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Isolation and characterization of six human hepatic isometallothioneins

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Human hepatic metallothionein (MT) was separated into six isoforms by using reversed-phase h.p.l.c. at the analytical and preparative levels. By comparison with the h.p.l.c. elution profiles of the charge-separable species MT-1 and MT-2 isolated by the procedure of Bühler & Kägi [(1974) FEBS Lett. **39**, 229–234], five of these isoproteins are identified as hitherto unresolved subforms of MT-1, and one is identical with MT-2. The six isoforms have distinct and reproducible retention times at neutral pH, where the metal remains bound to the protein, and at low pH, where the metal is removed. Their amino acid compositions display the high cysteine content and the lack of aromatic amino acids and of histidine typical of mammalian metallothioneins, but they differ significantly with respect to all other amino acids. A survey of autopsy material indicates that in adult human liver all six isoforms are usually expressed, albeit in somewhat variable relative proportions.

INTRODUCTION

MTs are small proteins (M_r approx. 6000) characterized by an extremely high sulphur and heavy-metal (zinc, copper and cadmium) contents. All mammalian forms sequenced thus far have 20 invariant cysteine residues and typically bind a total of seven bivalent metal ions (Hunziker & Kägi, 1985). Potentiometric and spectroscopic data indicate that the metals are bound through metal-thiolate linkages to the cysteine side chains and that the complexes formed are combined to two distinct oligonuclear metal–thiolate clusters (Otvos & Armitage, 1980; Vašák & Kägi, 1983). The biological function of MTs is still unclear. On account of the efficient induction of these proteins in many cells by exposure to salts of a number of toxic heavy metals they are thought to be involved in their detoxification. However, MT has also been implicated in homoeostatic adaption mechanisms bearing on the metabolism of essential d^{10} elements, as evidenced by its induction by hormones (Etzel & Cousins, 1981) and by conditions of physical stress (Oh *et al.*, 1978; Sobocinski *et al.*, 1978).

Since its discovery in horse kidney (Margoshes & Vallee, 1957), MT has been obtained from tissues of many species (Hunziker & Kägi, 1985). The organ distribution as well as tissue concentrations of the protein are thoroughly documented for mammals. The major sources for MT are liver and kidney of animals pretreated with large doses of cadmium or zinc salts.

By conventional methods (Bühler & Kägi, 1974) two isoprotein fractions differing in charge are resolved in most species. By accepted nomenclature, they are designated as MT-1 and MT-2 (Nordberg & Kojima, 1979). In certain tissues, such as mouse liver, these fractions were shown to be homogeneous by amino acid sequence analysis (Huang *et al.*, 1977, 1981). In contrast, the isoMT pattern of human liver is more complex. Whereas MT-2 was documented to be a single polypeptide chain (Kissling & Kägi, 1977), the MT-1 fraction was microheterogeneous by sequence analysis (Kissling & Kägi, 1979). From amino acid variations in a total of six positions it was inferred that the sequenced material contained at least three subforms. The basis for this heterogeneity was uncovered by Karin & Richards (1982*b*), who identified in the human genome about 11 genes for MT, including at least one non-functional pseudogene. Since these genes may be under separate control and since their expression may be serving quite different purposes, it is essential for the further elucidation of the biological roles of MTs to establish methods for the resolution and quantification of the corresponding gene products. The resolution of a number of closely related isoMTs from rabbit liver by reversed-phase h.p.l.c. (Klauser *et al.*, 1983) suggested that this method might be applicable to the isolation of the various human isoMTs. The present paper illustrates the usefulness of reversed-phase h.p.l.c. for this purpose and documents the existence of at least six isoforms of MT occurring in human liver. The procedure developed is suitable for comparing hepatic isoMT patterns in post-mortem samples of different individuals.

MATERIALS AND METHODS

Materials

Quartz distilled water was used for buffer preparation. Tris was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), trifluoroacetic acid ('zur Sequenzanalyse') and Perhydrol (30% H_2O_2, purum) were from Fluka (Buchs, Switzerland), Sephadex G-50 was from Pharmacia (Uppsala, Sweden) and acetonitrile was from J. T. Baker (Deventer, The Netherlands). All other reagents were of analytical grade and were obtained from Fluka or Merck (Darmstadt, Germany). Human liver samples frozen within 12 h *post mortem* were obtained from the Institute of Pathology of the University Hospital of Zürich.

