid have been used to derivatize the -SH group of amino-thiols prior to amino acid analysis [22, 25].

The need for high sensitivity amino acid analyses led to the development of procedures for the production of volatile derivatives suitable for separation by GLC. The sulphur amino acids did not derivatize well and more specific approaches were designed. Jellum *et al.* [26] formed volatile neopentylidine derivative by reaction with pivaldehyde (2,2-dimethylpropanal). A single step pertrimethylsilyation has been used for sulphur amino acids [27, 28] and D-penicillamine in plasma [29]. The use of NEM to block the reactive -SH group of captopril prior to methylation and gas chromatography with selected ion monitoring mass spectrometry has been demonstrated [30]. Various GLC derivatives have been employed for the determination of the mucolytic agents based on cysteine [31], esterification and acylation with heptafluorobutyric acid [32], acylation with heptafluorobutyric anhydride [33]. The flame photometric detector in the sulphur mode has been employed [34]. Most of these methods involved considerable sample preparation, and detection limits were high. In a detailed study, Smith concluded that the problems of irreproducibility and derivative degradation were too great for the practical application of GLC to the determination of thiols [35].

Some enzymatic assays, e.g. for cysteamine [36], have been developed. Few immunoassays for thiols exist. A system for D-penicillamine based on the direct coupling of the thiol to protein has been reported [37]. Specificity problems severely limit the application of both immunoassays and radio-immunoassays.

Because of these analytical difficulties and the problems of disulphide formation much of our detailed knowledge of the metabolism and pharmacokinetics of thiol drugs has depended on the use of radio-labelled thiols. ^{35}S, ^{14}C and ^{3}H have all been employed but few studies have employed even simple chromatography to determine the relative abundance of the metabolites.

The Determination of Free Thiols Using HPLC

As well as the problems associated with sample collection and stability the quantitation of free thiols presents two important technical difficulties. Chromatography is poor

because of the reactivity of the thiol group, and detection is difficult in the absence of strong chromophores and fluorophores.

Chromatography

Traditionally the amino-thiols have been separated on cation exchange resins. Even with the advent of HPLC some authors have continued to prefer sulphonated polystyrene resins, probably because of their high ion-exchange capacity. Cation-exchange coated silicas have been employed but their useful lifetime can be short, particularly when dealing with physiological extracts. The purine bases and their related thiopurines have been resolved on cation columns [38] while early studies on thio-nucleotides were performed on anion-exchange columns [39].

Many workers have chosen reversed-phase (RP-HPLC) systems for the separation of free thiols. Since purine bases are easily and reproducibly resolved on ODS columns using simple methanol/buffer gradients most assays for the mercaptopurines rely upon suitable isocratic mixtures of the same solvents. Narang *et al.* [40] resolved 6-mercaptopurine, thio-uric acid and thioguanine in 7 min on an ODS-ultrasphere column.

The amino-thiols possess three ionizable functional groups, -SH, $-NH_2$ and -COOH, with pK_a values of approximately 10, 8 and 2 respectively: in the range pH 4–7 these compounds are thus zwitterions. Resolution is only possible on ODS columns with either low pH eluents to suppress the ionization of the acid groups, or with ion pairing agents at pH values outside the zwitterion range. The use of low pH buffers has the advantage that it reduces the rate of disulphide formation: this may be the cause of the multiple peaks reported for some thiols at higher pHs [41, 42]. Various authors have attempted to separate cystine and cysteine using ion-paired RP-HPLC but in nearly every case both compounds eluted frontally [43, 44]. The same ion-pairs, however, gave good separations of D-penicillamine and its disulphides, and a detailed investigation of their chromatography was reported by Allison *et al.* [44]. The ion-pairs heptanesulphonic acid [45] and octane sulphonate [46] have been employed to improve the resolution of 6-MP from other plasma components. Although thiols, in general, interact strongly with metal ions few attempts at modifying separations by including metals in the eluent have been published. Wronski [47] reported that tributyltin chloride has a special affinity for sulphydryl groups and in chromatographic situations acts as a liquid anion exchanger, modifying the elution of thiols from polyamide columns.

Thiols in general do not chromatograph well, exhibiting poor column efficiencies, extreme peak tailing, and in some cases peak splitting. The first two faults occur with both ion-exchange and ODS columns, whereas peak splitting is confined to ODS columns and can be partially overcome by using low pH eluents [41, 42, 48]. Low chromatographic efficiencies affect both ion-exchange and reversed-phase systems, but efficiencies are significantly improved by the addition of EDTA [49], so they may be related to interactions between the -SH group and metal ions. The authors have also observed an unusual carry-over phenomenon when separating thiols on reversed-phase systems using eluents containing less than 20% methanol [49]. Thiols can be absorbed onto the silica packing materials and can then be displaced by other thiols, although blank injections do not give ghost peaks. Such situations arise particularly when thiol drugs are being assayed in physiological fluids containing relatively large amounts of naturally occurring thiols such as cysteine. Columns can be cleaned by injecting a strongly displacing thiol such as D-penicillamine and regular washing with methanol.

In an early study Nelson *et al.* [50] separated thiopurines using both DEAE-Sephadex and a pellicular anion exchanger and found that severe degradation of the thiols could occur in certain buffer systems. Inclusion of mercaptoethanol in the eluent prevented this degradation. Recently Jonkers *et al.* [46] reported that dithioerythritol also improved the RP-HPLC of thiopurines. Presumably other reducing agents such as dithiothreitol and cyanide could have the same effect if included in the eluent, and they might improve peak shape and recovery. Care would be needed to ensure that the conditions would not produce cleavage of disulphides, or interfere with detection.

Detection

Most thiols absorb in the 230–240 nm region with sufficiently large extinction coefficients to enable HPLC separations to be monitored. Disulphides also absorb at these wavelengths. Figure 2 shows the separation of a number of amino-thiols on an SCX column with detection at 232 nm. Sensitivities of 1, 1.5 and 2 nmol injected could be achieved for cysteine, D-penicillamine and thiomalate respectively. Most amino acids absorb in the far UV (e.g. cystine λ_{max} = 197 nm $E = 2.82 \times 10^{-3}$) and can be detected with limited sensitivity at these wavelengths [51]. Unfortunately such wavelengths are inadequate for determining thiols in biological fluids.

