^{203}Hg labelled PHMB as reagent for the determination of –SH groups in native and denatured proteins by hydrophobic interaction chromatography

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Aldolase, glyceraldehyde 3-phosphate dehydrogenase and ovalbumin were determined by hydrophobic interaction chromatography, which allows the evaluation of the number of –SH groups per molecule of protein in both the native and denatured form. These proteins were chosen as models to show the generality of use of the procedure for analytical and biochemical applications. The experiments were performed by using ^{203}Hg-labelled *p*-hydroxymercuribenzoate as reagent and known concentrations of proteins. The results were compared with FI-CV-ETAAS measurements and literature data.

Introduction

Thiol groups and disulfide bonds play a major functional and structural role in proteins. The determination of these functional groups is commonly performed by methods based on selective reactions in which sulfhydryl groups take part. A large number of –SH directed reagents have been proposed for this purpose and among them organic mercurial compounds such as *p*-chloromercuribenzoate (PCMB) and *p*-hydroxymercuribenzoate (PHMB) have been recognised as the most useful primarily because of their selectivity, related to the strong mercury–sulfur interaction. Mercury mercaptide formation is relatively rapid, modified proteins are stable and the chemical characterisation of proteins in either the native or denatured form is possible, provided that the method can be interfaced to a sufficiently sensitive and accurate detection system for the modified proteins.

Different methods of determining sulfhydryl groups and disulfide bonds in proteins modified with such compounds have been proposed, but these methods are characterized by poor sensitivity[1,2] or lengthy procedures[3,4] and only either native[5] or denatured[6] proteins have been considered.

The development of methods that use chemical modification of proteins by radioactively labelled reagents is advantageous since the labels can be detected with high sensitivity. In the determination process, these methods do not need an authentic standard that is identical with the protein under study. By combination of a radioactively labelled reagent method with high-performance liquid chromatography (HPLC) employing an appropriate stationary phase, the excess of the labelled reagent that is not consumed in the reaction with protein can be easily separated. In addition, chromatographic separation of more proteins that react with the same radioactively labelled reagent allows their direct and simultaneous quantification, including mixtures where the co-existence of other components gives hardly distinguishable UV peaks. A simple procedure of analysis which allows the quantification of individual active enzymes in terms of molarity makes up for the need to prepare a suitable radioactively labelled reagent, frequently not commercially available.

In a previous paper, the use of PHMB labelled with ^{203}Hg ([^{203}Hg]PHMB) (half-life 45.8 d; γ-energy 0.279 MeV) and hydrophobic interaction chromatography (HIC) to determine sulfhydryl groups of native and denatured (reduced or not) bovine serum albumin (BSA) was proposed.[7] HIC made possible the complete separation of the excess of organic mercurial reagent from mercury mercaptide BSA and gave a direct chromatographic signal of the protein. Radioactivity measurements of the modified protein permitted the evaluation of –SH groups by simply considering the relative specific activity and moles of protein used, without the need for standards. The mean RSD was of 3.0, 6.5 and 2.5% for native, denatured and reduced BSA, respectively, and the accuracy was in the range 1.9–5.0%.

In this study, we applied the method to three proteins of biological interest, aldolase (ALS) (EC 4.1.2.13), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) and ovalbumin (OVA). The radiochromatographic analysis of

Table 1 THGA electrothermal atomiser programme. Injection temperature = 20 °C

Step No.	Temperature/ °C	Ramp/s	Hold/s	Ar flow rate/ mL min^{-1}	Read
1	110	1	20	250	—
2	130	5	30	250	—
3	250	10	20	250	—
4	1300	0	5	0	Yes
5	2000	1	2	250	—
6		1	5	250	—

Table 2 FIAS-400 programme for CV-ETAAS. Injection volume = 100 μL. Further details are reported in ref. 13

Step No.	Time/s	Pump 1/ rev min^{-1}	Pump 2/ rev min^{-1}	Valve position
Pre-fill	12	100	0	Fill
1	12	100	80	Fill
2	8	0	0	Inject
3	99	0	30	Inject
4	8	0	0	Inject
5	20	0	100	Fill
6		0	120	Fill
7		0	120	Fill
8		0	120	Fill

proteins denatured by guanidine hydrochloride was also possible owing to the capability of HIC to separate denatured forms, as reported previously.[8] The results obtained by the radiochromatographic method were compared with those obtained by flow injection cold vapour electrothermal atomic absorption spectrometry (FI-CV-ETAAS).