Abbreviations used: MT, metallothionein; isoMT, isometallothionein; MT-1 and MT-2, charge-separable isoforms of metallothionein.

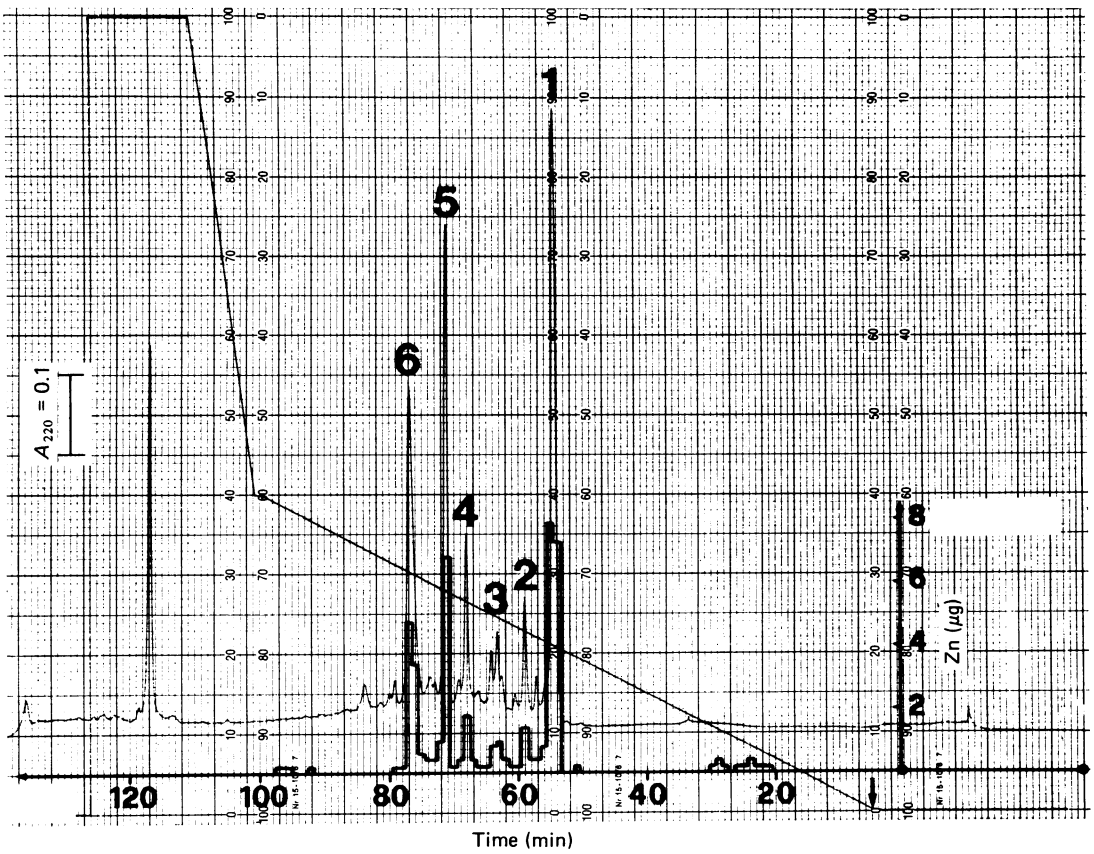

Fig. 1. Preparative h.p.l.c. at neutral pH of the MT-containing fraction obtained by Sephadex G-50 gel chromatography

One-fourth (18 ml) of the MT-containing Sephadex G-50 fraction was applied to a preparative LiChrosorb RP-18 (10 μm) column (21 mm internal diam. × 250 mm) through one of the pumps and chromatographed with a gradient (straight line) formed between buffer A (25 mM-Tris/HCl buffer, pH 7.5) and buffer B [as buffer A, containing 60% (v/v) acetonitrile] at a flow rate of 2 ml/min. The arrow indicates the start of the gradient. Fractions were collected every minute. IsoMTs were identified by measuring the zinc concentration in each fraction (block diagram) and by amino acid analysis. Referred to the total amount of zinc applied, the recoveries of zinc in the individual fractions were as follows: (1) 23%; (2) 2%; (3) 3%; (4) 3%; (5) 12%; (6) 13%.

