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Application of solid-phase extraction to the preconcentration of metallothionein and metals from physiological fluids[1]

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Abstract

Solid-phase extraction (SPE) with covalent affinity chromatography with thiol–disulphide interchange (CAC–TDI) gels as sorbents were used for the preconcentration of Cd–thionein (Cd–Th), Zn–thionein (Zn–Th) and Cu–thionein (Cu–Th) proteins and Cd, Zn or Cu bonded with metallothioneins (MTs) from water, plasma and human urine samples. An indirect method of quantitation of MTs, based on the analysis of metal content, for the determination of MT in human body fluids is described. In experiments, different types of CAC–TDI gels were tested. The results showed a satisfactory correlation between the concentration of MT proteins added to water or physiological fluids and the concentration of protein indirectly determined via the concentration of metals by atomic absorption spectrometry.

Keywords: Extraction methods; Metallothioneins; Metals; Thioneins; Proteins

1. Introduction

Covalent affinity chromatography with thiol–disulphide interchange (CAC–TDI) was introduced in 1973 when Brocklehurst et al. [1] used a Sepharose(glutathione-2-pyridyl disulphide) gel for the purification of papain. CAC–TDI, a method used to separate thiolproteins and metallothiolproteins, is based on the specific interaction between disulphide bridges immobilized on the insoluble support directly or via a spacer arm and the thiol groups of the proteins being separated. In 1977, Squibb and Cousins [2] used the CAC–TDI technique for the first time to separate Zn–thionein. The separation yields for different types of metallothioneins (MTs) using the CAC–TDI method were about 10–50% for applied protein and about 15–80% for metals bound to the MTs [3–7].

MT was discovered in equine renal cortex, as a cadmium-binding protein with an extremely high content of metals and sulphur [8]. MT was identified and characterized as a protein with a low molecular mass, of about $6.5 \cdot 10^3$, rich in –SH groups, of high metal molar content, of a very characteristic amino acid composition and with a characteristic UV–Vis spectrum due to metallothiolate chromophores [9–12]. Mammalian MTs have twenty cysteines and bind a total seven equivalents of bivalent ions such as Zn, Cd, Hg, Bi, Sn, Co, Ni, Rb or Tc. Higher stoichiometries (twelve equivalents) are observed with univalent d(10) ions such as: Cu(I), Ag(I) and Au(I). MTs have two metal-binding domains

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{−α}(31–61) and {−β}(1–29), with diameters of about 15–20 Å, which contain two metallothiolate clusters [A] and [B] of different structures and with different affinities to individual metals (A – three metal cluster, preference Cu, Zn, Cd; B – four metal cluster, preference Cd, Zn, Cu) [13–17]. MT plays many important biological functions in humans and animals. The most important of these are the uptake, storage or detoxication of heavy metals (Cd, Hg, Pb, Au, Ag, Pt, Co, Bi) and participation in the absorption, incorporation and administration of essential metals (Zn, Cu) [18]. The concentration of MT in human urine and plasma in the normal physiological state and after exposure to heavy metals shows a very good correlation with the metal content in physiological fluids and tissues; with a correlation coefficient ranging from 0.75–0.95 and are a good indicator of exposure to heavy metals [19,20].

In this paper, the aim was to test the application of solid-phase extraction (SPE) for the quantitative determination of the concentration of MTs in physiological fluids. A known amount of MT protein was added to water or human fluids, which then were preconcentrated by SPE on CAC–TDI gels with different lengths and modifications of the spacer arms and with different ligands. MT protein concentrations were indirectly determined (calculated) from the concentration of metals formerly bound to MT protein and adsorbed onto the SPE gel [21–25,41].

2. Experimental

2.1. Preparation of gel for the solid-phase extraction technique

The following commercial gels were used as the sorbents for SPE covalent affinity chromatography with thiol–disulphide interchange (CAC–TDI): activated Thiol–Sepharose 4B (ATS-4B) (1 μmol ligand/ml gel), Thiopropyl–Sepharose 6B (TS-6B) (20 μmol ligand/ml gel) (Pharmacia, Uppsala, Sweden) and Affi-Gel 401 (AF-401) (15 μmol ligand/ml gel) (Bio-Rad, Munich, Germany) with the following ligands: 2,2′-dithiobis(2-nitropyridine) (DTNP) (Sigma-Aldrich, Poznań, Poland) and 5,5′-dithiobis(2-nitrobenzoate) (DTNB-Ellman reagent, Sigma-Aldrich). Two gels synthesized in our laboratory, Sepharose–DTNB(A) and Sepharose–DTNB(B), were based on a Sepharose 4B support. Cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) 4-aminobutyric acid (Merck, Darmstadt, Germany), DTNB-Ellman reagent, DTNP and N-cyclohexyl-N′-[2-(N-methylmorpholino)ethyl]carbodiimide-*p*-toluenosulphonate (carbodiimide CMC, BDH, Promochem, Warsaw, Poland) were used for gel synthesis. The degree of substitution was about 0.17 mmol DTNB/g Sepharose and about 0.16 mmol DTNP/g Sepharose [26–29] (Fig. 1).

2.2. Preparation of metallothioneins

Cd–thionein (Cd–Th), Zn–thionein (Zn–Th) and Cu–thionein (Cu–Th) were obtained from the livers of female white rats (Wistar; 190–210 g) after exposure to salts of heavy metals ($CdCl_2$, $ZnSO_4$ and $CuSO_4$) at the following levels; 1.0 mg of Cd, 20.0 mg of Zn and 5.0 mg of Cu per kg body mass, administered subcutaneously in physiological salt solutions (for Cd, three times per week; for Zn and