Experimental

Instrumentation

An LKB-Pharmacia (Uppsala, Sweden) DfB HPLC System One, equipped with a Rheodyne (Cotati, CA, USA) Model 7125 injector, a 100 μL injection loop and an LC 2249 gradient pump was connected to a Model 2141 variable-wavelength UV detector operated at 250 nm. The output from the detector was displayed on a Model 2221 integrator (LKB-Pharmacia).

Chromatographic conditions for native proteins

An HRLC MP/HIC (Bio-Rad Labs., Richmond, CA, USA) column (50 × 7.8 mm id) was used with a 15 min linear salt gradient of buffer A (1.8 M ammonium sulfate in 0.1 M sodium phosphate, pH 7.0) and buffer B (0.1 M sodium phosphate, pH 7.0) using a solvent programmer at 20–25 °C. The flow rate was 1.0 mL min^{-1}.

Chromatographic conditions for denatured proteins

A Bio-Gel TSK Phenyl-5-PW (Bio-Rad Labs., Richmond, CA, USA) column (75 × 7.5 mm id) was used with 7 min of isocratic elution with buffer C (8.0 M urea, 1.1 M ammonium sulfate and 0.1 M sodium phosphate, pH 7.0) followed by a 30 min linear salt gradient from 0 to 100% buffer D (8.0 M urea in 0.1 M sodium phosphate, pH 7.0). The flow rate was 1.0 mL min^{-1}.

During all chromatographic experiments, the absorbance was measured at 250 nm, considering the possibility of obtaining the signal of PHMB at this wavelength. Exposure of the column to concentrated urea solutions for several months did not noticeably affect its performance or retention time reproducibility.

Radioactivity measurements

The eluted radioactive fractions were recovered using a Pharmacia (Uppsala, Sweden) Frac-100 collector connected to the detector outlet with carefully calibrated, low-volume PTFE tubing. A Berthold LB 2040 γ-counter (Laboratorium Prof. Berthold, Wildbad, Germany) was used. In all experiments, in particular with samples having low radioactivity, the measurements were performed so as to obtain a confidence limit of 95%.

Reagents

ALS and GAPDH from rabbit skeletal muscle and OVA, guanidine hydrochloride and PHMB were purchased from Sigma (St. Louis, MO, USA) and urea from Pharmacia Biotech (Uppsala, Sweden). Water de-ionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

A 0.01 M solution of PHMB was prepared by dissolving a weighed amount of the sodium salt in 0.1 M Na_2HPO_4 and adjusting the pH to 7.5 with 0.1 M NaH_2PO_4. The precise concentrations of PHMB solutions were determined from the absorbance at 232 nm (ε_{232} = 1.69 × 10^4 L mol^{-1} cm^{-1}).[1]

[^{203}Hg]PHMB was prepared as reported previously.[7] The measured relative specific activity of the resulting

