Simple methods for the detection and quantification of thiols from *Crithidia fasciculata* and for the isolation of trypanothione

Daniel J. STEENKAMP

Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, South Africa

Methods for the qualitative and quantitative analysis of thiols by means of the fluorogenic reagent 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin are described, with particular reference to the trypanosomatid metabolites glutathionylspermidine (GSH–spermidine) and trypanothione. Second-order rate constants for the derivatization of seven different thiols under defined experimental conditions and at 21 °C varied between 619 ± 34 and 10560 ± 236 $M^{-1} \cdot s^{-1}$. T.l.c. of the thiols from *Crithidia fasciculata* was used to monitor the purification of trypanothione from this organism in three steps involving adsorption, ion-exchange and reversed-phase chromatography. The yield was approx. 50 mg of pure trypanothione from 100 g (wet wt.) of trypanosomatids. The method for the quantitative analysis of biological thiols is based on fluorometric detection after separation by reversed-phase or ion-paired chromatography on a phenyl-silica column. Analysis of the thiol composition of cell lysates prepared under non-denaturing conditions point to the rapid degradation of the GSH–spermidine conjugates. In addition to GSH, GSH–spermidine and trypanothione, at least one other prominent thiol was detected, and the contribution of this thiol to the total thiol content in the various growth phases of *C. fasciculata* was investigated.

INTRODUCTION

During the past two decades, several biochemical peculiarities of the Trypanosomatidae have been delineated in studies aimed at the rational development of new anti-trypanosomal and anti-leishmanial drugs [1]. One of the most extraordinary deviations of their metabolism from that of their mammalian hosts involves the biosynthesis and utilization of conjugates of GSH and spermidine [2].

The reduction of GSH in these organisms is dependent on non-enzymic thiol–disulphide exchange from trypanothione, a bis(glutathionyl)spermidine [bis(GSH)–spermidine] adduct peculiar to trypanosomatids [3]. The reduction of trypanothione by NADPH is catalysed by trypanothione reductase [4], which fulfils a function similar to that of glutathione reductase in other organisms.

MonoGSH–spermidine also serves as a substrate of trypanothione reductase [5], and since the biosynthesis of trypanothione proceeds via N^1- and N^8-GSH–spermidine, both trypanothione and the two monoGSH–spermidine conjugates occur *in vivo* and are potential substrates of trypanothione reductase [6,7].

Enzyme kinetic studies of trypanothione reductase have been hampered by the limited availability of trypanothione. In their pioneering study, Fairlamb et al. [3] isolated only 300 μg of the substance from 118 g (wet wt.) of *Crithidia fasciculata*. Consequently monoGSH–spermidine, which can be isolated in significant amounts from *Escherichia coli* [8] was used, rather than trypanothione, in studies of the interaction of trypanothione reductase with 'turncoat' inhibitors [9]. More recently, El-Waer et al. [10] described the use of *N*-benzyloxycarbonyl-cysteinylglycine-3-dimethylaminopropylamide disulphide as a substrate of the enzyme from *C. fasciculata* and *Trypanosoma cruzi*. With this substrate the specificity parameter, $k_{cat.}/K_m$, is, however, only 18% of that observed with trypanothione. Moreover, GSH plays an important role in several important biochemical processes in the cell. These include the regulation of the thiol–disulphide redox balance, amino acid transport, the reduction of peroxide and of ribonucleotides, *cis/trans*-isomerization reactions and the conjugation of xenobiotics [11]. A trypanothione peroxidase activity has, for instance, been reported [12]. The extent to which trypanothione and GSH–spermidine are involved in these reactions can clearly not be evaluated with artificial substrates.

The relative concentrations of GSH, monoGSH–spermidine and trypanothione vary significantly during the growth phases of *C. fasciculata* [13]. These variations could possibly account for the fact that the presence of GSH–spermidine, in addition to trypanothione, was initially not detected [3]. Thus most of the GSH is present as trypanothione during exponential growth, but is largely converted into monoGSH–spermidine under conditions of increasing acidity which prevails in late exponential phase and in the stationary phase [13]. When stationary phase cells were resuspended in fresh medium a conversion of GSH–spermidine into trypanothione was observed.

Interestingly, the thiol and spermidine levels reported by Shim and Fairlamb [13] indicated that late exponential cultures of *C. fasciculata* contain about 3.5 mg of trypanothione/litre of culture medium and that the isolation of significant quantities of trypanothione from natural sources should be feasible.