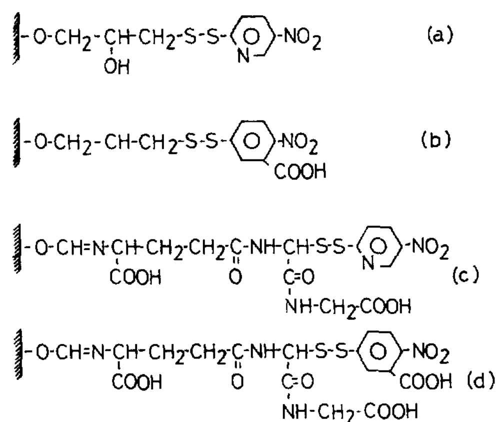

Fig. 1. Structures of covalent affinity chromatography with thiol–disulphide interchange (CAC–TDI) gels, used in the experiment as SPE sorbents. (a) Thiopropyl–Sepharose 6B–2,2′-dithiobis(2-nitropyridine) (TS-6B–DTNP). (b) Thiopropyl–Sepharose 6B–5,5′-dithiobis(2-nitrobenzoate) (TS-6B–DTNB). (c) Activated Thiol–Sepharose 4B–2,2′-dithiobis(2-nitropyridine) (ATS-4B–DTNP). (d) Activated Thiol–Sepharose 4B–5,5′-dithiobis(2-nitrobenzoate) (ATS-4B–DTNB). (e) Affi-Gel 401–2,2′-dithiobis(2-nitropyridine) (AF-401–DTNP). (f) Affi-Gel 401–5,5′-dithiobis-(2-nitrobenzoate) (AF-401–DTNB). (g) Sepharose–DTNB(A)-5,5′-dithiobis(2-nitrobenzoate) (S–DTNB(A)). (h) Sepharose–DTNB(B)-5,5′-Dithiobis(2-nitrobenzoate) (S–DTNB(B)).

Cu, seven times per week for two weeks). The animals were sacrificed three days after the last dose of salt solutions [5,6,30,31]. Liver homogenates [25% (w/v)] were prepared. After initial centrifugation (3000 g for 30 min), the supernatants were separated by low-pressure gel permeation chromatography (LPGPC) on Sephadex G-75 (column K 26/100, Pharmacia) with 0.1 mol/l ammonium formate buffer (pH 8.0, ionic strength μ=0.1) and with a flow-rate of 10.0 ml/h. Fractions (5.0 ml) were collected [5,6,30,31].

Molecular masses of the obtained MTs were about $10 \cdot 10^3$ ($M_{r(Cd-Th)}=10.0 \cdot 10^3$, $M_{r(Zn-Th)}=10.1 \cdot 10^3$ and $M_{r(Cu-Th)}=9.8 \cdot 10^3$). The concentrations of –SH groups were: 8.5 mol/mol for Cd–Th, 8.6 mol/mol for Zn–Th and 10.3 mol/mol for Cu–Th, and the concentrations of metals were: 4.397 mol Cd/mol MT, 5.428 mol Zn/mol MT and 6.727 mol Cu/mol MT. The characteristic ratios of absorption at 250 and 280 nm were: 6.155 for Cd–Th, 7.143 for Zn–Th and 8.161 for Cu–Th. All of these physicochemical parameters show the good quality of the obtained MTs.

2.3. Preparation of biological material

Human plasma and urine were collected from four healthy male non-smokers, after clinical control.

The samples of urine were pooled. The urine was centrifuged (3000 g for 10 min) and sodium azide (NaN_3) was added to a final concentration of 0.05%. The concentrations of protein, thiol groups and Cd, Zn and Cu in the urine were measured [22,23]. The concentrations in urine were: protein, 3.058 mg/l using the Lowry method and 0 mg/l using the tannin method; –SH groups, $2.6 \cdot 10^{-5}$ μmol/l; Cd, 3.398 μg/l; Zn, 5.128 μg/l and Cu, 4.981 μg/l.

Samples of blood (4.5 ml) were collected in dry plastic test-tubes with 0.5 ml of sodium citrate (3.2%, w/v). The samples were centrifuged (3000 rpm, 10 min). Plasma was collected and sodium azide (NaN_3) was added to a final concentration of 0.05%. The samples were pooled. The protein, thiol groups and Cd, Zn and Cu concentrations in the plasma were measured [24]. The concentrations in plasma were as follows: protein, ~32.531 g/l using the modified Lowry method and ~13.569 g/l using the tannin method; –SH groups, 22.34 μmol/l; Cd, 3.458 μg/l; Zn, 758.623 μg/l and Cu, 1556.877 μg/l.

2.4. Analysis of metallothionein concentration

For the determination of the recovery yields Cd–Th, Zn–Th and Cu–Th were added (50 μg/1.0 ml of sample; 5.0 ml) to three times distilled water and to human urine or plasma collected from male non-smokers who were in a good state of health.

Firstly, all the samples were separated by LPGPC on Sephadex G-75 (column K 26/100, Pharmacia) with 0.1 mol/l ammonium formate buffer (pH 8.0, ionic strength μ=0.1) as the eluent, at a flow-rate of 10.0 ml/h. Fractions (5.0 ml) were collected and monitored at the specific MT absorption wavelengths of 220, 250, 270 and 280 nm.

In the second step, MTs obtained by LPGPC (fraction M_r $10.0 \cdot 10^3$) were separated by CAC–TDI on gels with different structures, using a two-step elution. The unbound fractions (proteins and metals) were removed with 0.05 mol/l phosphate buffer (pH 7.0). The bound fractions were eluted with 0.05 mol/l β-mercaptoethanol in the same phosphate buffer. The CAC–TDI columns (column 130×10 mm I.D., v = 10.0 ml) were used. Fractions (2.0 ml) were collected at a flow-rate of 30.0 ml/h, at room temperature and with monitoring at 220, 250, 270, 280, 343 and 412 nm. In fractions with bound MTs, the protein concentration was determined using the modified Lowry method and the tannin method. Additionally, the concentrations of metals (Cd, Zn and Cu) were measured using atomic absorption spectrometry (AAS). The recoveries of MTs and metals adsorbed on CAC–TDI gels were determined using a chemical method and were calculated. Different types of CAC–TDI gels, with different structures and lengths of spacer arm and with different ligands, were tested in these experiments.