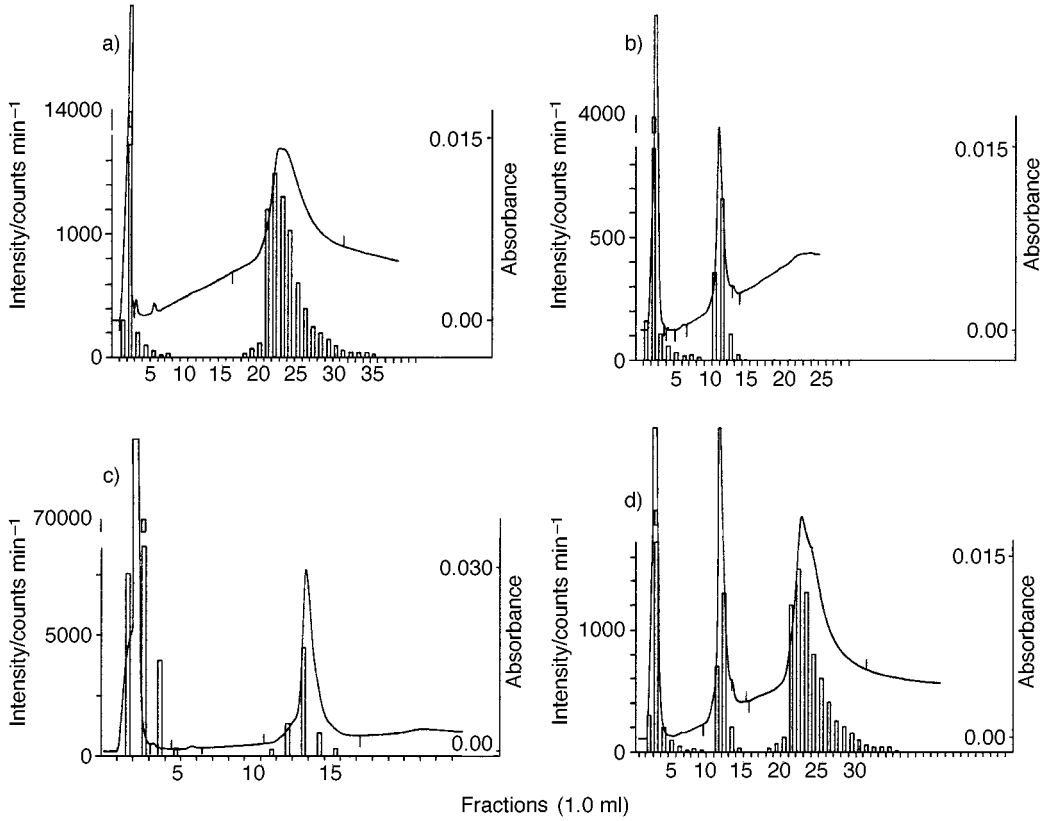

Fig. 1 Typical radiochromatograms from native (a) ALS, (b) GAPDH, (c) OVA and (d) a mixture of ALS (3.7 × 10^{-6} M) and GAPDH (5.8 × 10^{-6} M), treated with [^{203}Hg]PHMB. A methylic HIC column was used. See text for chromatographic conditions.

[^{203}Hg]PHMB solution was (9.0 ± 0.45) × 10^{12} counts min^{-1} mol^{-1}. It must be emphasised that the specific activity of the labelled PHMB must be known with the best accuracy possible; we followed the procedures described in ref. 9.

Procedures

(i) Native protein stock standard solutions were prepared in 0.1 M sodium phosphate (pH 7.0). The concentrations of OVA and ALS were determined from the absorbance at 280 nm (ε_{OVA} = 3.31 × 10^4 and ε_{ALS} = 1.46 × 10^5 L mol^{-1} cm^{-1}).[10,11] The purity of commercial enzymes was confirmed by the absence of other absorbing species in the chromatographic eluate at 280 nm. For GAPDH the concentration of apoenzyme was calculated from the absorbance ratio A_{260}/A_{280} according to Fox and Dandliker.[12] In our experiment, A_{260}/A_{280} was 1.16.