In the present paper a simple procedure for the isolation of trypanothione from *C. fasciculata* is described. I also report on convenient methods for the detection and quantitation of GSH, GSH–spermidine, trypanothione and other thiols by t.l.c. and h.p.l.c. of their 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) derivatives.

MATERIALS AND METHODS

Materials

The fluorogenic thiol reagent CPM was obtained from Molecular Probes, Eugene, OR, U.S.A. Cysteine and lipoic acid were from

Abbreviations used: CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; GSH and GSH–, glutathione and glutathionyl; solvents A, B and C have the various compositions given in the Table and Figure legends.

Fluka, Buchs, Switzerland; CoA, pantetheine, mercaptoethanesulphonic acid, mercaptoethylamine, glutamic acid, spermidine and dansyl chloride were from Sigma, St. Louis, MO, U.S.A.; glycine was from Merck, Darmstadt; and D-camphorsulphonic acid was from Aldrich.

Qualitative analysis of thiols

The thiols in extracts of whole cells were analysed as their CPM derivatives. Typically cells from 1 ml of a mid-exponential-phase culture were collected by centrifugation, resuspended in 50 μl of 40 mM sodium Hepes, pH 7.8, and treated with 50 μl of 1.25 mM CPM in acetone. After 5 min at room temperature the cellular debris was removed by centrifugation, and aliquots of the supernatant were applied to a silica-gel 60 F_{254} t.l.c. plate (Merck). The plate was developed with butanol/acetic acid/water (2:1:1, by vol.). Since CPM itself is non-fluorescent, it is revealed as a dark spot near the solvent front when the plate is observed with a short-wavelength (254 nm) u.v. light. The presence of excess reagent indicates that the amount of reagent was sufficient to achieve complete derivatization. The presence of thiols in column effluents during the preparation of trypanothione was detected by a similar procedure. Where necessary, the pH of the solution was adjusted to about 8.0. The thiols in 100 μl of a solution were then reduced by incubation with 40 mM β-mercaptoethanol at 70 °C for 10 min. The solution was extracted three times with 200 μl ethyl acetate in order to remove most of the β-mercaptoethanol and an aliquot was then mixed with an equal volume of 1.25 mM CPM in acetone. Since the CPM derivative of β-mercaptoethanol is well separated from the GSH–spermidine conjugates, the excess which remains does not interfere with their detection.

Quantitative analysis of thiols

Total thiols were estimated after reduction with $NaBH_4$ as described by Brown [14]. Up to 100 μl of a solution of thiols were incubated with 250 μl of a 25 mg·ml^{-1} solution of $NaBH_4$ for 1 h at 40 °C. The remaining $NaBH_4$ was destroyed by the addition of 250 μl of 1 M acetic acid, and, after adding water to obtain 1 ml, the thiol content was estimated as described in [15]. Purified trypanothione to be used as a chromatographic marker was also reduced enzymically in a mixture containing 20 nmol of trypanothione, 100 nmol of NADPH, 2 μmol of sodium Hepes buffer, pH 7.6, and 15 munits of trypanothione reductase in a final volume of 60 μl. After allowing 20 min at 37 °C for the reaction to go to completion, the reduced trypanothione was derivatized by the addition of 60 μl of 1.25 mM CPM.

CPM derivatives of thiols were separated on a phenyl-silica reversed-phase column (0.46 cm × 25 cm) (Vydac 219 TP) using a Kontron model 325 ternary gradient h.p.l.c. pump, a Waters model 481 detector and Waters model 420 fluorescence detector. Data acquisition was by means of DAPA software (DAPA Scientific, Kalamunda, Western Australia, Australia). The conditions of chromatography are given in the Figure legends.

Rates of reaction of thiols with CPM

The rates of reaction of thiols with CPM were monitored fluorimetrically at 21 °C using an Aminco model SPF 500 spectrofluorimeter. The excitation wavelength was 387 nm and emission was measured at 465 nm. Thiols were prepared in distilled water, and the concentration estimated by the procedure of Grasetti and Murray [15]. Reaction mixtures contained 0.25 nmol of CPM in 2 ml of acetone/40 mM sodium Hepes, pH 7.8 (1:1, v/v). The reaction was initiated by the addition of a 40–400-fold excess of the thiol. Under these conditions, pseudo-first-order kinetics was observed for all the thiols except for cysteine, which showed biphasic kinetics. The fluorescence changes were analysed by means of the Enzfitter program (Elsevier/Biosoft).