The concentrations of MTs were calculated according to Eq. (1) [22–25,41]:

$$c = (AM_{MT})/(M_M mR) \cdot 100\% \quad (1)$$

where c is the calculated content of MTs' protein in the sample (μg), A is the content of metals bound to the covalent affinity support (μg), M_{MT} is the molecular mass of MT protein (g/mol), M_M is the

molecular mass of the determined metal (g/mol), m is a coefficient determined experimentally that characterizes the metal content on the MT molecule (mol of metal/mol of protein) and R is the mean recovery of adsorption of the metals during the separation of MTs by CAC–TDI (%). To determine the correlation (r) between the concentration of MTs added to the water or human fluids and the concentration determined by an indirect method using SPE, and based on analysis of metals adsorbed on CAC–TDI gels and determined by AAS methods, were used. MTs were added at concentrations of 10, 25, 50, 75, 100 and 150 μg/ml. The correlation was tested for five gels, Sepharose–DTNB(A), Sepharose–DTNB(B), Affi-Gel 401, activated Thiol–Sepharose 4B and Thiopropyl–Sepharose 6B, with DTNB and DTNP as ligands. The correlation was calculated for all five supports and the mean correlations were calculated for the most specific supports with the best recoveries of MT proteins and metals.

The detection limits for the indirect method were calculated according to Eq. (1) and were based on detection limits for detected metals (Cd, Zn and Cu). The detection limits for Cd, Zn and Cu that were obtained in these experiments (and were similar to literature values) were about 0.0001 μg/ml for all three metals [22–25,41].

2.5. Analytical methods

The following determinations were carried out: protein concentration using a modified Lowry method that is specific for thiolproteins [32–34] and the tannin method [35]. HS-group concentration [36,37],

Table 1
Separation yields for Cd–thionein and Cd on SPE sorbents for covalent affinity chromatography with thiol–disulphide interchange

Number	SPE gel[a]	Medium	Mean recovery of Cd–thionein protein		Mean recovery of cadmium	
			(μg)	(%)	(μg)	(%)
1	ATS-4B–DTNP	W	56.3	22.5	22.218	20.7
2	ATS-4B–DTNP	P	107.3	42.9	72.188	54.9
3	ATS-4B–DTNP	U	69.3	27.7	31.426	23.9
4	ATS-4B–DTNB	W	62.3	24.9	28.402	21.6
5	ATS-4B–DTNB	P	119.0	47.6	73.371	55.8
6	ATS-4B–DTNB	U	69.5	27.8	32.478	24.7
7	TS-6B–DTNP	W	30.7	12.3	23.274	17.7
8	TS-6B–DTNP	P	91.8	36.6	65.076	50.1
9	TS-6B–DTNP	U	36.9	14.7	24.457	18.6
10	TS-6B–DTNB	W	35.8	14.3	24.326	18.5
11	TS-6B–DTNB	P	104.8	41.9	65.088	49.5
12	TS-6B–DTNB	U	37.6	15.0	25.641	19.4
13	AG-401–DTNP	W	55.5	22.2	28.402	21.6
14	AG-401–DTNP	P	112.4	45.0	76.001	57.8
15	AG-401–DTNP	U	64.0	25.6	34.450	26.2
16	AG-401–DTNB	W	63.3	25.3	29.848	22.7
17	AG-401–DTNB	P	109.3	43.7	74.818	56.9
18	AG-401–DTNB	U	69.8	27.9	30.637	23.3
19	S–DTNB(A)	W	62.3	24.9	29.059	22.1
20	S–DTNB(A)	P	119.3	47.7	73.503	55.9
21	S–DTNB(A)	U	67.3	26.9	30.637	23.3
22	S–DTNB(B)	W	12.8	5.1	21.170	16.1
23	S–DTNB(B)	P	27.8	11.1	63.351	49.7
24	S–DTNB(B)	U	19.0	7.6	29.332	22.3

[a] ATS-4B, activated Thiol–Sepharose 4B; TS-6B, Thiopropyl–Sepharose 6B. AG-401, Affi-Gel 401; S–DTNB(A), Sepharose–DTNB(A); S–DTNB(B), Sepharose–DTNB(B); DTNP, 2,2′-dithiobis(2-nitropyridine); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); W, water; U, urine and P, plasma.
[b] The mean values were calculated for $n=5$ samples.

Cd, Zn and Cu were determined by atomic absorption spectrometry using a Beckman AAS Model 1248 with a graphite Massman cuvette (Model 1268) with a non-specific correction deuterium lamp (Model CNA 1275 and Model AA-660 V-3; Shimadzu) and measuring the absorbance at 220, 250, 270, 280, 343 and 412 nm (Phillips UV–Vis spectrophotometer PU-8700) [5,6,30,31].

3. Results

The separation yields of Cd–Th, Zn–Th and Cu–Th proteins and Cd, Zn and Cu, separated by SPE on CAC–TDI gels from water, urine and plasma are given in Tables 1–3. The mean recoveries for different CAC–TDI gels were about 5–48% for Cd–Th, 5–53% for Zn–Th and 8–58% for Cu–Th. The mean recoveries of metals for all types of CAC–TDI gels were about 16–58% for Cd, 18–88% for Zn and 23–144% for Cu. The mean recoveries of MTs and metals for different mediums and for two types of ligands (DTNB and DTNP) are given in Tables 4 and 5. The relation between the length of the spacer arm and the recovery yield for MT proteins and metals are shown in Fig. 2. The correlation coefficients (r) between the concentrations of MTs added to the distilled water, human plasma or urine and the concentrations calculated from the content of metals bound to covalent affinity chromatography gels with different lengths of spacer arm [Affi-Gel 401, Sepharose–DTNB(A), Sepharose–DTNB(B), activated Thiol–Sepharose 4B and Thiopropyl–Sepharose 6B] were about 0.54–0.97

Table 2
Separation yields for Zn–thionein and Zn on SPE sorbents for covalent affinity chromatography with thiol–disulphide interchange