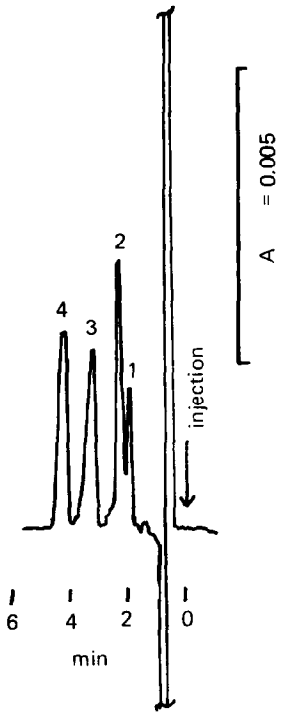


Figure 2
UV detection of amino-thiols at 232 nm following cation-exchange HPLC. A 100 × 4 mm SCX column was used with 0.03 mol/l ammonium acetate pH 2.5 as eluent at a flow rate of 0.5 ml/min. Sample 10–20 nmol of (1) thiomalic acid, (2) cysteine, (3) D-penicillamine and (4) penicillamine disulphide.

All thio-purines absorb strongly in the 300–350 nm range with extinction coefficients sufficiently large to allow their detection in plasma. 6-Mercaptopurine and 6-thioguanine have been quantitated in serum using absorption maxima of 322 and 342 nm respectively [38]. This characteristic absorption band is retained by both their metabolites and their nucleosides and nucleotides, which can thus be determined in tissue extracts using HPLC. Such studies were first performed by Brown [39] using a pellicular ion-exchanger.

Many of the current systems for the monitoring of 6-mercaptopurine [40] and 6-thioguanine [52] in plasma combine spectrometric detection with separation on microparticulate ODS packings and offer simple, relatively specific and reasonably sensitive (10 nmol/l) assays.

The electrochemical activity of thiols has been known since the 1920s. The anodic oxidation of cysteine–cystine was reported in 1923 [53] and in 1933 Brdicka [54] observed a catalytic polarographic wave for thiols in the presence of cobalt(II), which he employed to determine cysteine in proteins. Of more practical benefit was the characterization of the electrochemistry of cysteine at the dropping mercury electrode by Kolthoff and Barnum [55], who found that the wave obtained corresponded to a specific one-electron reaction (Reaction 1). Although satisfactory for many purposes the utility of this reaction in biochemical studies was limited by the mixture of thiols found in physiological fluids.

$$(1) \qquad 2\,RSH + Hg \longrightarrow (RS)_2Hg + 2H^+ + 2e$$

By combining electrochemical determination with chromatography Rabenstein and Saetre produced a major improvement in the assay of thiols in complex mixtures [56]. They constructed a mercury-pool electrochemical detector to monitor the eluate from a cation exchange column. This arrangement was capable of separating amino-thiols and using a working potential of +0.05 V (vs SCE) could detect less than 1 nmol of various thiols. These authors and their collaborators subsequently detailed many applications of this assay; D-penicillamine in blood and urine [8, 57, 58], cysteine and homocysteine in plasma and urine [59] and cysteine and glutathione in fruit [60]. Later Kucharczyk and Shaninian [29] reported using a laboratory polarograph with a dropping mercury electrode as an HPLC detector to determine D-penicillamine.

The introduction of a commercially available (Bioanalytical Systems) gold electrode, on which a mercury amalgam could be formed, widened the availability and thereby the application of these procedures. The use of a static electrode also increased the sensitivity of the system and allowed meaningful studies on the pharmacokinetics of a number of thiol drugs to be performed. Wiesner *et al.* [61] mentioned that a gold/mercury amalgam electrode was used to determine penicillamine levels following both intravenous and oral administration of the drug. A similar determination following cation exchange separation was described in more detail by Bergstrom and colleagues [62] and later applied to characterize protein binding and plasma kinetics for penicillamine [10]. Various other authors have also used this electrode with penicillamine [35, 63], and sensitivities of less than 100 fmol injected have been achieved.

Few other applications to either biological thiols or other drugs have been reported. The number of publications concerning penicillamine reflects not only interest in the drug but also the fact that its plasma levels are above those of similar compounds, so the difficulties of the assay are reduced. Perrett and Drury [42] used a gold/mercury electrode to detect captopril in plasma following reversed-phase chromatography (Fig. 3A) and the present authors have also shown that the method could be applied to 6 mercaptopurine. Bergstrom *et al.* [62] showed that at high sensitivity the electrode can lose sensitivity, and noise levels can increase due to fouling of the mercury amalgam. This necessitates both frequent injections of standards and tedious cleaning of the electrode plus re-making of the amalgam. Nevertheless, with due care, the Au/Hg electrode is precise, extremely sensitive and specific towards thiols.

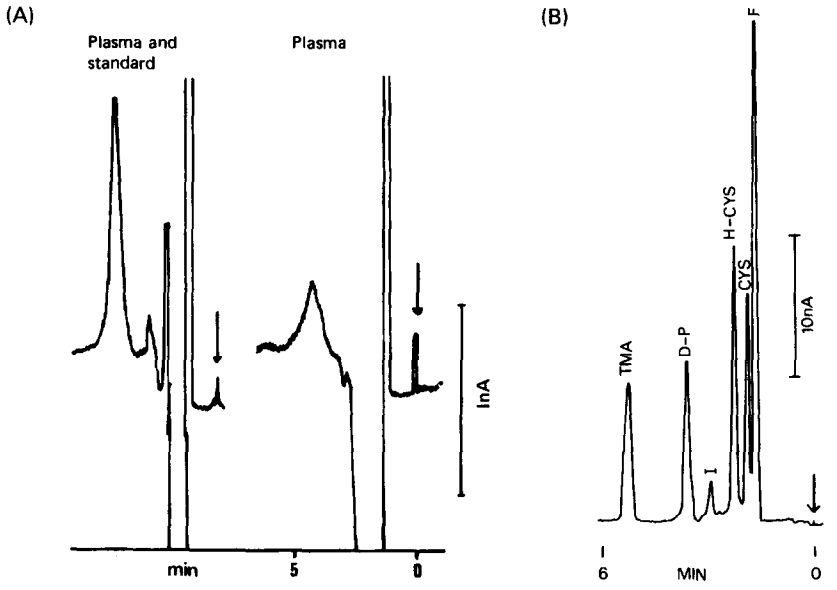


Figure 3
Electrochemical detection of thiols. (A) Determination of captopril in plasma (approx. 250 nmol/l) and with added standard at a Au/Hg electrode at +0.08 V. (B) Detection at a gold electrode at +0.7 V TMA = thiomalic acid, D-P = D-penicillamine, H-CYS = homocysteine, CYS = cysteine, and F = frontal artefact. Sample size — 50 pmol injected. Both separations were by reversed-phase chromatography as in [48].