Growth and harvesting of *C. fasciculata*

C. fasciculata was cultured in the medium described by Shames et al. [4], but with amikacin (50 μg/ml) as the antibiotic. Large-scale culture of the organism was conducted in 20-litre carboys, using a 4% inoculum. Organisms reached an A_{600} of approx. 1.7 after 42 h of growth at room temperature. The cells were harvested in a Beckman JCF-Z continuous-flow rotor at 10000 rev./min to give about 3.8 g (wet wt.) of packed cells/litre of medium. The harvested cells were suspended in a solution containing 1% glucose, 0.85% NaCl and 50 mM potassium phosphate buffer, pH 7.8 (PBS/glucose) to obtain about 25 g of cells/litre. The pH was adjusted to 7.5 with 1 M K_2HPO_4 and the cells stirred gently for 30 min to obtain a homogeneous suspension. The cells were pelleted by centrifugation, washed once more with PBS/glucose and then stored at −80 °C.

Purification of trypanothione from *C. fasciculata*

Trypanothione was prepared from 67 g (wet wt.) of *C. fasciculata* which had been washed with PBS/glucose to maximize the trypanothione content. The frozen cells were thawed in 2 vol. of 0.75 M $HClO_4$, then stirred for 30 min on ice before collecting the supernatant by centrifugation. The pellet was extracted with a further 2 vol. of 0.75 M $HClO_4$. The supernatants were pooled and applied to a 16 mm × 220 mm column of Florisil which had been washed with 5% (v/v) acetic acid. The column was washed with 5% acetic acid and then with water, in both cases until the A_{280} was < 0.1. Trypanothione was subsequently eluted with 20% pyridine and could be detected in the effluent by t.l.c. of a β-mercaptoethanol-reduced aliquot derivatized with CPM as described above.

Fractions containing trypanothione were dried on a rotary evaporator. The yellow residue was dissolved in a small amount of 5 mM ammonium bicarbonate buffer, pH 8.3, and was applied to a 40 cm × 2.2 cm column of DEAE-cellulose which had been equilibrated with the same buffer. Trypanothione was poorly retained and was recovered in a volume of 175 ml by washing the column with the starting buffer. The solvent was removed on a rotary evaporator and the white crystalline solid redissolved in 0.1% trifluoroacetic acid.

Further purification of trypanothione was achieved by h.p.l.c. using a Vydac C_{18} protein and peptide column (Vydac 218 TP 1022). Conditions of chromatography are described in the Figure legends. The identity of trypanothione was verified by determination of the composition of the isolated material after hydrolysis in 6 M HCl at 110 °C, followed by dansylation and analysis essentially as described in [16].

Preparation of trypanothione reductase

Trypanothione reductase was prepared from *C. fasciculata* by following the procedure of Shames et al. [4] until after the DEAE-Sephacel step. The preparation had a specific activity of 4.7 μmol·min^{-1}·mg^{-1} when assayed with 190 μM NADPH and 178 μM trypanothione in 50 mM sodium Hepes, pH 7.6, at 30 °C.

RESULTS AND DISCUSSION

Rates of reactions of thiols with CPM

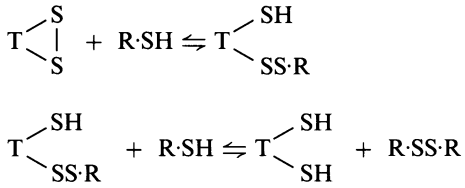
CPM has decided advantages for the analysis of thiols. The addition reaction to the maleimido double bond has a high degree of selectivity for thiols, and few undesirable side reactions are observed compared with the more widely used monobromobimanes, which undergo hydrolytic displacement of bromide in water. Second-order rate constants for the reaction of a number of dissimilar thiols with CPM were estimated under the conditions employed for the derivatization of thiols in cell extracts (Table 1). At a CPM concentration of 0.625 mM, as used in the analytical methods described in the present study, and assuming a large excess of reagent, the $t_{\frac{1}{2}}$ for the addition reaction ranged between 0.105 and 1.79 s and is therefore essentially complete within 30 s at room temperature. The rapid reaction rate is desirable, since it minimizes the contribution of side reactions, such as enzymic degradation of naturally occurring thiols during and after extraction.