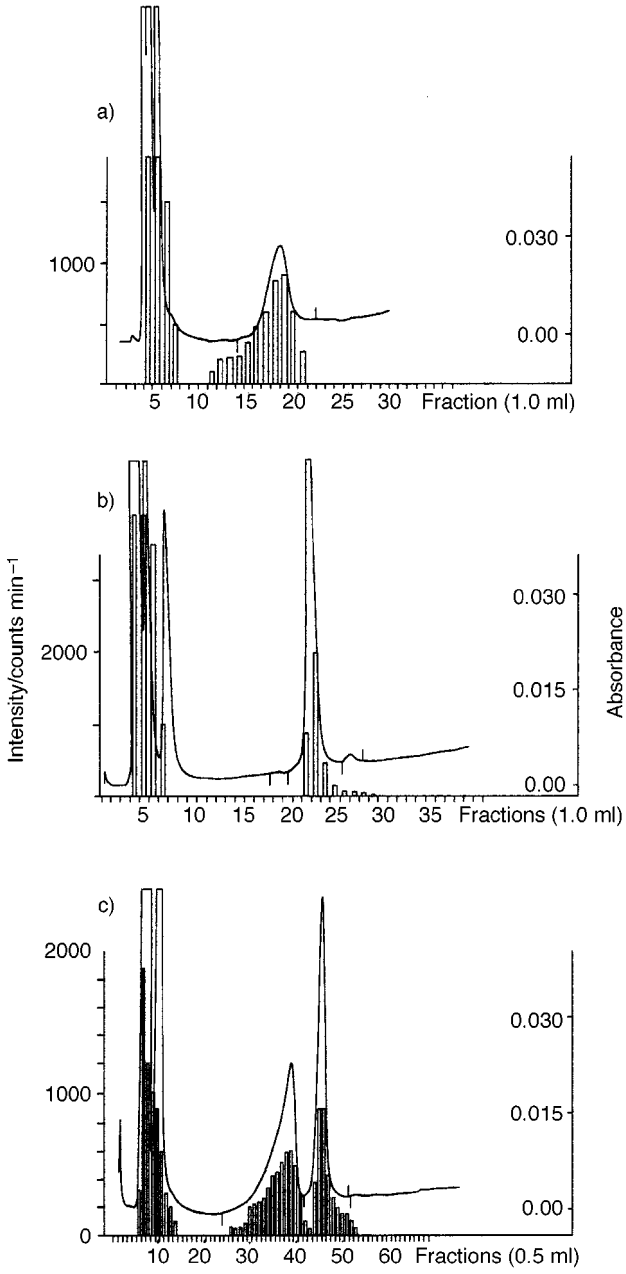

Fig. 2 Typical radiochromatograms from denatured (a) ALS, (b) GAPDH and (c) their mixture (ALS 1.8 × 10^{-6} M; GAPDH 2.0 × 10^{-6} M), treated with [^{203}Hg]PHMB. A phenylic HIC column was used. See text for chromatographic conditions.

(ii) Denaturation of proteins was performed by diluting a small volume (10–60 μL) of protein stock standard solution in 6.0 M guanidine hydrochloride, 0.9 M ammonium sulfate and 0.1 M sodium phosphate (pH 7.0) (1–2 mL) under a stream of helium.

^{203}Hg-labelled PHMB having an exactly known relative specific activity (from 1000 to 9000 counts min^{-1} $nmol^{-1}$) was added to protein solutions. The molar concentration of labelled organic mercury was 100 times that of native OVA, ALS and GAPDH and 50 times that of denatured ALS and GAPDH. For each experiment [see (i) and (ii)], five samples (1.0 mL) were prepared and after incubation for 90 min at 21 °C a 100 μL aliquot was injected on to the HPLC column. Elution fractions (1.0 or 0.5 mL) were collected and their γ-activities measured. As a reference, a sixth sample containing an identical amount of [^{203}Hg]PHMB only was used.

The number of thiol groups in the examined protein could be calculated directly from the radioactivity of the eluted peak, considering the relative specific activity (counts min^{-1} $nmol^{-1}$) used.

FI-CV-ETAAS measurements

Thiol groups of native OVA, ALS and GAPDH and denatured ALS modified with unlabelled PHMB were also determined by FI-CV-ETAAS. A Perkin-Elmer (Norwalk, CT, USA) Model 4100 ZL atomic absorption spectrometer with a transverse-heated graphite atomizer (THGA) and longitudinal Zeeman-effect background correction and an AS-71 autosampler were employed in ETAAS studies. The spectrometer was interfaced with a Perkin-Elmer FIAS-400 flow injection system. The optimised parameters have been reported previously.[13] Standard THGA graphite tubes with integrated platforms were pretreated with a carbide-forming element (W) and then with a noble metal (Ir). The mercury electrodeless discharge lamp (EDL) (Imaging and Sensing Technology, Series 4000, New York, USA) were powered at 27.09 MHz (5 W, wavelength 253.7 nm) by means of a Model DS345 30 MHz synthesised function generator (Stanford Research System, Sunnyvale, CA, USA), a Model 150C wideband rf amplifier (Kalmus Engineering International, Bothell, Washington, USA) and a Model MFJ-94 1E Versa Tuner II (MFJ Enterprises, MS, USA). The temperature programmes used for PHMB detection are given in Table 1. In Table 2 the FIAS-400 programme, which is synchronised with the THGA electrothermal atomiser programme, is given.

Fractions of eluate were collected and treated by a digestion procedure based on the action of bromine in concentrated hydrobromic acid,[14] and mercury was determined. Working standard solutions of samples for ETAAS measurements were prepared before use in 3% v/v HNO_3. A stock standard solution

Table 3 Number of sulfhydryl groups of native and denatured OVA, ALS and GAPDH determined by the radiochromatographic method ([^{203}Hg]PHMB-HIC) and FI-CV-ETAAS

		mol –SH mol^{-1} protein[b]	
Protein	Concentration/ M[a]	[^{203}Hg]PHMB-HIC	FI-CV-ETAAS
Native OVA	1.6 × 10^{-5}	1.05 ± 0.05 (90 min)	1.1 ± 0.09
		2.50 ± 0.13 (24 h)	—
Native ALS	3.8 × 10^{-6}	11.5 ± 0.57	11.8 ± 0.10
Denatured ALS	1.8 × 10^{-6}	22 ± 1.10	22.5 ± 0.5
Native GAPDH	2.6 × 10^{-6}	1.53 ± 0.08	2.1 ± 0.10
Denatured GAPDH	2.3 × 10^{-6}	8.95 ± 0.45	—