The use of carbon electrodes was introduced by Mefford and Adams [64] who separated GSH and cysteine by cation-exchange chromatography and detected the thiols by electrochemical detection at +1 V. They were able to determine as little as 5 pmol of the thiols in acid extracts of animal tissue. They also reported retention data and electrochemical responses for six related thiols. The ready availability of glassy-carbon electrodes with electrochemical detectors has led some workers [43, 65, 66] to use these to detect D-penicillamine. The maximal voltagraphic response for simple thiols occurs at a substantially higher voltage, e.g. +1.0 − +1.5 V vs Ag/AgCl, than with the Au/Hg electrode. The nature of the oxidation reaction, for reasons to be discussed later, is not clear. The high working potential means that many other electroactive materials in plasma and urine interfere, so the demands on the chromatographic separation are increased. Stulik and Pacakova [67] determined thiouracil at +1.4 V using a carbon paste electrode but did not apply their findings to biological materials.

Metal electrodes are commonly used in classical electrochemistry but such electrodes are not readily obtainable for commercial HPLC detectors. Kreuzig and Frank [68] oxidized D-penicillamine on a gold electrode at +0.8 V following cation exchange chromatography and showed that the system possessed comparable sensitivity to the Au/Hg electrode. Rudge *et al.* [48] used a gold electrode and RP-HPLC to measure picomole levels of thiomalate in the plasma and urine of rheumatoid arthritis patients receiving sodium aurothiomalate injections. The same system has recently been applied to captopril [69] (Fig. 3B).

Perrett [70] investigated the use of various electrode materials for the determination of electroactive compounds including a number of thiols. Many thiols were found to react in a semi-specific manner with metal electrodes. All thiols could be detected at working

potentials above 1 V on both metal and carbon surfaces. Some, including thiomalate and penicillamine, also exhibited a second maximum in the region 0.7–0.8 V with the gold electrode. Of the thiols investigated using a platinum electrode, this secondary peak only occurred with cysteamine and extended to such low voltage (+0.4 V) that the possibility of a highly specific assay was suggested. If, as has been suggested [65], the peak at voltages greater than 1 V is due to the direct oxidation of the thiol to the disulphide, the reason for some thiols giving two peaks is unclear. Possibly the lower voltage is associated with an intermediate oxidation product or the formation of an unstable mercaptide. With physiological fluids, metal electrodes are less specific than mercury electrodes but are more specific than carbon electrodes, which are maintained at even higher voltages: the higher the voltage the larger the number of endogenous compounds detected in samples. We have found the gold electrode to be more robust than the mercury electrode when crude physiological samples are being analysed.

The formation of electro-active derivatives of thiols and the determination of disulphides following electrolytic reduction will be discussed later.

Derivatization Procedures for Thiols

The comparative advantages of pre-column and post-column derivatization methods for HPLC have been discussed in detail by others [71, 72]. Both approaches have been used to determine thiols. Although derivatization of the -SH group would be the obvious method, many workers, particularly those measuring amino-thiols, have modified other functional groups.

Derivatization of the amino-group with ninhydrin is a standard technique and has been frequently described [73]. Cysteine and the other amino-thiols not only give poor responses with ninhydrin [22] but also with the new fluorogenic reagents for amines, *o*-phthalaldehyde/mercaptoethanol (OPA/ME) [74] and fluorescamine [75], an effect apparently due to the presence of the -SH group [76]. Oxidation of the thiol by on-line addition of hypochlorite or *N*-chlorosuccinimide [77, 78] gives a substantially increased fluorescence.

To determine glutathione, Reed *et al.* [79] formed the *S*-carboxymethyl derivative by reaction with iodoacetic acid followed by chromophore formation of the amino-group with 1-fluoro-2,4-dinitrobenzene. The DNP derivatives have been separated on amino-propyl packings [79] and -NH_2 columns [80]. Similarly Cooper and Turnell [81] reduced cystine with ME and reacted it with iodoacetic acid prior to derivatization with OPA/ME. The derivatives were then separated by RP-HPLC. Mackey and Beck [82] oxidized amino acids with performic acid prior to the formation of the dimethylamino-naphthalene sulphonyl (DNS)-amino acids, which can be separated by RP-HPLC with detection at 254 nm. All these systems are non-specific and require the resolution of the amino-thiol from all other amino compounds, which can number many hundreds in physiological fluids. Such difficulties are highlighted by the extensive sample clean-up needed in a recent method to measure cystamine using OPA/ME [83].

Derivatization of the thiol group offers an equally wide choice of reagents, more selectivity, and therefore simpler and quicker chromatography. Pre-column derivatization of the -SH group can offer a number of potential advantages since blockage of the active thiol should reduce losses during storage as well as improving the chromatography. Disadvantages could be (a) slow reaction times giving rise to disulphide formation; (b) the need to remove other thiols, in particular proteins, prior to reaction;

and (c) the possibility of partial reactions and/or side reactions. With post-column derivatization the problems of poor chromatography and storage loss experienced with thiols would persist, but incomplete reaction and length of reaction time could be less important.

Post-column derivatization of the -SH group

Some of the earliest applications of this technique are to be found in the amino acid literature, the determination of amino acids with ninhydrin being an obvious example of post-column derivatization. A number of authors used split-stream techniques after cation-exchange separation to apply more specific reagents.

The selective reaction of thiols and metal ions offers a simple reaction which is readily adapted for post-column detection. Barber [84] used the iodoplatinate reagent of Winegard *et al.* [85] to monitor specifically the effluent from an amino acid analyser. The composition of the reagent, which is bleached by sulphur-containing compounds, was improved by Awward and Adelstein [86]. Fowler and Robins [87] later applied it to studies of sulphur amino acids in metabolic disorders. A modification of this reaction to give a fluorescent end-point will be discussed later. Recently Witt and Wilson [88] measured penicillamine by a continuous flow procedure based on an earlier colorimetric method [89]. The column eluate was mixed with ferric chloride, which forms a blue complex absorbing at 600 nm with thiols (Fig. 4a). Urine from subjects receiving penicillamine gave two peaks, one for the drug and another for endogenous cysteine.