T.l.c. of CPM–thiols

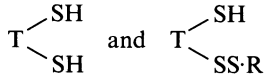
R_F values for the separation of a number of derivatized thiols by t.l.c. on silica-gel plates are reported in Table 2. Conditions for the separation of the GSH–spermidine conjugates from GSH and other thiols were optimal in solvent A. The progress of the separation can be monitored by virtue of the yellow–green fluorescence of the CPM–thiols when irradiated with a long wavelength (365 nm) u.v. lamp. When the plates are dry, the fluorescence changes to a deep-blue colour which fades only gradually over a period of days upon exposure to ambient light.

T.l.c. analysis of column fractions proved to be a convenient method by which the purification of trypanothione could be monitored. The reduction of trypanothione with β-mercaptoethanol, however, resulted in the formation of two fluorescent CPM derivatives, one migrating with the CPM derivative of enzymically reduced trypanothione and the other with the CPM derivative of GSH–spermidine. The reduction of a disulphide such as trypanothione

with β-mercaptoethanol occurs by the thiol–disulphide exchange reaction:



The relative amounts of the species



present at equilibrium may be expected to vary with the redox potential of the disulphide compared with that of β-mercaptoethanol and with the excess of β-mercaptoethanol used. Both mono- and di-CPM derivatives of trypanothione are, therefore, likely to be formed.

Table 1 Second-order rate constants for the derivatization of thiols with CPM at 21 °C

Thiol	10^{-3} × Rate constant ($M \cdot s^{-1}$)
CoM	0.813 ± 0.013
β-Mercaptoethanol	1.4 ± 0.053
GSH	1.048 ± 0.018
GSH–spermidine	10.56 ± 0.236
Trypanothione	1.1 ± 0.027
CoA	0.619 ± 0.034
Dithiothreitol	2.02 ± 0.038

Table 2 R_F values of CPM–thiols on silica gel

Two solvent systems were used: solvent A was Butanol/acetic acid/water (4:2:2, by vol.); solvent B was propan-2-ol/pyridine/water (7:1:2, by vol.). The stationary phase was silica-gel 60 F_{254}.

Thiol	R_F	
	Solvent A	Solvent B
CoA	0.34	0.145
CoM	0.56	0.62
Cysteamine	0.52	0.4
Pantetheine	0.62	0.61
Glutathione	0.45	0.39
Homocysteine	0.53	0.4
L-Cysteine	0.53	0.52
Trypanothione	0.23	0
GSH–spermidine	0.11	0
Lipoic acid	0.68	0.57
β-Mercaptoethanol	0.63	0.59

H.p.l.c. of CPM–thiols

Although most CPM–thiols can be resolved satisfactorily by reversed-phase chromatography on octadecylsilane, the CPM derivatives of trypanothione and GSH–spermidine are not eluted from C_{18} columns with methanol or acetonitrile. Phenyl-silica columns were found to afford excellent separation of a variety of CPM-thiols (Figure 1). Very low limits for the detection of CPM thiols by h.p.l.c. was reported by others, and the linearity of the fluorescence of CPM–thiols with concentration has been amply documented [17]. Under conditions which were optimal for the separation of several CPM–thiols, the GSH–spermidine conjugates, however, showed significant tailing. More satisfactory chromatography of GSH–spermidine and trypanothione was achieved by ion-paired reversed-phase h.p.l.c. using a modification of the method described by Fairlamb et al. [18] (Figure 2). The CPM derivatives of GSH and cysteine are, however, not resolved by ion-pair reversed-phase chromatography at pH 2.6, and the CPM derivative of CoA failed to be eluted from the column. These observations point to the advisability of basing the analysis of an unknown mixture of thiols on more than one set of chromatographic conditions. It was previously reported that washing *C. fasciculata* cells can have a pronounced effect on their thiol composition [13].

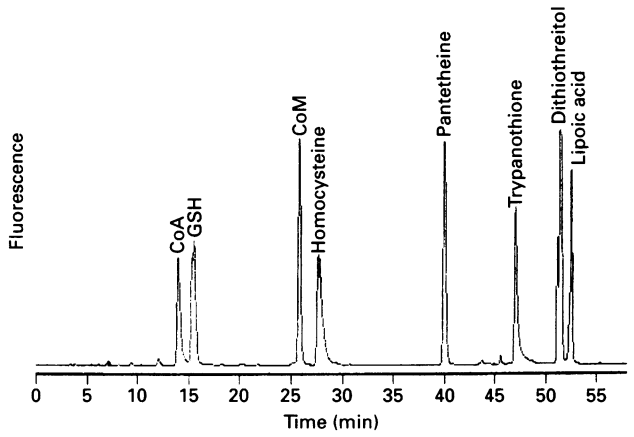


Figure 1 H.p.l.c. separation of a mixture of eight thiols (as indicated on the Figure) after derivatization with CPM