Number	SPE gel[a]	Medium	Mean recovery of Zn–thionein		Mean recovery of zinc	
			(μg)	(%)	(μg)	(%)
1	ATS-4B–DTNP	W	59.2	23.7	26.391	27.9
2	ATS-4B–DTNP	P	106.3	42.5	81.631	86.3
3	ATS-4B–DTNP	U	72.0	28.8	28.188	29.8
4	ATS-4B–DTNB	W	61.3	24.5	29.796	31.5
5	ATS-4B–DTNB	P	131.5	52.6	82.388	87.1
6	ATS-4B–DTNB	U	70.7	28.3	29.701	31.4
7	TS-6B–DTNP	W	36.8	14.7	22.323	23.6
8	TS-6B–DTNP	P	95.2	38.1	78.983	83.5
9	TS-6B–DTNP	U	39.3	15.7	26.674	28.2
10	TS-6B–DTNB	W	38.5	15.4	22.985	24.3
11	TS-6B–DTNB	P	109.5	43.8	79.926	84.5
12	TS-6B–DTNB	U	44.7	17.9	26.958	28.5
13	AG-401–DTNP	W	57.8	23.1	27.336	28.9
14	AG-401–DTNP	P	116.7	46.7	77.280	81.7
15	AG-401–DTNP	U	66.2	26.5	29.134	30.8
16	AG-401–DTNB	W	67.1	26.8	29.985	31.7
17	AG-401–DTNB	P	130.6	52.3	83.333	88.1
18	AG-401–DTNB	U	72.3	28.9	30.742	32.5
19	S–DTNB(A)	W	66.2	26.5	29.228	30.9
20	S–DTNB(A)	P	124.5	49.8	82.776	87.5
21	S–DTNB(A)	U	69.5	27.8	27.904	29.5
22	S–DTNB(B	W	13.2	5.3	16.648	17.6
23	S–DTNB(B)	P	30.1	12.0	49.849	52.7
24	S–DTNB(B)	U	17.2	6.9	19.769	20.9

[a] ATS-4B, activated Thiol–Sepharose 4B; TS-6B, Thiopropyl–Sepharose 6B; AG-401, Affi-Gel 401; S–DTNB(A), Sepharose–DTNB(A); S–DTNB(B), Sepharose–DTNB(B); DTNP, 2,2′-dithiobis(2-nitropyridine); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); W, water; U, urine and P, plasma.

[b] The mean values were calculated for $n=5$ samples.

Table 3
Separation yields for Cu–thionein and Cu on SPE sorbents for covalent affinity chromatography with thiol–disulphide interchange

Number	SPE gel[a]	Medium	Mean recovery of Cu–thionein protein		Mean recovery of copper	
			(μg)	(%)	(μg)	(%)
1	ATS-4B–DTNP	W	67.3	26.9	35.657	31.3
2	ATS-4B–DTNP	P	130.8	52.3	156.754	137.6
3	ATS-4B–DTNP	U	80.3	32.1	41.467	36.4
4	ATS-4B–DTNB	W	71.5	28.6	36.340	31.9
5	ATS-4B–DTNB	P	144.8	57.9	158.463	139.1
6	ATS-4B–DTNB	U	83.0	33.2	42.834	37.6
7	TS-6B–DTNP	W	41.3	16.5	31.556	27.7
8	TS-6B–DTNP	P	108.8	43.5	137.615	120.8
9	TS-6B–DTNP	U	48.2	19.3	36.682	32.2
10	TS-6B–DTNB	W	46.7	18.7	32.011	28.1
11	TS-6B–DTNB	P	123.3	49.3	138.868	121.9
12	TS-6B–DTNB	U	61.2	24.5	37.594	33.0
13	AG-401–DTNP	W	66.2	26.5	37.138	32.6
14	AG-401–DTNP	P	122.7	49.1	159.146	139.7
15	AG-401–DTNP	U	74.0	29.6	42.378	37.2
16	AG-401–DTNB	W	80.3	32.1	38.163	33.5
17	AG-401–DTNB	P	133.0	53.2	163.703	143.7
18	AG-401–DTNB	U	91.8	36.7	43.973	38.6
19	S–DTNB(A)	W	83.0	33.2	37.480	32.9
20	S–DTNB(A)	P	130.7	52.3	157.210	138.8
21	S–DTNB(A)	U	74.7	29.9	41.695	36.6
22	S–DTNB(B)	W	20.8	8.3	25.860	22.7
23	S–DTNB(B)	P	36.8	14.7	117.907	103.5
24	S–DTNB(B)	U	24.5	9.8	30.189	26.5

[a] ATS-4B, activated Thiol–Sepharose 4B; TS-6B, Thiopropyl–Sepharose 6B; AG-401, Affi-Gel 401; S–DTNB(A), Sepharose–DTNB(A); S–DTNB(B), Sepharose–DTNB(B); DTNP, 2,2′-dithiobis(2-nitropyridine); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); W, water; U, urine and P, plasma.
[b] The mean values were calculated for $n=5$ samples.

Table 4
Mean recoveries of Cd–Th, Zn–Th and Cu–Th from all media using 2,2′-dithiobis(2-nitropyridine) and 5,5′-dithiobis(2-nitrobenzoate) as the ligands for CAC–TDI gels

Number	Samples	Mean recoveries of MTs (%)	
		DTNP[a]	DTNB[a]
1	Cd–Th/water	19.0±1.9	22.5±1.3
2	Cd–Th/urine	22.1±2.3	24.6± 2.1
3	Cd–Th/plasma	39.5±5.5	44.4±6.1
4	Zn–Th/water	20.5±1.9	23.2±1.4
5	Zn–Th/urine	21.1±2.3	26.0±2.2
6	Zn–Th/plasmsa	40.4±5.5	45.6±6.1
7	Cu–Th/water	23.3±2.1	26.5±1.9
8	Cu–Th/urine	25.0±2.7	29.5±2.7
9	Cu–Th/plasma	43.8±6.0	53.5±6.2

[a] DTNP, 2,2′-dithiobis(2-nitropyridine); DTNB, 5,5′-dithiobis(2-nitrobenzoate).