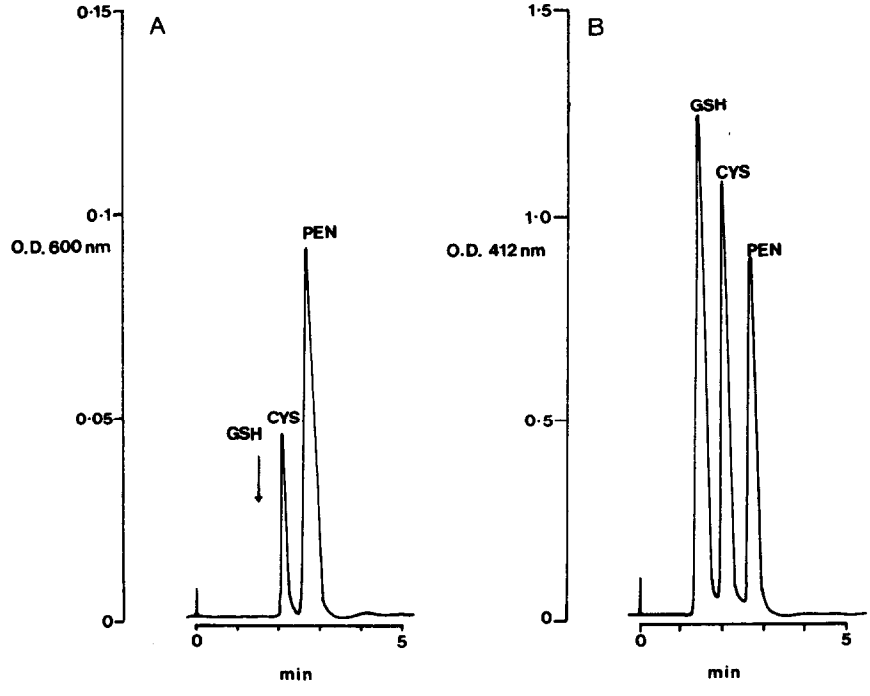
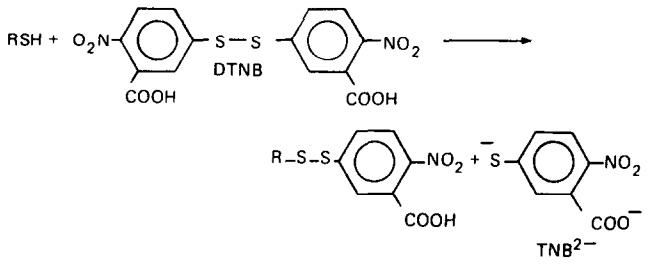


Figure 4
Post-column detection of thiols using (A) ferric chloride and (B) Ellman's reagent with colorimetric detection following separation on a cation exchange column. Chromatogram by courtesy of Dr I.D. Wilson.

The reagent is certainly economical but care is needed to avoid the formation of precipitates of ferric hydroxide. The low sensitivity (1 nmol on column) limits the application to the determination of urinary levels.

In the above-mentioned study [84], Barber employed a triple manifold to monitor the sulphur amino acids, one line using ninhydrin, another iodoplatinate and the third nitroprusside. Disulphides were reduced with dithiothreitol, rather than the more usual cyanide, prior to reaction with nitroprusside. Walsh *et al.* [90] monitored preparative scale chromatography columns with both a cyanide/nitroprusside reagent and a dithioerythritol/arsenite/DTNB reagent. Ellman's reagent (DTNB) was used as a post-column reagent with HPLC [91]. With a reaction time of 17 s the disulphide exchange went to 90% completion and the resulting chromophore was monitored at 412 nm (Reaction 2). The reaction had a wide linear range and was very robust. Penicillamine could be detected in urine but the sensitivity is probably insufficient to measure the drug in plasma (Fig. 4b).

(2)

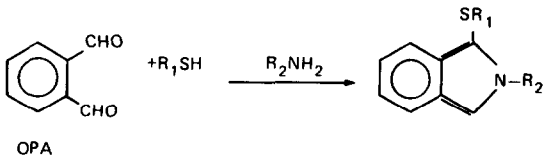


6,6′-Dithiodinicotinic acid (TNA) is superior to DTNB both because the product forms at a lower pH (6.5–7.5) and the absorption maximum is 344 nm, close to a mercury line commonly used in HPLC detectors [92]. Nishiyama and Kuninora used TNA in a post-column reactor after separating seven common thiols on an ODS column [93]. For cysteine sensitivities of 100 pmol were achieved but cyclic thiols such as thiouracil and thiolhistidine gave 80% less sensitivity. Since the products are stable TNA would appear to be a good candidate as a pre-column derivatization reagent.

Although direct UV monitoring of thiopurines is possible, sensitivity can be poor and more sensitive fluorimetric techniques have been developed. Thiopurines can be oxidized by chromate to purine 6-sulphonates, which fluoresce in alkaline solution [94]. Jonkers *et al.* [46] performed this oxidation on-line following ion-pair reversed-phase HPLC achieving a sensitivity of 2 ng/ml of plasma.

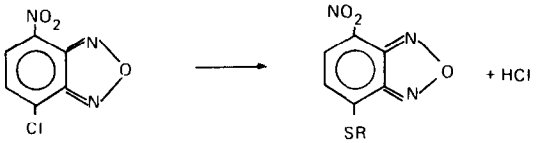
Although the OPA/ME method is not well suited to measuring amino-thiols the reagent is not restricted to ME; ethanethiol is frequently used and OPA has been used to determine GSH [19]. Nakamura and Tamura [95] turned the assay about and investigated the use of OPA to measure thiols (Reaction 3) in the presence of a fixed concentration of an amino acid (taurine). Using a flow injection technique some 34 thiols

(3)



and related compounds were investigated. In general the simpler thiols, e.g. ME, ethanethiol, gave the most intense fluorescence, whilst dithiols and disulphides gave some 100-fold less. The thiopurines did not give any fluorescence. It was found that β-mercaptopropionic acid gave the most fluorescence (30% more than ME); a finding also reported in two amino acid analysis studies using OPA [96, 97]. The Japanese workers used a post-column reaction system to monitor the elution of a mixture of thiols from an anion exchange column and achieved sensitivities in the range 30–100 pmol. Data from amino acid analysis studies [98] suggest that sensitivity could be improved three-fold by replacing taurine with alanine, leucine or several more reactive amino acids.

Recently interest has re-focused on a group of benzofurazans, first described in 1968 [99] as possible anti-cancer agents, and their chemistry has been reviewed [100]. The reagent orginally described was 4-chloro-7-nitrobenzofurazan (NBD-Cl) and it was shown to form highly fluorescent derivatives with amino-, mercapto- and imino groups (Reaction 4). The thio derivatives are formed at a lower pH than the amino derivatives

(4) RSH + 

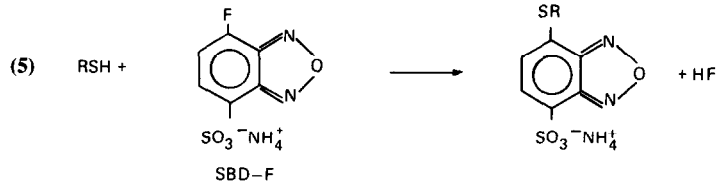
and absorb at lower wavelengths (425–435 nm). NBD-Cl has had only limited use as a reagent for amino acid analysis since the reaction conditions (65°C for 5–15 min) are rather unfavourable for post-column reactors [101, 102]. In an attempt to modify these conditions Imai and Watanabe [103] synthesized the fluoro-derivative NBD-F, which was found to give faster reactions and a more intense fluorescence. These authors [104] recently used NBD-F in two post-column reactors connected to an amino acid analyser: one optimized for amino plus imino acids and the other for thiols. The major difference between the two systems was the pH, which for amino groups was *ca* 10 and for thiols was *ca* 4. In both cases the reaction time was *ca* 14. The difference in pH produced a dramatic change in selectivity of the reagent. Excitation was at 450 nm and emission at 520 nm, with detection limits of about 50 pmol. Umagat *et al.* [102] measured the NBD-amino acids with increased sensitivity by excitation at 220 nm with the emission measured using a 370 nm cut off filter. Unfortunately NBD-F possesses several disadvantages. It has poor solubility in aqueous buffers, is possibly both mutagenic and cytotoxic [105] and, particularly with thiols, may undergo a number of secondary reactions.