A phenyl Vydac column (25 cm × 0.46 cm) was used as the stationary phase. Separation was achieved at a flow rate of 0.8 ml · min^{-1}, employing a ternary solvent system which consisted of 50 mM ammonium acetate, pH 5.5 (solvent A), 60% acetonitrile (solvent B) and 100% acetonitrile (solvent C). The following gradient was used: 5 min at 50% solvent A/50% solvent B; 30 min linear gradient to 30% solvent A/70% solvent B; 15 min gradient to 10% solvent A/90% solvent C, 5 min at 10% solvent A/90% solvent C, followed by a return to initial conditions.

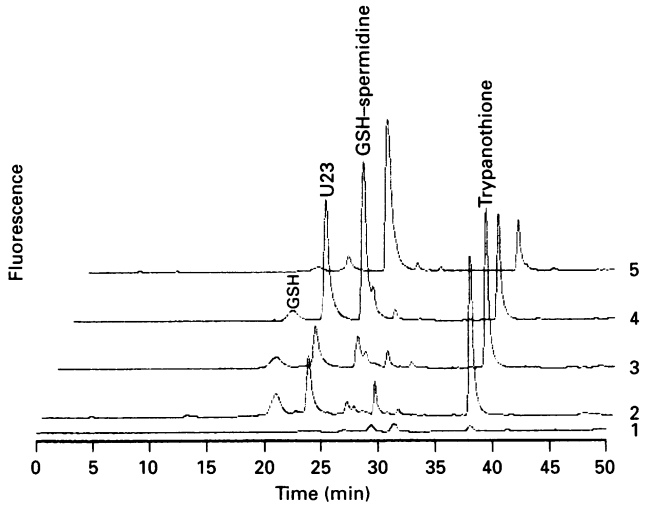


Figure 3 Analysis of the changes in the thiol composition of *C. fasciculata* using CPM as fluorescent label

Conditions of chromatography were as described in the legend to Figure 2. The column effluent was monitored using a fluorescence detector. Trace 1 represents the elution profile of a 25 μl aliquot prepared by derivatizing the growth medium (i.e. the supernatant when the cells are harvested in the late exponential phase) with CPM. Traces 2, 3, 4 and 5 represent the elution pattern when cells obtained from aliquots of a *C. fasciculata* culture were analysed at absorbance readings (A_{600}) of 1.1, 1.25, 1.76 and 1.8 respectively. The results were used for the quantitative analysis presented in Table 4.

Consequently the presence of thiols in the culture medium, which remains as a retention volume when the cells are harvested by centrifugation, must be taken into consideration. In practice the amounts of thiols derived from the medium present in unwashed pellets is, however, small relative to the high concentrations of endogenous thiols, the intracellular levels of which typically approach millimolar concentrations in most cells, including trypanosomes [18]. This is illustrated in Figure 3, where the elution pattern of thiols present in the medium is included for comparison.

Since at least one previously unreported thiol component was

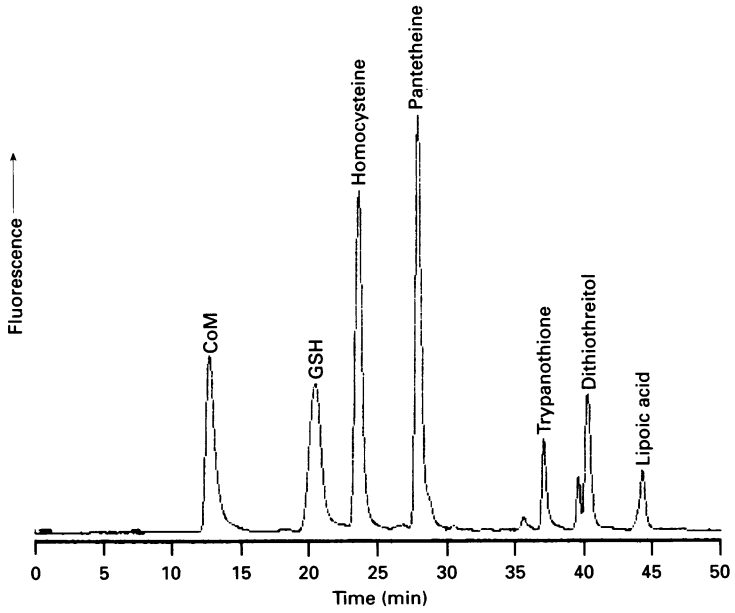


Figure 2 Separation of CPM thiols in the presence of an ion-pairing reagent

A phenyl Vydac column (0.46 cm × 25 cm) was used. Separation was achieved at a flow rate of 0.7 ml · min^{-1} employing a binary solvent system consisting of 0.25% (w/v) camphorsulphonic acid buffered at pH 2.65 with LiOH (solvent A) and 60% acetonitrile containing 0.25% camphorsulphonic acid which had been buffered similarly (solvent B).