MT isolation

For each preparation, 10 g wet wt. of frozen liver was thawed, cut into small pieces and homogenized in a Waring blender twice at 30 s intervals with 2 vol. (v/w) of buffer (250 mM-sucrose in 10 mM-Tris/HCl buffer, pH 8.6, containing 2 mM-2-mercaptoethanol). The homogenate was centrifuged at 100000 *g* for 90 min at 4 °C. After filtration through four layers of gauze the supernatant was chromatographed on a Sephadex G-50 column (3.5 cm × 75 cm) in 10 mM-Tris/HCl buffer, pH 8.6, at a flow rate of 43.4 ml/h. Metal concentrations in the effluent were monitored by atomic-absorption flame spectrophotometry. MT-containing fractions were pooled and used for h.p.l.c. without prior concentration.

H.p.l.c.

The h.p.l.c. equipment described previously (Klauser *et al.*, 1983) was used for analytical and preparative chromatography. Separations were achieved on Hyperchrom columns (LiChrosorb RP-18, 10 μm particle size; Bischoff Analysentechnik, Leonberg, Germany) and on an Aquapore RP-300 column (RP-8, 10 μm particle size, 30 nm pore size; Brownlee Laboratories, Santa Clara, CA, U.S.A.). Solvent systems were adapted from Klauser *et al.* (1983).

Amino acid analysis

Amino acid analysis after performic acid oxidation (Hirs, 1967) and hydrolysis at 110 °C for 22 h were carried out either on a Durrum D-500 analyser or by h.p.l.c. after dansylation (5-dimethylaminonaphthalene-1-sulphonylation) (De Jong *et al.*, 1982).

RESULTS

The analytical h.p.l.c. of a sample of the MT-containing Sephadex G-50 fraction at neutral pH on a C_{18} support demonstrated the presence of several zinc-containing protein fractions, with retention times comparable with those reported previously for rabbit and fish MTs (Klauser *et al.*, 1983). The elution diagram of a preparative h.p.l.c. run (Fig. 1) shows the resolution of six zinc-containing fractions. The total recovery of zinc was 50–58%.

Each metal-containing fraction was rechromatographed with the same buffer system on an Aquapore RP-300