Pre-column Derivatization

The first colorimetric reagent to be used for pre-column derivatization was Ellman's reagent (Reaction 2). Reeve *et al.* [106] used it to measure GSH in rat liver. Following reduction with dithioerythritol, mixed disulphides were formed with DTNB and separated by RP-HPLC with an isocratic ammonium formate pH 5–methanol (90:10 v/v) eluent. The eluate was monitored not at 412 nm but at 280 nm and the disulphides of GSH, cysteine and γ-glutamyl-cysteine detected with a sensitivity of 5 μmol/l. In a more detailed study, Kuwata *et al.* [107] used the same technique to quantitate alkyl-thiols in a variety of environmental and food samples. Atmospheric thiols were trapped by

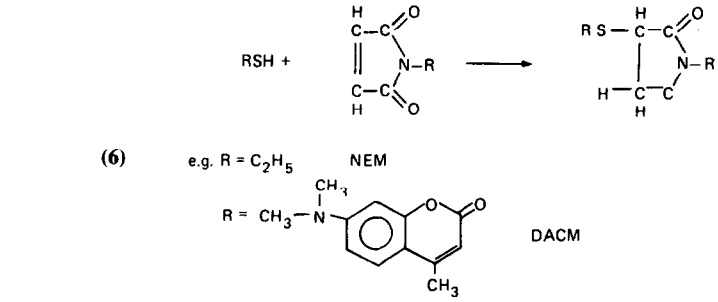
bubbling up to 150 l of air through a DTNB solution buffered to pH 8. Using gradient elution from a reversed-phase column two sets of conditions were necessary; one to separate C1–C4 thiols and the other for C5–C7 thiols. Altogether some 13 thiols were resolved. For most thiols the absorption maximum was at 330 nm and the derivatives were stable for at least three weeks.

Andrews *et al.* [108] reduced the problems associated with the use of NBD-C1 and NBD-F, mentioned earlier, when they introduced ammonium 4-chloro-7-sulfobenzofurazan which is water-soluble, stable, non-toxic and can be highly specific for thiols. It also gives greater relative fluorescent intensities (excitation 380 nm, emission 510 nm) than NBD-C1 and the fluorogens are more stable. As in the case of NBD-F, Imai *et al.* [109] synthesized the fluoro derivative (SBD-F) which was again superior to the chloro-derivative but the optimum reaction conditions (60°C, pH 9.5, 1 h) proved unsuitable for post-column derivatization (Reaction 5). The reagent was, however, suitable for pre-



column derivatization, the derivatives being separated by reversed-phase HPLC. Toyo'oka and Imai [110] determined cysteine, homocysteine, cysteamine, reduced glutathione and *N*-acetyl-cysteine directly with detection limits of 0.07–1.4 pmol. Cystine and oxidized glutathione were determined by reducing them to the corresponding thiol using tri-*n*-butylphosphine.

In 1949 Friedmann and co-workers [111] showed that, at pH 8, thiols attached to the C = C double bond of certain maleimides such as *N*-ethyl maleimide (NEM) (Reaction 6).



NEM has remained an invaluable reagent for thiol determinations ever since but, unfortunately, its adjuncts are only weak chromophores. Most workers therefore used radio-labelled NEM to achieve sufficient sensitivity, and employed paper or TLC to separate the products formed in biological extracts. Many substituted maleimides have been synthesized to produce histochemical stains for -SH groups in proteins. Although not directly developed for quantitative analytical work many of them have proved useful reagents for pre-column derivatization of thiols [for a review, see 112]. Maleimides that

can be directly determined by UV detection, fluorescence or electrochemistry have now been synthesized.

Kawahara *et al.* [113] used maleimides containing UV chromophores to quantitate captopril in plasma but it was necessary to extract and concentrate the derivatives to gain the necessary sensitivity. They employed *p*-bromophenacyl bromide (BPB) and *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM); both reagents reacted rapidly (*ca* 10 min) with the drug at neutral pH. Values of λ_{max} for the two derivatives were *ca* 260 nm and *ca* 245 nm respectively. Compared with the fluorescent maleimides the chromatograms obtained using these reagents with RP-HPLC and 254 nm detection were complex and the chromatography was therefore more exacting. Even so the method has been applied by both the original workers [114] and others [115] to measure captopril's pharmacokinetics. Recently, Chang *et al.* [116] synthesized two thiol-specific reagents 4-dimethylaminoazobenzene-4′-*N*-maleimide (DABMA) and 4-dimethylaminoazobenzene-4′-iodoacetamide (DABIA) with the aim of improving the chromatography of cysteine-containing peptides by the introduction of a hydrophobic chromophore. Both reagents reacted with the peptides at room temperature within 1 h and the resulting derivatives could be separated by RP-HPLC with detection at 436 nm. Both reagents could apparently be adapted to more quantitative studies.

By blocking the -SH group of mercaptopurine with NEM the characteristic absorption band at 330 nm is lost and a new maximum at 280 nm is produced: this wavelength is the same as that for azathioprine. Using this derivatization, followed by ethyl acetate extraction and RP-HPLC, a method for the simultaneous measurement of both drugs in plasma has been developed [117].

Kanaoko and co-workers [118, 119] synthesized a series of non-fluorescent maleimides with imidazole side chains that produced highly fluorescent thiol adducts. Nara and Tuzimura [120] found that *N*-(9-acridinyl)maleimide (NAM) gave strongly fluorescent products (excitation wavelength 362 nm, emission wavelength 426 nm) when reacted with cysteine at pH 3–6. Later they separated the reaction products with glutathione, homocysteine, coenzyme A and cysteine by RP-HPLC [121]. NAM has also been used as a post column reagent to detect cysteamine, cysteine, reduced glutathione and *N*-acetyl cysteine following separation on an amino-silica column. Heating the reactor to 70°C with a dwell-time of about 4 min gave sensitivities of 100 pmol [122].