Table 3 Comparison of integrated peak areas for the fluorescence of CPM–thiols separated by h.p.l.c.

Thiol concentrations were estimated as described in the Materials and methods section prior to derivatization with CPM. Known amounts were subsequently injected and separated using the elution conditions described in the legend to Figure 3 (System 1) or Figure 2 (System 2). Peak areas are given relative to that of GSH.

	Relative peak area	
Thiol	System 1	System 2
GSH	1	1
CoA		0.69
CoM	0.81	0.94
Homocysteine	1.15	0.91
Pantetheine	1.28	1.094
Trypanothione	0.96	0.97
Dithiothreitol*	0.77	0.67
Lipoic acid	0.837	0.85

* Dithiothreitol was eluted in two peaks the areas of which were in the ratio 5:1. For the purpose of the calculation these areas were summed.

Table 4 Percentage distribution of fluorescence amongst four endogenous thiols from *C. fasciculata* after separation by h.p.l.c. as their CPM derivatives

Aliquots of a culture of *C. fasciculata* were withdrawn at the times indicated and their absorbance recorded as a measure of growth. The thiol composition of the cells was determined by h.p.l.c. of CPM-derivatized aliquots. Abbreviations: GSH–SPD, GSH–spermidine; $T(SH)_2$, trypanothione.

		Distribution (%)*						
	Time (h)...	20	22	24	27.5	33.5	41.5	65
Thiol	A_{600}†...	0.72	0.82	1.1	1.3	1.67	1.76	1.8
Glutathione		13.6	18	18.4	10.92	15.5	5.4	3.7
U23		20.7	21.8	25.8	25.6	24.4	34.3	7.1
GSH–SPD		4.56	8.2	5.2	8.9	9.66	38.5	72.1
$T(SH)_2$		61.1	51.8	50.62	54.6	50.4	21.8	17.1

* Percentage of the fluorescence measured compared with that in GSH + U23 + GSH–spermidine + trypanothione.

† Turbidity.

observed in extracts of *C. fasciculata*, it was of interest to establish whether the fluorescence yield, as reflected in the integrated peak areas of CPM–thiols separated by h.p.l.c., varied significantly. Such variations may be due to the changing solvent composition or the nature of the thiol component. Initially the separation of CPM–dithiols by h.p.l.c. gave low and variable fluorescence peak areas. This problem was traced to the poor solubility of CPM–dithiols in 50% acetone, especially when stock solutions of these are stored at −20 °C. The integrated peak areas observed for a number of different CPM–thiols relative to CPM–GSH are presented in Table 3. In either of the two chromatographic systems the integrated peak areas varied by a factor of about 1.6. Considering that both mono- and di-thiols were analysed, this variation is relatively small, and the observed peak areas are therefore, to a first approximation, a reasonable reflection of the relative concentrations of thiols in a mixture. When CPM–thiols were stored for a week at −20 °C, the integrated peak areas remained essentially unaltered.

It was previously reported that growth-dependent changes in the thiol composition of *C. fasciculata* can be described in terms of the relative contributions of trypanothione, GSH–spermidine and free GSH to the total thiol content of the cells [13]. While about 10% of the total GSH was present as free GSH throughout the various growth phases, a marked shift from trypanothione as the major thiol during exponential growth to GSH–spermidine as the major thiol in the stationary phase was observed [13]. When analysed as the CPM derivatives, the conversion of trypanothione into GSH–spermidine as the principal thiol was also the most pronounced growth-dependent alteration. Several other significant changes were, however, observed (Figure 3). These changes are represented in Table 4 as percentages of the total fluorescence recovered in GSH, its spermidine conjugates and a prominent unknown thiol which was eluted at approx. 23 min (U23). The CPM derivatives of the latter component and of GSH co-migrate on t.l.c. with butanol/acetic acid/water as the mobile phase. Cells harvested in the late exponential phase of growth at an A_{600} of 1.67 contained predominantly trypanothione, which is eluted at about 38 min, while 15.5% of the fluorescence could be ascribed to GSH and 24.4% to U23 (Figure 3, trace 3, and Table 4). Trace 4 of Figure 3 shows the thiol distribution 10 h later at an A_{600} of 1.76 when the culture had reached the stationary phase. GSH–spermidine is now the major thiol, while U23 represented 34.3% of the total and GSH accounted for only 5.4%. During the next 17 h the contribution of GSH–spermidine increased further to 72.1% of the total, while U23 and, to a lesser extent, trypanothione and GSH, declined.