[a] Protein concentration in the incubation mixture. [b] Mean of five experiments ± *s*.

of 5% $NaBH_4$ in 0.3% m/v NaOH was prepared by dissolving sodium tetrahydroborate pellets (for AAS, Spectrosol; BDH, Poole, Dorset, UK) in an aqueous solution of NaOH (30% solution, Suprapur; Merck, Darmstadt, Germany) and filtration through a 0.45 μm membrane filter. This solution was stored refrigerated and was diluted before use daily as required (typically 2% m/v $NaBH_4$–1% m/v NaOH). The schematic diagram of the flow injection system has been reported previously.[13]

Results and discussion

Fig. 1 shows typical radiochromatograms from incubation of [^{203}Hg]PHMB (1000 counts min^{-1} $nmol^{-1}$) with (a) native ALS, (b) GAPDH, (c) OVA and (d) a mixture of ALS and GAPDH. Fig. 2 shows analogous radiochromatograms for denatured (a) ALS, (b) GAPDH and (c) their mixture. By employing an HIC column, the excess organic mercurial reagent can easily be separated because it is eluted at the start of the chromatographic profile. The elution of modified native proteins easily occurs using a methylic (HIC) column (Fig. 1). With the methylic column, modified denatured proteins elute too fast and overlap with the organic mercury peak, owing to the presence of urea. To avoid these problems, a phenylic (HIC) column was used and the elution programme was modified (Fig. 2). The experimental conditions described for denaturing proteins make it possible to maintain the solubility of the protein, provided that a high concentration of chaotropic reagent in the chromatographic mobile phase is retained.

Treatment of native proteins with a moderate excess of PHMB modifies part of the free sulfhydryl groups theoretically calculated on the basis of the number of cysteines in the primary structure (32 in ALS, 4 in OVA and 16 in GAPDH).[15] The thiol groups of proteins could have different reactivities.[16] The arrangement of –SH groups in the protein structure (hydrophobic or hydrophilic site, steric hindrance, *etc*.), the type of reagent used and the reaction conditions influence their reaction time with titrating agents. The results obtained for native proteins were in agreement with those obtained by other workers.[2,4,5,10,17–22]

[^{203}Hg]PHMB reacts with a larger number of –SH groups in denatured proteins than native proteins. This result is expected considering that denaturation would unfold the protein, thus exposing buried –SH groups. However, denaturation by guanidine hydrochloride does not make possible titration of all 'free' –SH groups (not involved in disulfide bridges) present in the proteins examined. This means that also in the denatured state several thiol groups are not accessible to the organic mercurial reagent, showing that denatured proteins may retain substantial structures.[23] Theoretically, the radiochromatographic method could be applied to the separation and characterization of different 'unfolded' states induced by chaotropic agents.

Table 3 shows the number of –SH groups accessible to the organomercurial reagent determined by radiochromatography and FI-CV-ETAAS for the proteins examined. Table 4 summarises the values reported in the literature obtained by using other procedures.[2,4,5,10,17–22] A comparison of the results obtained by different methods is of interest and shows a good agreement. However, because of the different reactivities of

Table 4 Average moles of accessible –SH groups per mole of protein determined by different methods