Klasen [123] used *N*-(7-dimethylamino-4-methylcoumarinyl) maleimide (DACM) to detect cysteinyl peptides in peptide maps. Kagedal and Kallenberg [124] employed both DACM and *N*-(1-pyrene) maleimide (PM) to derivatize mercaptoacetate and *N*-acetyl cysteine prior to ion-pair RP-HPLC (Fig. 5). Derivatization required approx. 20 h at 37°C (pH 9) but the products were stable for up to 4 days. Detection limits were better for DACM (50 fmol) than PM (400 fmol), but different excitation wavelengths were required. PM was also the derivatization reagent chosen by Jarrott *et al.* [125] for the determination of captopril in plasma: reaction products were separated by RP-HPLC. Miners *et al.* [126] based an assay for D-penicillamine on derivatization with *n*-[*p*-(2-benzoxazolyl)-phenyl] maleimide (BOCM) followed by isocratic RP-HPLC. The fluorescent derivative was excited at 319 nm and the emission was measured with a 360 nm cut-off filter. The reaction time could be reduced to 2 h by heating the mixture to 60°C but the authors used 20 h at 37°C (pH 5) for convenience. Concentrations of 250 nmol/l of D-penicillamine in plasma could be determined.

Although thiols are inherently electroactive they can be unstable in physiological fluids. Shimada *et al.* [127] used derivatization with *N*-(4-anilinophenyl)maleimides

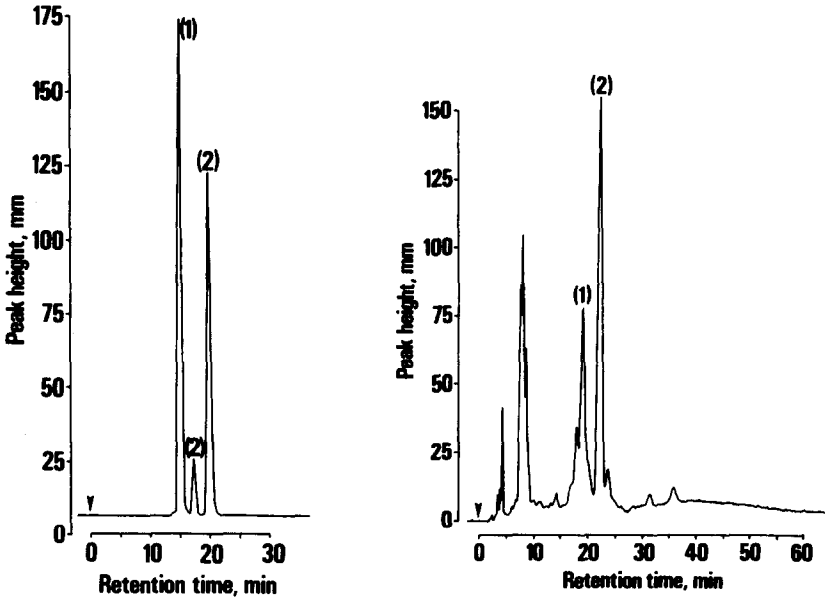


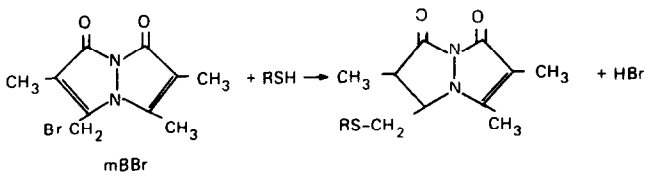
Figure 5
Pre-column derivatization with maleimides. (A) Determination of mercaptoacetate (1) and *N*-acetylcysteine (2) following derivatization with DACM. (B) Chromatography of urinary thiols following derivatization with PM. Separations used ion-pair reversed-phase chromatography. Reproduced with permission from ref. [124].

(APM) to confer both stability and electrochemical reactivity. They tested DAPM, APM and ANM with *N*-acetyl cysteine, cysteine, glutathione and D-penicillamine. The reaction was complete in 10 min at 0°C. Maximum sensitivities achieved for DAPM, ANM and APM were 60, 40 and 20 pg injected respectively. All four thiol derivatives could be separated in 12 min using isocratic elution from a C18 column. Under certain conditions two peaks were obtained with both D-penicillamine and cysteine due to a rearrangement of the adduct's sidechain. Despite giving the worst sensitivity DAPM was used for the determination of captopril and a detection limit of 10 ng/ml was claimed [128].

The use of maleimides is increasing but there have been reports that some maleimide–thiol adducts are not stable with time, and that succinimide ring cleavage can give rise to two fluorescent products [127, 129]. Tests are therefore necessary to establish that each thiol-maleimide combination is stable during both storage and analysis.

Syn-9,10-Dioxabimanes are a class of fluorescent compounds recently synthesized by Kosower *et al.* [130]. The bromo-derivatives (monobromo-, dibromo- and monobromotrimethylammoniobimane) are an exception since they are non-fluorescent but are able to react specifically and rapidly (2–60 min, room temperature, pH 7.4) with thiols to produce stable highly fluorescent compounds (Reaction 7). These new reagents

(7)



were applied to the labelling of thiols and the free -SH groups of proteins [131]. Later the -SH groups of haemoglobin were labelled before hydrolysis and peptide mapping [132]. Monobromobimane (mBBr) and dibromobimane (dBBr) were shown to cross cell membranes and label the intra-cellular thiols. Excitation maxima for the bimanes varied with both the thiol and the solvent system but were in the range 370–396 nm, with emission maxima between 475 and 484 nm. In a later study [133], the bimane derivatives of 14 biological thiols were separated by two-dimensional TLC/electrophoresis. Picomole sensitivity was obtained by separating bimane derivatives by RP-HPLC [134]. Using a methanol–dilute acetic acid gradient some 20 thiols could be separated in 30 min. Problems with the formation of hydrolysis products and impurities were noted.

To date few analysts have used bimanes but mBBr is now commercially available (Thiolyte, Calbiochem) and its use should increase rapidly. Vogel and Lumper [135] labelled NADPH-cytochrome P-450 reductase and separated the cysteine hydrolysis products by HPLC. To reduce side reactions derivatives were formed at 4°C. A similar reaction procedure (4°C overnight) has been employed by Burton *et al.* [136] in a recently developed assay for 6-mercaptopurine in plasma (Fig. 6).