The amount of CPM–GSH detected depends not only on the stage of growth, but is critically dependent on the integrity of the cells. This is illustrated in an experiment using cells which had been harvested at an A_{600} of 1.7 and which were then frozen at −80 °C (Table 5). A comparison of the thiol composition of cells which were subsequently thawed under non-denaturing conditions before derivatization with CPM indicates a rapid breakdown of several endogenous thiols and a large increase in the percentage contribution of free GSH. U23 was barely detectable, and the amounts of trypanothione and GSH–spermidine significantly diminished. The effectiveness with which thiols are released from the cells in 50% acetone was compared with the amount released when the cells were suspended in $HClO_4$. Analysis of the supernatants after centrifugation indicated that the extraction of thiols from the cells by the two methods were comparable. The difference in the results presented in Table 5 can, therefore, not be ascribed to a differential extraction of the thiol components. The rapid breakdown in cell lysates of GSH–spermidine conjugates to free GSH account to a large extent for the very poor recovery of trypanothione when this compound was first isolated from extracts of *C. fasciculata* [3].

At least one other unknown thiol, apart from U23, was detected. This species was eluted at approx. 29 min (Figure 3) and was prominent in the exponential phase of growth, but disappeared once the cells entered the stationary phase. Since conjugates of GSH and spermidine have been shown to play such an important and novel role in the metabolism of the Kinetoplastida, a further investigation of the identity and physiological function of these unknown thiol species is certainly warranted.

Isolation of trypanothione

A substantial improvement in the recovery of trypanothione from *C. fasciculata* could be achieved by the procedure described in the Materials and methods section (Table 6). It was established that large amounts of cells could be washed in a volume of PBS/glucose equivalent to approx. one-tenth of the volume of

Table 5 Effect of extraction under denaturing and non-denaturing conditions on the thiol composition of *C. fasciculata*

C. fasciculata was grown to late exponential phase (A_{600} 1.7), harvested and stored at −80 °C, until used for thiol analysis. In a first sample 100 mg (wet wt.) of the cells were thawed in 200 μl of 0.5 M $HClO_4$ acid, left on ice for 5 min and the solution clarified by centrifugation. The supernatant was adjusted to neutrality with 4 M KOH containing 0.4 M Hepes, and an aliquot was allowed to react with CPM. In a second sample the cells were thawed by vortex mixing with 200 μl of 50 mM sodium Hepes, pH 7.6. An aliquot was immediately mixed with an equal volume of 1.25 mM CPM in acetone and, after leaving the mixture for 15 min at room temperature, the suspension was clarified by centrifugation for 3 min in an Eppendorf centrifuge. The thiol composition was determined by h.p.l.c.

	Thiol composition (%)*			
Sample	GSH	U23	GSH–spermidine	Trypanothione
1	4.51	13.4	29.2	52.84
2	58.0	3.3	11.14	27.55

* Percentage of the fluorescence measured compared with that in GSH + U23 + GSH–spermidine + trypanothione.

Table 6 Recovery of thiols during the purification of trypanothione from *C. fasciculata*

The data relate to the recovery of thiols from 67 g (wet wt.) of *C. fasciculata* which had been harvested as described in the Materials and methods section. Thiol estimations were done after reduction of aliquots with borohydride.