Table 5
Mean recoveries of Cd, Zn and Cu from all media using 2,2′-dithiobis(2-nitropyridine) and 5,5′-dithiobis(2-nitrobenzoate) as the ligands for CAC–TDI gels

Number	Samples	Mean recoveries of metals (%)	
		DTNP[a]	DTNB[a]
1	Cd/water	20.0±2.1	22.9±2.1
2	Cd/urine	22.5±2.4	24.5±2.3
3	Cd/plasma	52.3±3.3	55.1±3.4
4	Zn/water	26.8±3.1	29.2±3.1
5	Zn/urine	28.5±3.5	32.8±3.5
6	Zn/plasma	81.8±5.2	86.6±5.5
7	Cu/water	29.5±2.5	31.2±2.5
8	Cu/urine	33.3±3.2	36.4±3.2
9	Cu/plasma	128.7±5.4	134.9±5.6

[a] DTNP, 2,2′-dithiobis(2-nitropyridine); DTNB, 5,5′-dithiobis(2-nitrobenzoate).

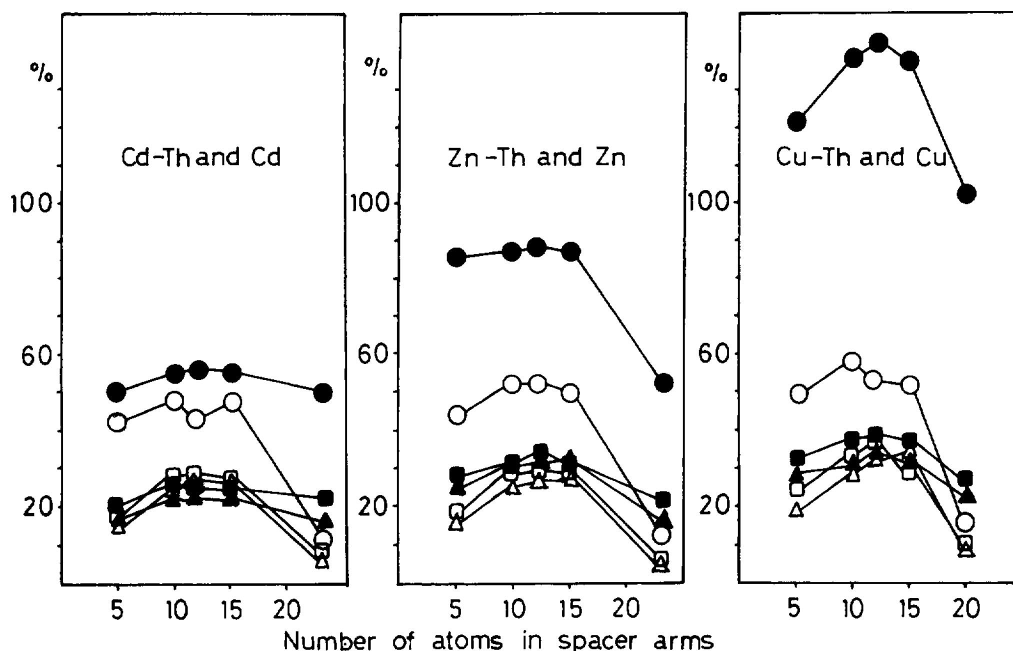

Fig. 2. Relation between adsorption yields of metallothionein protein and metals, and the number of atoms in the spacer arm in the CAC–TDI gels used as SPE sorbents. (△) Metallothionein from water; (○) metallothionein from plasma; (□) metallothionein from urine; (▲) metal from water; (●) metal from plasma; (■) metal from urine.

from water, 0.51–0.93 from urine and 0.42–0.89 from plasma (Table 6, Figs. 3–5). The mean correlation coefficients (r) calculated for the best three CAC–TDI gels with the highest recoveries and with the highest (r) coefficients [Affi-Gel 401, Sepharose–DTNB(A) and activated Thiol–Sepharose 4B], with DTNB as the ligand, are presented in Table 7. The mean general detection limits for different MTs in water, urine and plasma, calculated by the indirect method, based on the detection limits of metals and obtained using the AAS method are presented in Table 8.

4. Discussion

Previous results showed that the mechanism of separation and immobilization of polythiolproteins and metallopolythiolproteins is very complicated and that the native structure of the separated proteins is changed [26–29,38–40]. The yields of protein were between 10–50% of the amount applied and depended on the type of MT proteins [3–7]. Based on these results, we propose a new type of SPE sorbent, based on CAC–TDI gels, for the preconcentration of MTs and metals for quantitation of MT protein from water, urine and plasma. The concentration of MT was calculated according to Eq. (1) [22–25,41].

The data in Tables 1–3 show the differences in recoveries of MT proteins and metals depending on the type of CAC–TDI gel used. The separation yield depended on the length of the CAC–TDI spacer arm (Fig. 2) and on the type of modifications (which influence their hydrophobicity). The highest recoveries of proteins and metals, and the lowest dispersion, were obtained for Affi-Gel 401, Sepharose–DTNB(A) and activated Thiol–Sepharose 4B, with spacer arms containing 10 to 15 atoms. Similar and very constant results were obtained for all three types of MTs and metals. The highest recoveries were for Cu–Th and Cu from plasma. The lowest absorptions found were for Cd–Th and Cd (Tables 4 and 5). The recoveries for Sepharose–DTNB(B) and Thiopropyl–Sepharose 6B (very short and very long spacer arms, respectively) were very low for all types of MTs and metals. Even modifying the spacer

Table 6
Correlation coefficients (r) between the concentration of metallothionein added to water, urine and plasma and the concentration calculated from the content of metals bound to the covalent affinity chromatography gels