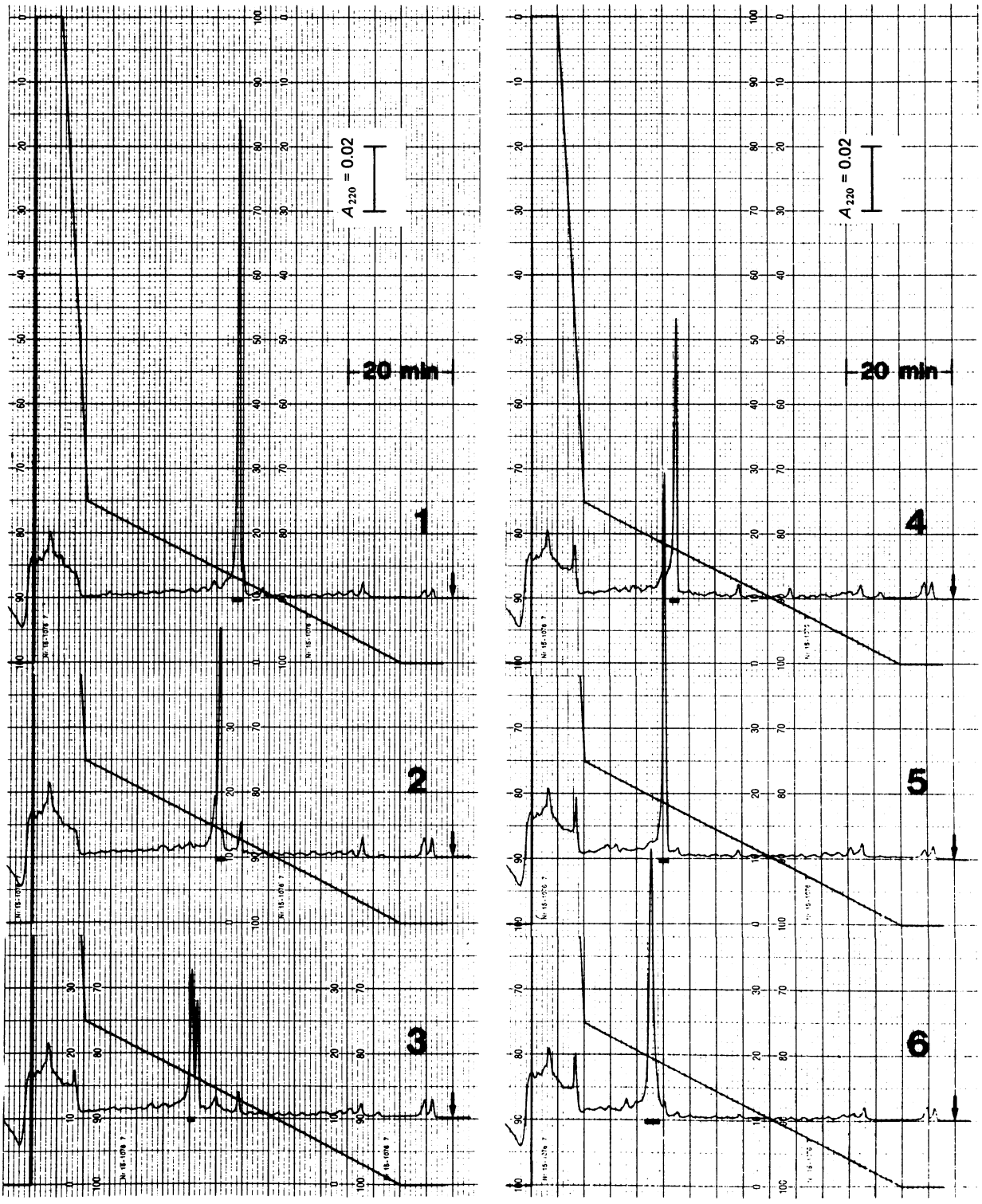

Fig. 2. Rechromatography at neutral pH of isoMT fractions obtained by preparative h.p.l.c. (Fig. 1)

H.p.l.c. was performed on an Aquapore RP-300 column (4.6 mm internal diam. × 250 mm) at 1 ml/min, with the same gradient (straight line) as in Fig. 1. At the arrow, protein samples containing 1 μg of zinc were injected. For rechromatography at low pH (Fig. 3), MT fractions were collected as indicated by the horizontal bars. Numbers 1–6 correspond to the numbered isoMT fractions of Fig. 1.