Purification step	Total thiols (μmol of SH groups)
$HClO_4$ extract	270
Florisil	
Fraction 1 (not retained)	21
Fraction 2 (eluted with water)	33
Fraction 3 (eluted with 20% pyridine)	129
DEAE-cellulose of fraction 3 from Florisil	121
H.p.l.c. on a Vydac C_{18} Protein and Peptide Column	70

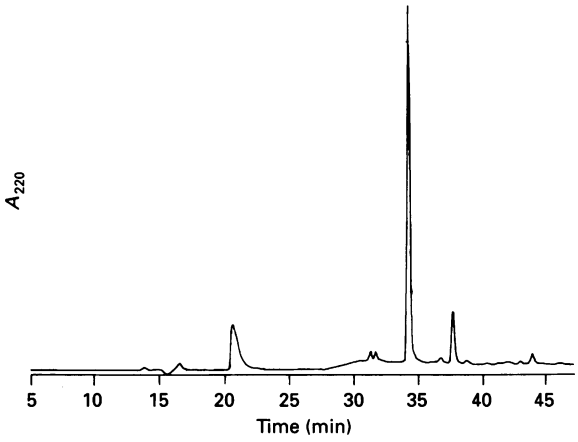


Figure 4 Final step in the purification of trypanothione

The trypanothione-containing fractions from DEAE-cellulose chromatography were further purified in batches on a Vydac C_{18} Protein and Peptide column (2.2 cm × 25 cm) using a binary solvent system comprising 0.1% trifluoroacetic acid (solvent A) and 80% methanol containing 0.1% trifluoroacetic acid (solvent B). The following gradient was employed: 10 min at 0% solvent B, 30 min gradient to 60% solvent B, 5 min gradient to 80% solvent B, 5 min at 80% solvent B and return to initial conditions. The flow rate was 4 ml·min^{-1}. Trypanothione was eluted at 34 min.

Table 7 Composition of the product from Table 6

A weighed aliquot was oxidized with performic acid as described in [19], hydrolysed with 6 M HCl *in vacuo* for the indicated times and analysed after dansylation [16]. Dansylated samples were diluted with water to lower the acetonitrile concentration to 15% and were then analysed on a Novapak C_{18} cartridge using a binary solvent system. The column was eluted for 5 min with 5% solvent B, for 20 min with a gradient to 90% solvent B, for 10 min with 90% solvent B followed by a return to initial conditions. (Solvent A: 0.57% acetic acid/0.088% triethylamine in water; solvent B: 100% acetonitrile). A formula weight of 952 was assumed for the trifluoroacetic acid salt of trypanothione.

		Composition (mol/mol of trypanothione)	
Component	Period of hydrolysis (h)...	18	38
Cysteic acid		1.2	1.05
Glycine		1.06	0.85
Glutamic acid		0.96	1.0
Spermidine		0.5	0.474

culture medium harvested, to achieve a significant conversion of GSH–spermidine into trypanothione. As shown above, the method of cell breakage is of paramount importance if rapid enzymic degradation of trypanothione is to be avoided. In the present study frozen cells were thawed in $HClO_4$ to achieve rapid inactivation of the enzymes. The subsequent isolation of trypanothione from $HClO_4$ extracts is greatly facilitated by the fact that it forms an internal disulphide in preference to mixed disulphides. Initial attempts to adsorb trypanothione and other thiols from *C. fasciculata* on to activated thiopropyl-agarose resulted primarily in the oxidation of reduced trypanothione concomitant with the release of pyridine-2-thione.

Chromatography on florisil afforded a much more convenient, inexpensive and effective method for the purification of trypanothione. Approx. 70% of the thiols applied to a florisil column was recovered in the effluent fractions. At least part of the remaining 30% unaccounted for can be ascribed to the irreversible adsorption of GSH–spermidine to florisil, probably by chemisorption. Trypanothione, however, is eluted from the column in a fraction which contained 48% of the total thiols applied. Subsequent recovery of thiols from DEAE-cellulose was almost quantitative (Table 6). Final purification of trypanothione from the remaining contaminants was achieved by chromatography on a preparative C_{18} Vydac Protein and Peptide column (Figure 4). Trypanothione was eluted at 34 min as the major component detected at 220 nm, while a minor thiol species, which co-migrated with trypanothione on t.l.c., was eluted at 38 min. When this latter component and trypanothione were rechromatographed on the same column, they retained their respective elution positions, indicating that they are not interconvertible. This final purification step involved successive applications of the DEAE-cellulose eluate in order to avoid possible overloading and entailed a significant loss in total thiols. The yield in this step could possibly be improved by attention to handling losses which result from adsorption to glass surfaces [8]. The material isolated contained glutamic acid, glycine and

spermidine in a 2:2:1 stoichiometry (Table 7) and a MH^+ ion with a mass of 722 Da was observed by fast-atom-bombardment m.s. Moreover, the substance served as a substrate for the partially purified preparation of trypanothione reductase.

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