Number	MTs	SPE support	Correlation coefficient (r)		
			Water	Urine	Plasma
1	Cd–Th	TS-6B–DTNP	0.68	0.61	0.53
2	Cd–Th	TS-6B–DTNB	0.73	0.65	0.59
3	Cd–Th	ATS-4B–DTNP	0.91	0.86	0.81
4	Cd–Th	ATS-4B–DTNB	0.97	0.92	0.85
5	Cd–Th	AF-401–DTNP	0.89	0.85	0.79
6	Cd–Th	AF-401–DTNB	0.96	0.92	0.87
7	Cd–Th	S–DTNB(A)	0.96	0.91	0.89
8	Cd–Th	S–DTNB(B)	0.74	0.70	0.63
9	Zn–Th	TS-6B–DTNP	0.64	0.63	0.55
10	Zn–Th	TS-6B–DTNB	0.71	0.69	0.61
11	Zn–Th	ATS-4B–DTNP	0.87	0.85	0.73
12	Zn–Th	ATS-4B–DTNB	0.96	0.93	0.85
13	Zn–Th	AF-401–DTNP	0.92	0.90	0.84
.14	Zn–Th	AF-401–DTNB	0.95	0.93	0.86
15	Zn–Th	S–DTNB(A)	0.95	0.91	0.88
16	Zn–Th	S–DTNB(B)	0.69	0.65	0.53
17	Cu–Th	TS-6B–DTNP	0.54	0.51	0.43
18	Cu–Th	TS-6B–DTNB	0.68	0.63	0.56
19	Cu–Th	ATS-4B–DTNP	0.85	0.82	0.74
20	Cu–Th	ATS-4B–DTNB	0.92	0.89	0.77
21	Cu–Th	AF-401–DTNP	0.89	0.85	0.74
22	Cu–Th	AF-404–DTNB	0.94	0.90	0.83
23	Cu–Th	S–DTNB(A)	0.93	0.90	0.85
24	Cu–Th	S–DTNB(B)	0.59	0.53	0.42

arm and changing its hydrophobicity did not help this situation. A spacer arm that is too short gives the effect of "steric hindrance" and one that is too long gives rise to "conformational acclusion" effects, which contributed to the very low yields and unspecific adsorption. Similar results were obtained in the experiment where different structures of the spacer arm and of the ligand were used for fast immobilization of proteins and enzymes [26–29].

The best ligand was DTNB. With this ligand, the recoveries for all types of MTs and metals from water, plasma and urine were higher than obtained using DTNP (Tables 4 and 5). For two soft bases such as DTNB and DTNP, the thionitrobenzoic acid is better stabilized by a resonance effect. These results confirm the greater ability of metals (soft acids) to create the mercaptide bonds or disulphide bridge between ligands and sulfhydryl groups of separating thiolproteins. In this situation, the adsorption yields (recovery of proteins) decrease for better stabilized ligand [38,40]. In later experiments, testing the correlation between the concentration of MTs added to samples and the concentration calculated using an indirect method, CAC–TDI gels, such as Sepharose–DTNB(A), Affi-Gel 401 and activated Thiol–Sepharose 4B, were used, with DTNB as the ligand.

Tables 1–3 show a big variation in the separation

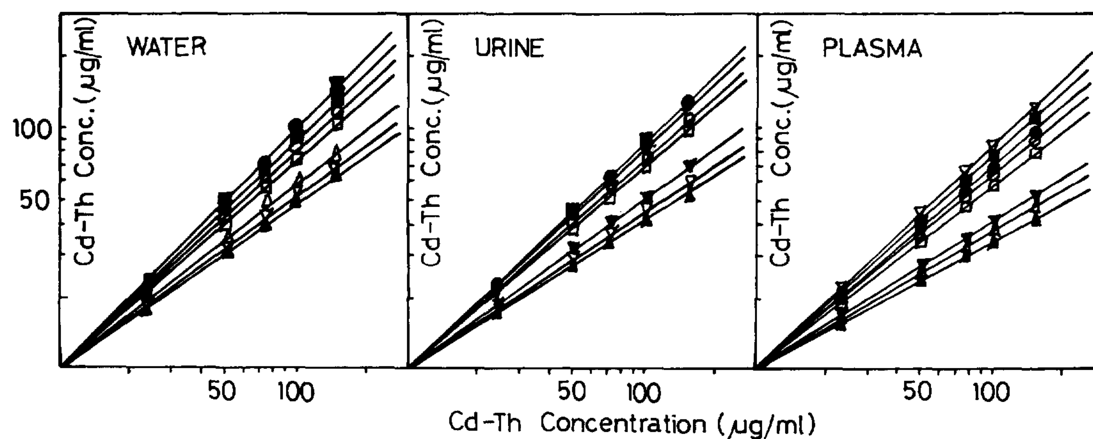

Fig. 3. Correlation between the concentration of Cd–Th added to the samples and the concentration of Cd–Th calculated from the Cd content. (left) Cd–Th added to water; (middle) Cd–Th added to urine; (right) Cd–Th added to plasma. (▲) TS-6B/DTNP; (△) TS-6B/DTNB; (○) ATS-4B/DTNP; (●) ATS-4B/DTNB; (□) AF-401/DTNP; (■) AF-401/DTNB; (▼) S–DTNB(A); (▽) S–DTNB(B).

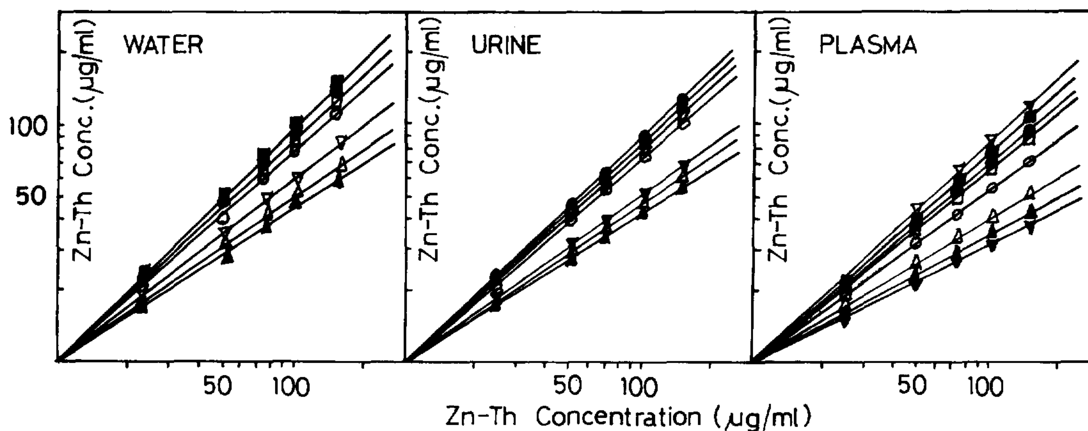

Fig. 4. Correlation between the concentration of Zn–Th added to the samples and the concentration of Zn–Th calculated from the Zn content. (left) Zn–Th added to water; (middle) Zn–Th added to urine; (right) Zn–Th added to plasma. (Symbols are the same as in Fig. 3).