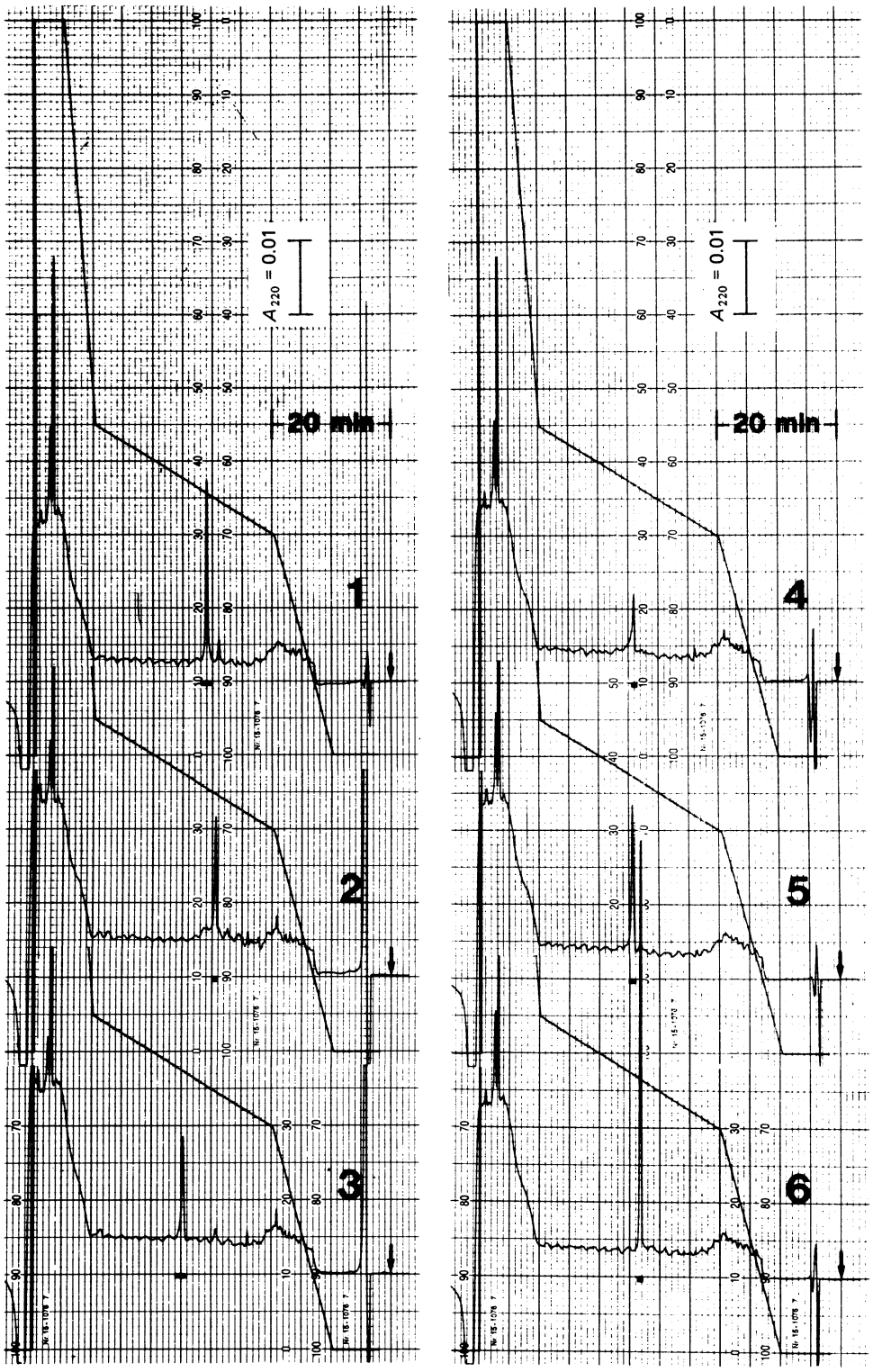

Fig. 3. H.p.l.c. at low pH of isoMT fractions obtained as indicated in Fig. 2

Chromatography was performed at a flow rate of 1 ml/min on an Aquapore RP-300 column with a gradient (straight line) formed between buffer A [0.1% (v/v) trifluoroacetic acid] and buffer B [0.1% trifluoroacetic acid in 60% (v/v) acetonitrile]. At the arrow, samples containing 0.5 μg of zinc were injected. For amino acid analysis (Table 1), MTs were collected as indicated by the horizontal bars. Numbers 1–6 correspond to the numbered isoMT fractions of Fig. 1.

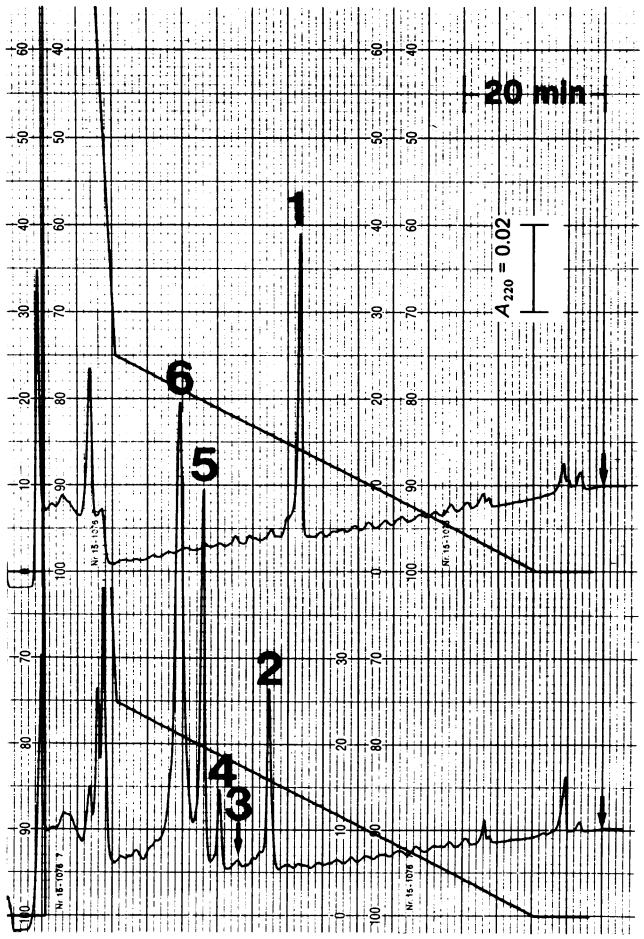

Fig. 4. H.p.l.c. analysis of charge-separable MT-2 (top) and MT-1 (bottom) fractions