Dansylaziridine (5-dimethylaminonaphthaline-1-sulfonylaziridine) was used by Scouten *et al.* [137] to label thiols, and this reagent was adapted for the pre-column derivatization of thiols by Lankmayr *et al.* [138]. Maximum yield was achieved at pH 8.2 after 1 h at 60°C with an excess of reagent: under such conditions only free -SH groups were shown to react. Weaker nucleophiles such as amines and alcohols were not derivatized. The reaction products were stable and could be separated by RP-HPLC in the presence of ethylenediamine as an ion-pair. Fluorescence determination used excitation at 338 nm and emission at 540 nm. The method was also applied to the measurement of D-penicillamine in plasma [139]. Although a detection limit of 12 pmol was claimed, chromatography was relatively slow in order to resolve the drug from a number of endogenous reactants and from the excess dansylaziridine, which eluted with a retention time twice that of the D-penicillamine product.

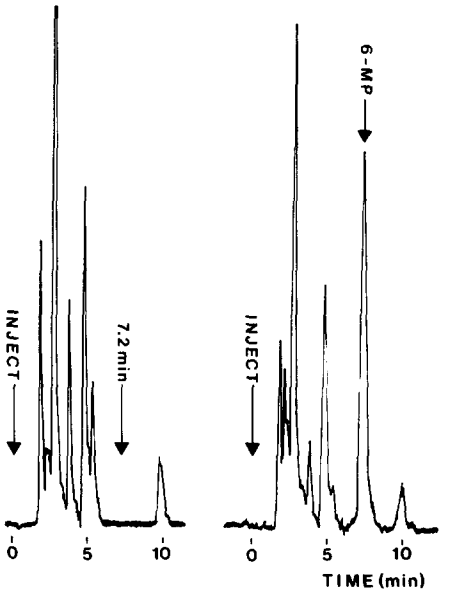


Figure 6
Determination of 6-mercaptopurine in plasma by pre-column derivatization with monobromobimane followed by reversed-phase chromatography (150 × 5 mm ODS Hypersil; 80:20 v/v 1% acetic acid: Acetone; 1 ml/min; excitation at 395 nm, fluorescence at 455 nm). (A) Blank plasma and (B) plasma from patient receiving 6-mercaptopurine. Chromatogram by courtesy of N. Burton, University of Surrey.

To measure 2-mercapto-propionylglycine in urine Springolo *et al.* [140] introduced a novel reagent, 2-furonyl chloride. The two compounds reacted quantitatively at pH 7 within 1 min and the resultant products, 2-furoyl-MPG and 2-furoyl chloride, could be rapidly separated by RP-HPLC and monitored at 290 nm with a detection limit of 1 μg/ml. Since the detection system was relatively non-specific some sample clean-up was required.

Alkaline permanganate oxidation of 6-thiopurines and its derivatives gives highly fluorescent compounds that can be separated by anion exchange chromatography [141]. Herbert *et al.* [142] have modified this method to include a dual column (anion and RP) separation of the oxidation products for improved resolution, and dual monitoring (fluorescence and UV) for improved identification.

Disulphides and Related Compounds

There are two approaches to the analysis of disulphides using HPLC, direct determination by detection of the disulphide bond or another group, and determination after reduction to the corresponding thiol(s): the second approach is more often chosen. The thiols are liberated from both disulphides and, particularly in the case of pharmacological studies, from binding sites on proteins. Subsequent chromatography of thiols liberated off-line also supplies information on the nature of mixed disulphides.

Disulphides can be reduced to the corresponding thiols either chemically or electrochemically. Electrochemical reduction is probably the more successful approach since disulphides can be completely reduced to thiols at voltages of approximately −1 V (Reaction 8). Saetre and Rabenstein [8, 59] reduced penicillamine disulphide in protein-precipitated plasma and blood at an Hg pool electrode before chromatography and electrochemical detection. Samples, placed in a number of electrolysis cells connected in series, were reduced at −6 V prior to HPLC injection. Comparison of peak size before and after electrolysis was used to calculate the concentration of the disulphide. The same procedure was followed by Bergstrom *et al.* [62].

$$\text{RSSR} + 2\text{H}^+ + 2\text{e} \longrightarrow 2\text{RSH} \qquad (8)$$

For quantitative analytical work, such procedures assume that only one disulphide is present and that it is totally reduced. In contrast to the released thiols no chromatographic identification of the disulphides is achieved. To overcome this disadvantage Eggli and Asper [143] introduced a dual electrode flow-through detector for the determination of both thiols and disulphides. The first electrode (reduction cell) consisted of a column of amalgamated silver grains through which the eluate containing the thiols and disulphides flowed. The disulphides (in this case cystine) were reduced and immediately entered a mercury pool electrode (detection cell). The dual cell was placed at the outlet of a cation exchange column. In the experimental conditions (pH 2.6, 0.09 M phosphate/citrate, flow-rate 0.3 ml/min) complete reduction of cystine was achieved at below −1.1 V. Simultaneous separation and detection of cysteine, cystine and penicillamine was demonstrated. In a detailed study [144], Allison and Shoup adapted the dual electrode procedure to use two commercially available gold/mercury amalgam cells as the reducing and detecting cells with reduced and oxidized glutathione as the model compounds. They employed the same system to study the chromatographic behaviour of thiols and disulphides [44]. The efficiency of the flow-through reducing

electrode is limited by a number of factors, including diffusion coefficient, pH, applied potential and (most importantly) the structure of the disulphides. Penicillamine disulphide reduction is sterically inhibited by its two methyl groups and although it can be detected the sensitivity is greatly reduced compared with cystine [145]. A cysteine–penicillamine mixed disulphide would be expected to have an intermediate level of sensitivity (Fig. 7).

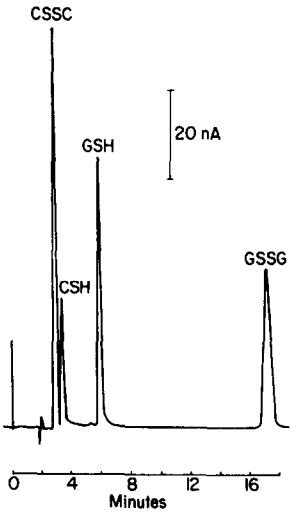


Figure 7
On-line electrochemical reduction of disulphides at a Au/Hg electrode at −1 V followed by detection of the formed thiols at a second Au/Hg electrode at +0.15 V following ion-paired reversed phase chromatography. CSSC = cystine, CSH = cysteine, GSH = reduced glutathione and GSSG = glutathione. By courtesy of Bioanalytical Systems.