yields for Cd–Th, Zn–Th and Cu–Th proteins and for metals, such as Cd, Zn and Cu, from urine and especially from human plasma. In general, the lowest and more constant results were obtained for Cd. The highest variation in recoveries was observed for Cu (Table 3). Figs. 3–5 and Tables 6 and 7 show the correlations between the concentrations of all three MT proteins added to water or human fluids and the concentrations calculated from the content of metals. The worst results were obtained for plasma. The best correlation was obtained for Cd–Th and the worst for Cu–Th. In this situation, when the correlation coefficient (r) are so low, the calculation of Cu–Th and Zn–Th concentrations in plasma, by the indirect method is more difficult than for Cd–Th. The detection limits calculated for Cd–Th, based on the results from ATS-4B, S-DTNB(A) and AG-401, were 7.7–7.9 ng/ml for Cd–Th, 8.5–8.7 ng/ml for Zn–Th and about 9.3–9.5 ng/ml for Cu–Th (Table 8).

This method can be used for the determination of low concentrations (~10 ng/ml; 7–9 ng/ml depending on the type of CAC–TDI gel used) of MTs in physiological fluids (plasma, urine) and in tissues

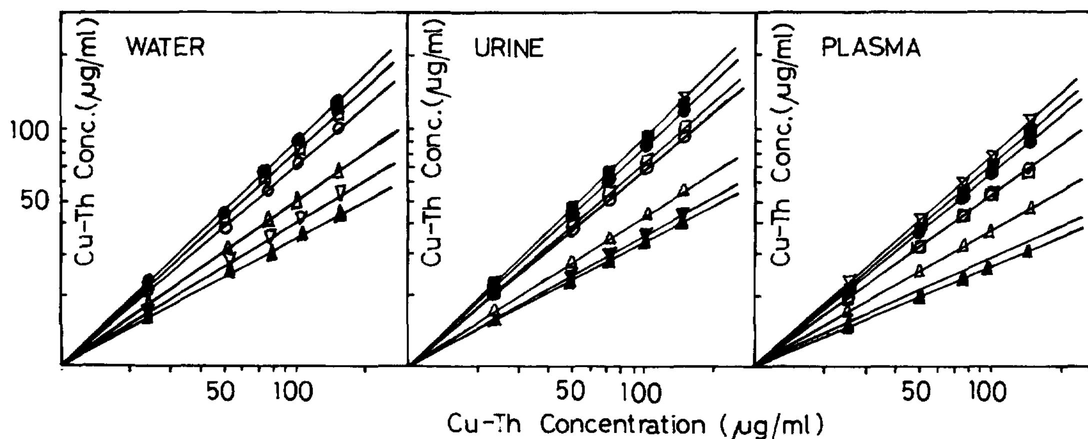

Fig. 5. Correlation between the concentration of Cu–Th added to the samples and the concentration of Cu–Th calculated from the Cu content. (left) Cu–Th added to water; (middle) Cu–Th added to urine; (right) Cu–Th added to plasma. (Symbols are the same as in Fig. 3).

Table 7
Mean correlation coefficients (r) between the concentration of metallothionein added to water, urine and plasma and the concentrations calculated from the content of metals bound to covalent affinity chromatography gels

Number	Metallothionein	Mean correlation coefficient (r)[a]		
		Water	Urine	Plasma
1	Cd–thionein	0.96	0.92	0.87
2	Zn–thionein	0.95	0.92	0.84
3	Cu–thionein	0.93	0.90	0.82

[a] The mean correlation coefficients were calculated for Sepharose–DTNB(A), Affi-Gel 401 and activated Thiol–Sepharose 4B, with DTNB as the ligand.

similar to latex immunoassay (LIA) or radioimmunoassay (RIA) techniques. The results obtained were higher than those reported in the literature for immunological methods (LIA and RIA, detection limits of about 0.1–0.01 ng/ml), but were lower than the detection limits for MT protein concentration obtained using chemical methods (Lowry, BCA, CBB, tannin method, etc.), which were about 1000–10 000 ng/ml.

The presented indirect method for the determination of MT protein concentration in human fluids using CAC–TDI supports can be used for the determination of MTs after exposure to heavy metals, when the concentration of MT ranged from 20–2500 μg/g creatinine [42–44]. The MT concentration level in fluids (urine, plasma) in the physiological state ranges from 20 ng/g–2.5 μg/g creatinine [42,43,45] and use of the indirect method of analysis, with a detection limit of ~10 ng/ml (higher than 0.5 μg/g creatinine), is more problematic. The proposed method must be continually modified for the determination of MT concentration in the physiological state in the human body.

5. Conclusions

(1) The supports for covalent affinity chromatography can be used as SPE sorbents for the preconcentration of metallothionein protein and metals.

(2) The concentration of metallothionein can be determined by an indirect method, based on calculation of the MT concentration from the content of metals bound to a covalent affinity chromatography column and the detection limits for these methods are about 7–9 ng/ml for all three types of MTs.

(3) The optimal lengths of spacer arms used in experiments were from 10 to 15 atoms in chain (Sepharose–DTNB(A), Affi-Gel 401 and activated Thiol–Sepharose 4B) and gave the maximum absorption of metals and proteins with correlation coefficients of about 0.8–0.9.

(4) The best ligand for CAC–TDI gels was 5,5′-dithiobis(2-nitrobenzoate) (DTNB), which was stabilized by resonance structures, and gave very constant thiol complexes with heavy metals.

(5) The indirect method of determination of metallothionein concentration in physiological fluids can be used only for human fluids from a population exposed to heavy metals, but not in the normal physiological state.