MT-2 and MT-1 separated by ion-exchange chromatography (Bühler & Kägi, 1974) were applied at the arrow to a RP-18 column (4.6 mm internal diam. × 250 mm). Conditions for chromatography were as given in Fig. 2. The numbers correspond to the isoMT fractions of Fig. 1.

column (Fig. 2). In each case the zinc-containing material was recovered as a single well-resolved fraction, the order of elution being identical with that in the case of preparative h.p.l.c. The recoveries of zinc for the individual fractions were (1) 67%, (2) 33%, (3) 26%, (4) 46%, (5) 60% and (6) 49%.

That the zinc-containing fractions are essentially homogeneous was also demonstrated by rechromatography on an Aquapore RP-300 column at low pH (Fig. 3). Under these conditions MT is eluted as the metal-free or apo form. With respect to protein, the recoveries of the various fractions were (1) 80%, (2) 34%, (3) 20%, (4) 20%, (5) 30% and (6) 66%.

Table 1 compares the amino acid compositions of each fraction obtained by h.p.l.c. at low pH. As is typical for mammalian MTs, all fractions exhibit high cysteine and lysine contents and completely lack aromatic amino acids and histidine. Fraction 1 differs from all other forms by a higher aspartate/asparagine content and a lower valine content, features previously shown to be diagnostic of isoform MT-2 separated by ion-exchange chromatography (Bühler & Kägi, 1974). This correspondence is verified independently in Fig. 4, where the MT-1 and MT-2 fractions prepared from human liver by the conventional method (Bühler & Kägi, 1974) were subjected to analytical h.p.l.c. on RP-18 at neutral pH. In contrast with MT-2, which is known to be homogeneous (Kissling & Kägi, 1977) and is eluted as a single peak in the position of fraction 1, MT-1 yields the expected five remaining fractions.

All normal human livers examined contained these six isoMTs. As shown in Fig. 5, material from three different livers gave h.p.l.c. fractions with corresponding retention times, but the relative amounts in these fractions varied widely.

DISCUSSION

The present data show that, by using reversed-phase h.p.l.c., MT of human liver can be resolved into its components (Fig. 1). By using the pH 7.5 buffer system, nearly complete separation of six native isoMTs was achieved. The high sensitivity of h.p.l.c. should allow analytical screening of isoMT content in human liver samples as small as 0.1 g.

The elution profile (Fig. 1) of the MT-containing Sephadex G-50 fraction of cytosolic preparations of the same liver was reproducible, with respect to both yield and retention times. On rechromatography on Aquapore RP-300 at neutral pH (Fig. 2), fractions 1, 4, 5 and 6 could not be resolved further, but fractions 2 and 3 were each separated into two closely adjacent fractions of which only one contained detectable amounts of zinc. The same high resolution was achieved when rechromatography was performed at low pH, but the order of elution of the isoproteins was changed (Fig. 3). In both analytical h.p.l.c. systems the recoveries of each isoform were constant but differed substantially from one another. Since neither metal nor protein were found to be eluted at other positions, the incomplete recovery must be attributed either to partial precipitation of protein within the column or to its irreversible adsorption on the support. Comparable variations in yield have been reported during the reversed-phase h.p.l.c. of a number of proteins (Wilson *et al.*, 1982).