Determination method	Reactant	Protein	pH	Protein concentration/M	Reaction time	Number of –SH groups	Ref.
Spectrophotometric (250 nm)	*p*-Mercuribenzoate	OVA	Phosphate, 0.05 M, 7.0	10^{-5}	≥ 24 h	3.2	2
CVAAS after gel filtration and digestion	*p*-Hydroxymercuribenzoate	OVA	TRIS, 0.1 M, pH 7.5	10^{-6}	24 h	2.66 ± 0.11	4
		OVA	Phosphate, 0.1 M, pH 7.5	10^{-5}	24 h	3.73 ± 0.14	
Spectrophotometric (250 nm)	*p*-Hydroxymercuribenzoate	OVA	TRIS, 0.1 M, pH 7.5	5×10^{-5}	23 h	2.81	10
CVAAS after:							
Sephadex(20 min)+ digestion		OVA	TRIS 0.1 M, pH 7.5	5×10^{-5}	23 h	2.75 ± 0.03	
dialysis (3 days)+digestion	*p*-Hydroxymercuribenzoate	OVA	TRIS 0.1 M, pH 7.5	5×10^{-5}	23 h	2.86	
	p-Hydroxymercuribenzoate Mercuric chloride	OVA	TRIS 0.1 M, pH 7.5	5×10^{-5}	23 h	2.31 ± 0.04	
Back-titration with cysteine (nitroprusside as indicator)	*p*-Chloromercuribenzoate	OVA	Acetate, pH 5.3 and 3.2	10^{-3}	5–15 min	2.92 ± 0.18	18
			Acetate pH 5.3 and 3.2, Gdm·HCl	10^{-3}		4.06 ± 0.15	
HPLC-ICP-MS	Ethylmercuric chloride	OVA	Phosphate, 0.3 M, pH 7.0	10^{-5}	30 min	2.8	5
	p-Chloromercuribenzoate				24 h	2.8	
Spectrophotometric (250 nm)	*p*-Mercuribenzoate	GAPDH	Phosphate, 0.1 M, pH 7.0	10^{-6}	—	7.4	19
			Glycine, 0.3 M, acetate, 0.3 M, pH 6.8	10^{-6}		8.1	
			Acetate, 0.5 M, pH 4.6	10^{-6}		11.8	
			Phosphate, 0.1 M, pH 5.6	10^{-6}		8.2	
			Acetate, 0.5 M, pH 5.6	10^{-6}		8.9	
			Acetate, 0.5 M, pH 4.6	10^{-6}		14	
Spectrophotometric (250 nm)	*p*-Mercuribenzoate	ALS	Phosphate, 0.05 M, pH 7.0	2.6×10^{-6}	15 s 90 min	5-7 8-9	17
Amperometric titration	$AgNO_3$	OVA	TRIS, pH 7.4	10^{-6}	—	5 ± 0.1	20
		ALS	TRIS, pH 7.4	10^{-6}		23	
		GAPDH	TRIS, pH 7.4	10^{-6}		11	
		OVA	TRIS, pH 7.4, urea, 8 M	10^{-6}		4.3	
		ALS	TRIS, pH 7.4, urea, 8 M	10^{-6}		28.5	
		GAPDH	TRIS, pH 7.4, urea, 8 M	10^{-6}		11	
Enzymatic assay after chemical modification	Iodoacetamidonaphthol	GAPDH	TRIS, 0.05 M, pH 8	$(3–5) \times 10^{-6}$	—	2 3.9	21
	N-(4-Dimethylamino-3,5-dinitrophenyl)-maleimide						
Redox titration	*o*-Iodosobenzoate	GAPDH	—	10^{-7}	90 s	10-11	22

–SH groups, 'no single analytical procedure based on one or the other reaction of –SH groups with "–SH reagents" can actually be regarded as conclusive by itself'.[24] The precision of the procedure, on the basis of the response with native OVA, ALS and GAPDH and denatured ALS and GAPDH, was evaluated and the results are reported in Table 3. The precision (mean RSD) was about 5%. The results obtained from comparing the amount of radioactivity applied to the column with that recovered from the complete eluates of residual organometallic reagent and of modified protein showed recoveries in the range 98–101%. The specificity of the method has been reported previously.[7]

On the basis of the repeatability of these measurements, the simultaneous determination and separation of native and denatured thiolic proteins can also be performed as reported in Fig. 1(d) and 2(c), respectively.

Conclusion

A radiochromatographic method has been successfully applied to several native thiolic proteins and proteins denatured by guanidine hydrochloride, chosen as a model to show the generality of use of the procedure for analytical and biochemical applications. FI-CV-ETAAS analysis of chromatographic fractions also gave values of moles of –SH per mole protein in agreement with those obtained by radiochromatography and by other methods reported in the literature.

However, the HIC-radiochromatographic method should be preferred to FI-CV-ETAAS analysis because no digestion procedure of the sample is required, the sample handling being reduced. Furthermore, the procedure proposed, based on metal-sensitive detection, is a simple, fast and selective method to determine sulfhydryl groups in native and denatured proteins, not requiring the use of a standard for quantitative determinations and not suffering from any interference. Owing to the repeatability of measurements, simultaneous determination and separation of native and denatured thiolic proteins can also be performed. The very high specific activity of [^{203}Hg]PHMB could allow low protein concentrations to be determined.

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