Table 8
Mean theoretical detection limits for Cd–thionein, Zn–thionein and Cu–thionein determined in water, plasma and urine by SPE

Number	Medium	Mean detection limits (ng/ml)[a]		
		Cd–thionein[b]	Zn–thionein[b]	Cu–thionein[b]
1	Water	7.712±0.071	8.534±0.085	9.348±0.099
2	Plasma	7.889±0.097	8.731±0.135	9.527±0.157
3	Urine	7.776±0.089	8.627+0.091	9.422±0.112
4	Mean	7.792±0.086	8.631±0.104	9.432±0.123

[a] The mean detection limits were calculated for Sepharose–DTNB(A), Affi-Gel 401 and activated Thiol–Sepharose 4B, with DTNB as the ligand.
[b] The detection limit used for the calculation of metallothionein concentration was 0.0001 μg/ml for Cd, Zn and Cu.

References

[1] K. Brocklehurst, J. Carlsson, M.P.J. Kiersten and E.M. Crook, Biochem. J., 133 (1973) 573.
[2] K.S. Squibb and R.J. Cousins, Biochem. Biophys. Res. Commun., 75 (1977) 806.
[3] L. Ryden and H.F. Deutsch, J. Biol. Chem., 253 (1978) 519.
[4] A.K.M. Kabziński and T. Paryjczak, Chem. Anal., 36 (1991) 111.
[5] A.K.M. Kabziński and T. Paryjczak, Zesz. Nauk. P. L., 43 (1991) 456.
[6] A.K.M. Kabziński and T. Paryjczak, Chem. Anal., 36 (1991) 123.
[7] A.K.M. Kabziński and T. Paryjczak, Chem. Anal., 36 (1991) 699.
[8] M. Margoshes and B.L. Vallee, J. Am. Chem. Soc., 79 (1957) 4813.
[9] J.H.R. Kagi and B.L. Vallee, J. Biol. Chem., 235 (1960) 3460.
[10] J.H.R. Kagi and B.L. Vallee, J. Biol. Chem., 236 (1961) 2435.
[11] J.H.R. Kagi, S.R. Himmelhoch, P.D. Whanger, J.L. Bethune and B.L. Vallee, J. Biol. Chem., 249 (1974) 3537.
[12] Y. Kojima, C. Berger, B.L. Vallee and J.H.R. Kagi, Proc. Natl. Acad. Sci. USA, 73 (1976) 3413.
[13] W. Braun, W. Wagner, E. Worgotter, M. Vasak, J.H.R. Kagi and K. Wuthrich, J. Mol. Biol., 187 (1986) 125.
[14] A. Arseniev, P. Schultze, E. Worgotter, W. Braun, G. Wagner, M. Vasak, J.H.R. Kagi and K. Wuthrich, J. Mol. Biol., 201 (1988) 637.
[15] J.H.R. Kagi and A. Schaffer, Biochemistry, 27 (1988) 8509.
[16] J.H.R. Kagi and Y. Kojima, Experientia, Suppl., 52 (1987) 25
[17] I.Y. Huang, M. Kimura, A. Hata, H. Tsunoo and A. Yoshida, J. Biol. Chem., 89 (1991) 1839.
[18] J.H.R. Kagi, Methods Enzymol., 205 (1991) 613.
[19] Z.A. Shaikh and C. Tohyama, Environ. Health Perspect., 54 (1984) 171.
[20] Z.A. Shaikh, K.M. Harnett, S.A. Perlin and P.C. Huang, Experientia, 45 (1989) 146.
[21] A.K.M. Kabziński, presented at the 7th Symposium on Handling of Environmental and Biological Samples in Chromatography. Lund, 8–10 May 1995, Conference Materials, p. 24.
[22] A.K.M. Kabziński and T. Takagi, Biomed. Chromatogr., 9 (1995) 123.
[23] A.K.M. Kabziński and T. Paryjczak, Chem. Anal., 40 (1995) 831.
[24] A.K.M. Kabziński, Chem. Anal., 40 (1995) 847.
[25] A.K.M. Kabziński, T. Takagi and K. Tanaka, Eur. J. Clin. Chem. Clin. Biochem., 33 (1995) A56.
[26] A.K.M. Kabziński and T. Paryjczak, Przem. Chem., 71 (1992) 309.
[27] A.K.M. Kabziński and T. Paryjczak, Przem. Chem., 72 (1993) 202.
[28] A.K.M. Kabziński and T. Paryjczak, Przem. Chem., 75 (1996) 338.
[29] A.K.M. Kabziński, Chromatographia, 43 (1996) 513.
[30] A.K.M. Kabziński, Chem. Anal., in press.
[31] A.K.M. Kabziński, Chem. Anal., in press.
[32] O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[33] M. Higuchi and F. Yoshida, Anal. Biochem., 77 (1977) 542.
[34] E. Ross and G. Schatz, Anal. Biochem., 54 (1973) 304.
[35] W. Mejbaum-Katzenellenbogen, Acta Biochim. Polon., 11 (1955) 279.
[36] G.L. Ellman, Arch. Biochem. Biophys., 62 (1959) 70.
[37] P.H.W. Butterworth, H.Baum and J. Porter, Arch. Biochem. Biophys., 118 (1967) 716.
[38] A.K.M. Kabziński, Chromatographia, 35 (1993) 439.
[39] A.K.M. Kabziński and T. Takagi, Acta Chromatogr., 5 (1995) 60.
[40] A.K.M. Kabziński and T. Paryjczak, Acta Chromatogr., 5 (1995) 34.
[41] A.K.M. Kabziński, Polish J. Environ. Stud., 5 (1996) 217.
[42] G.F. Nordberg, J.S. Garvey and C.C. Chang, Environ. Res., 28 (1982) 179.
[43] F.Y. Falck, L.J. Lin, R.G. Smith, J.S. Garvey. A. Schork, B. England, K.D. McClathey and H.J. Linton, Brit. J. Ind. Med., 40 (1983) 305.
[44] H. Roels, R.R. Lauwery, J.P. Buchet, A. Bernard, J.S. Garvey and H.J. Linton, Int. Arch. Occup. Environ. Health, 52 (1983) 159.
[45] C.C. Chang, R.J.V. Mallie and J.S. Garvey, Toxicol. Appl. Pharmacol., 55 (1980) 94.