The amino acid compositions substantiate the high homology of the different isoMTs (Table 1). They also document their relationship to the isoforms separated by conventional ion-exchange chromatography (Bühler & Kägi, 1974; Kissling & Kägi, 1979). The composition of fraction 1 was identical with that of the electrophoretic species MT-2 as deduced from protein and cDNA sequencing (Kissling & Kägi, 1977; Karin & Richards, 1982*a*). By contrast, the electrophoretic species MT-1 is now documented to be a mixture of five isoproteins, which, besides their lower negative charge, also contain two valine residues. Some of their mutual differences with respect to most of the other amino acids are in keeping with the sequence heterogeneity encountered with unresolved human MT-1 (Kissling & Kägi, 1979), and have also been confirmed by preliminary sequence analysis of the tryptic peptides of isolated MT-1 isoforms (P. E. Hunziker, A. Kern, B. Looser & J. H. R. Kägi, unpublished work). The observed variations in cysteine content are within the limits of the method of measurement. However, the observation that as much as three MT-1 isoforms (fractions 2, 3 and 4) contain up to

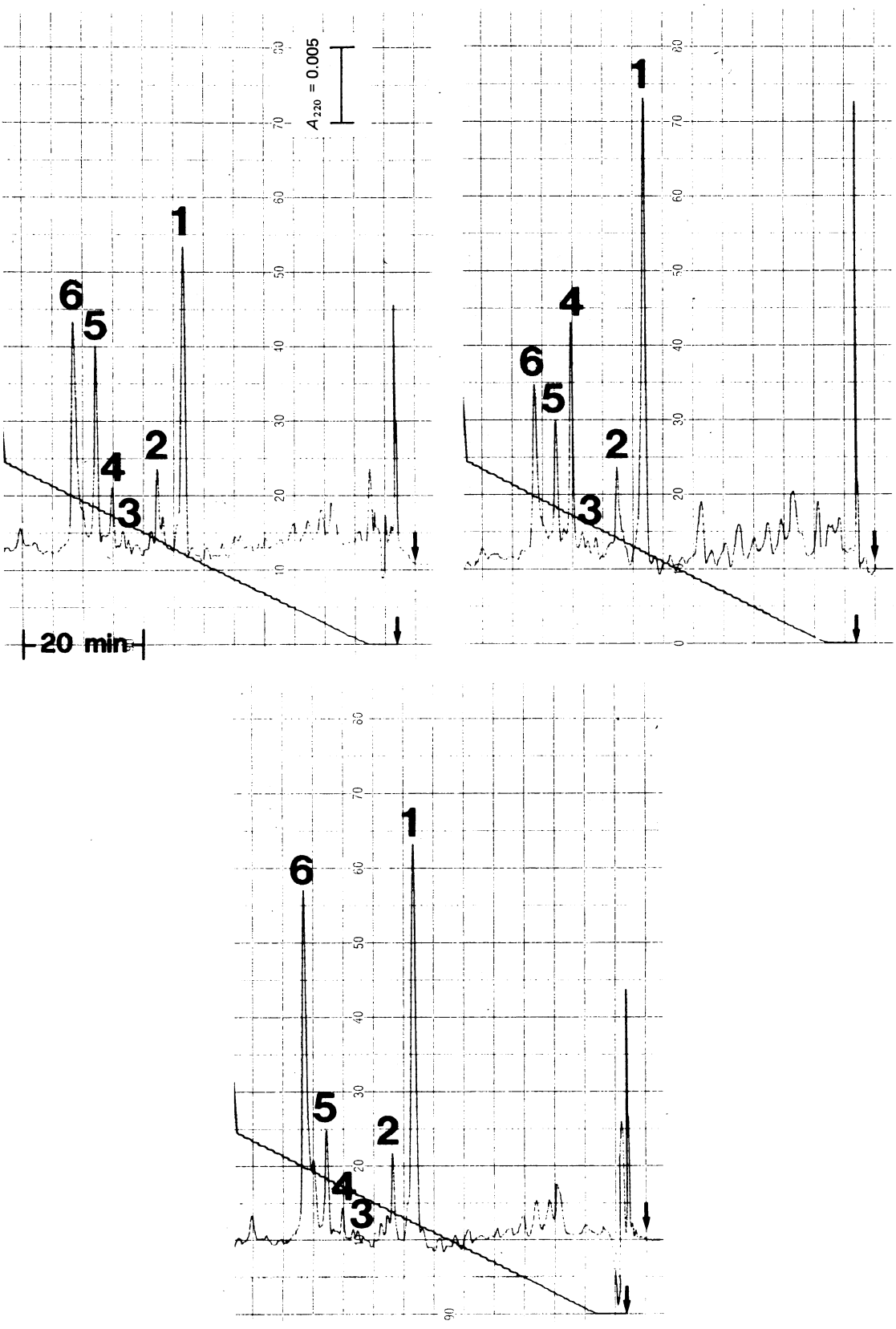

Fig. 5. Comparison of isoMT h.p.l.c. patterns of different human livers

MT-containing fractions of cytosols from 10 g of three normal human livers were prepared by gel filtration (see the Materials and methods section). Samples containing a total of 0.64, 0.77 and 0.63 μg of zinc respectively were chromatographed on a RP-18 column (4.6 mm internal diam. × 250 mm), with the chromatographic conditions described in Fig. 2. The numbered fractions correspond to the isoMT fractions of Fig. 1.

Table 1. Amino acid compositions of human hepatic isoMTs separated by h.p.l.c.: comparison with MT fractions separated by ion-exchange chromatography

The number of amino acids per polypeptide chain was calculated from the composition of the fractions resolved by h.p.l.c. at low pH (Fig. 3) assuming a chain length of 61 amino acid residues. All samples were oxidized before hydrolysis and analysis. Cysteine was determined as cysteic acid, methionine as methionine sulphone. — indicates less than 0.1. The composition of MT-2 refers to the known sequence (Kissling & Kägi, 1977; Karin & Richards, 1982*a*). The composition of MT-1 was taken from Bühler & Kägi (1974).

	Amino acid composition (mol of residues/mol)							
Amino acid	1	2	3	4	5	6	MT-2	MT-1
Asx	4.0	2.6	2.3	3.1	2.4	3.2	4	3