Studebaker [146, 147] developed a number of solid-phase reaction systems capable of reducing disulphides in the eluate from an HPLC column. In one scheme, the eluate passed through a short column packed with thiol–Sepharose, which reduced cystine. Cysteine was then detected with a standard post-column reactor using DTNB. Alternatively [147] the DTNB reaction was replaced with polymer-bound chromophore (i.e. *N*-dinitrophenylcysteine bound to Sepharose) which was released in the presence of thiols. By coupling the DNP–Sepharose column after a thiol–Sepharose column it was also possible to detect disulphides in HPLC eluents. The system was tested using cystine and cysteamine: sensitivities in the low nanomole range were achieved but no practical application of this promising approach have been reported. Possibly sensitivity could be enhanced by coupling fluorigenic compounds to the polymer.

The most common chemical agents used to reduce disulphides are mercaptoethanol, dithiothreitol, dithioerythritol, cyanide and sodium borohydride. It has long been recognized that their ability to reduce disulphide bonds can vary considerably, particularly for mixed disulphides. For instance cyanide completely reduces cystine in 90 min at room temperature but requires over 24 h to reduce cysteine–penicillamine disulphide completely: penicillamine disulphide is not reduced at all [148]. Bir *et al.* [149] reported the same difficulties when attempting to reduce sterically hindered disulphides with borohydride. Abounassif and Jefferies [43] claimed that an 89% yield of penicillamine from its homodisulphide could be achieved by incubation with a 10-fold excess of DTT at 60°C for 3–4 h. Comparative studies with the drug's major metabolite, cysteine–penicillamine disulphide, or with protein-bound penicillamine were not quoted. Large amounts of DTT can produce both chromatographic and detection problems. Both DTT and its oxidized form are readily separated by RP-HPLC and can

be determined by detection at 254 nm [150]. These products can also be detected electrochemically but in the authors' experience injection of excessive amounts of DTT rapidly degrades electrodes, particularly gold/mercury amalgam surfaces. Some workers, e.g. [84], have used DTT and cyanide in post-column reaction systems with amino acid analysers but this approach has apparently not been applied to HPLC systems.

The other option available for the measurement of disulphides is direct determination. Disulphides, like thiols, can be detected with limited sensitivity at *ca* 240 nm (Fig. 2) or in the case of the thiopurines at *ca* 330 nm. Since disulphides are electroactive they may be detected at glassy-carbon electrodes maintained at high potentials. If the chromatographic conditions are correct systems designed to detect thiols may also detect disulphides. Abounassif and Jefferies [43] found that cystine and cysteine–penicillamine disulphide gave a maximum response at +0.9 to 1.1 V and penicillamine disulphide a maximum response at +1.2 V. However, the sensitivity was 1000-fold less than for the corresponding thiols. The chemistry of the electrochemical oxidation was not discussed.

Few colorimetric methods are available that are specific for either the disulphide bond or for sulphur. The ability of sulphur-containing compounds to bleach iodoplatinate solutions has already been mentioned [85]. Palladium(II) can quench the fluorescence of the indicator calcein but in the presence of thio-compounds the Pd(II) is partially complexed with sulphur so releasing fluorescent calcein [151]. Incorporating this principle into a post-column reactor Werkhoven-Gowrie *et al.* [152] were able to detect a number of sulphur-containing pesticides as well as cystine, cysteine, methionine and reduced glutathione in physiological fluids (Fig. 8). Even at the elevated temperature used (65°C, 7 min) the displacement reaction was only 12% complete and detection limits were therefore only in the range 0.5–5 ng. Since this method should detect all the metabolites of a sulphur-containing drug including *S*-methyl derivatives, it possesses great potential, though the authors have achieved less than one-tenth of the sensitivity claimed [152], possibly due to differences between fluorescence spectrometers.

Conclusions

Although many procedures are now available for the assay of thiols, difficulties still exist in the measurement of thiols in physiological fluids. All the HPLC methods discussed are capable of good resolution and sufficient sensitivity for many studies. It is clear, however, that sample preparation plays a paramount role in attaining good results. Many thiols, particularly the aliphatic thiols, are unstable in solution unless precautions against oxidation are taken. This problem is considerably greater in physiological fluids. Binding to proteins both *in vivo* and *in vitro* is so rapid that some thiols can be completely bound within a few minutes. Steps to prevent this must therefore be taken. Where sample numbers are large and assays can be performed within a few days of sample collection the combination of HPLC and electrochemical detection with metal electrodes offers both speed and sensitivity. Good column maintenance is essential to avoid poor chromatography, 'late runners' and 'ghost' peaks.

Fluorescent derivatization methods permit sensitive determinations without chromatographic problems, but no ideal reagent exists as yet. Derivatization procedures that involve either long periods at or below room temperature or short periods at elevated temperatures all risk oxidation of the sample to undeterminable disulphides. For the analysis of thiol drugs sufficient reagent must be added not only to derivatize the drug but also all endogenous thiols. This excess reagent may cause chromatographic

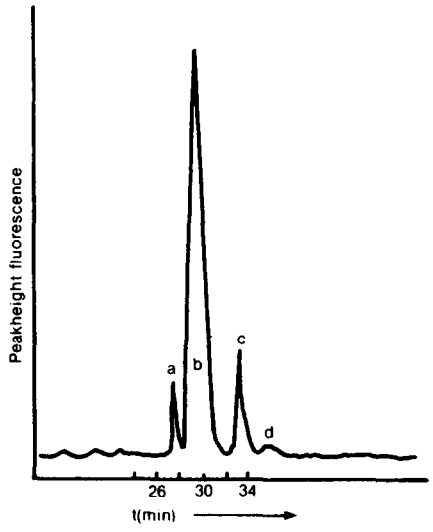


Figure 8
Post column detection of sulphur containing amino acids by reaction with a palladium(II)-calcein reagent following cation-exchange separation: (a) glutathione, (b) cystine, (c) cysteine and (d) methionine. Reproduced from ref. [152] with permission.

problems. Doubts about the stability of some thiol–maleimide combinations have been raised but some maleimides give high sensitivity. The bimanes offer rapid derivatization but side reactions limit their use at present. A wider choice of purer fluorogenic reagents will be needed for their more general applicability to be tested.

For true pharmacokinetic and metabolic studies it is essential that thiols and disulphides are not measured in isolation. Pre-column reduction procedures partially overcome this problem but qualitative data on the disulphides is missing. Post-column reduction can be restrictive since sensitivity depends on the ability of the reactor to reduce disulphides: in some cases this is impossible. Only one study investigating the kinetics of a thiol drug has measured both the free thiol and its disulphides. Kyogoku and colleagues [66] found it necessary to employ two different chromatographic methods to study D-penicillamine metabolism. Until an HPLC equivalent to a GLC sulphur-specific detector is developed, multiple HPLC analyses seem the only workable approach to complete qualitative and quantitative analyses.

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