Thr	2.0	1.9	1.0	2.0	1.9	2.0	2	2
Ser	7.7	7.9	7.9	8.7	7.9	8.7	8	8
Glx	2.4	3.4	3.5	3.1	3.4	2.0	2	3
Pro	2.0	1.9	2.0	1.9	1.9	2.7	2	2
Gly	5.2	7.0	7.1	5.1	4.8	5.2	5	5
Ala	7.1	6.5	6.8	6.6	7.4	5.2	7	6
Cys	20.1	18.7	17.6	18.9	19.8	20.2	20	18
Val	1.1	1.8	1.7	2.1	2.0	1.9	1	2
Met	0.9	0.9	0.7	0.7	1.0	0.9	1	1
Ile	0.9	0.4	1.1	1.1	0.9	0.9	1	1
Leu	—	—	1.0	—	—	—	0	1
Tyr	—	—	—	—	—	—	0	0
Phe	—	—	—	—	—	—	0	0
His	—	—	—	—	—	—	0	0
Lys	7.9	8.0	8.5	8.0	7.6	8.0	8	8
Arg	—	—	—	—	—	—	0	0

10% less cysteine than the others supports our initial findings (Bühler & Kägi, 1974). It seems possible, therefore, for certain subforms of the MT-1 family to be lacking in some of the invariant cysteine residues.

The identification of six distinct isoMTs in human livers is of particular interest in view of the report by Karin & Richards (1982*b*) that the human genome contains 10–12 genes coding for MT. Two genes, one coding for MT-2 and one for an MT-1 isoform, have been sequenced and shown to be expressed upon induction by zinc and/or cadmium. The possible translation product of the MT-1 gene differs from those reported here by the complete absence of valine and by an additional methionine residue in the polypeptide chain (Richards *et al.*, 1984). Our failure to recover this isoform implies that this gene is not expressed in significant amounts in normal adult human liver.

The total amounts of MT in adult human liver can vary appreciably. In general, livers with readily detectable quantities of MT (> 10 mg) invariably contained all the six isoproteins identified in the present study (Fig. 5). However, there are marked quantitative differences among the subforms. Although the physiological significance of these variations is not understood, they may reflect differential regulation of expression. Differences in the promoter region of the different MT genes have been postulated to account for the unequal inducibility and function of the various isoforms by metals and hormones (Richards *et al.*, 1984). In conclusion, the comparative study of isoMT profiles in human tissues by h.p.l.c. is expected to be useful in complementing the molecular-genetics approach in the search for the physiological functions of the various isoMTs in man. Preliminary studies also indicate that massive changes in isoMT h.p.l.c. profile are associated with various forms of liver pathology (P. E. Hunziker, E. van Wieringen & J. H. R. Kägi, unpublished work).

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