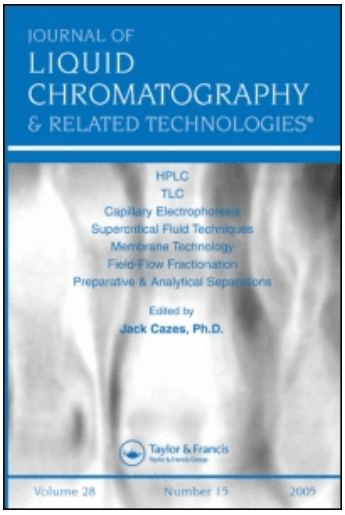

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Quantification of Basal Amounts of Isometallothioneins in Cultured Cells by Reversed-Phase HPLC

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QUANTIFICATION OF BASAL AMOUNTS OF ISOMETALLOTHIONEINS IN CULTURED CELLS BY REVERSED-PHASE HPLC

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ABSTRACT

A reversed phase HPLC method was developed to separate and quantify basal amounts of the major isometallothionein, i. e. MT-2, in cell extracts of human liver cells. To separate MT-2 from interfering proteins an ultrafiltration step was included in the preparation of the sample. Calibration was performed with varying amounts of pure human MT-2 quantified by amino acid analysis. Using MT-2 diluted with 25 mM Tris/HCl a non-linear calibration curve with a detection limit of 70 pmol was obtained. Linear correlation between the amount applied and the integrated area resulted when MT-2 was diluted with cytosol equivalent to 5.5 x 10^6 cells. The detection limit under these conditions was 5 pmol, sufficient to monitor basal metallothionein concentrations and to follow changes in the accretion of these proteins under physiological conditions.

INTRODUCTION

Metallothioneins (MT's) are cysteine- and metal-rich polypeptides identified in all animal phyla, in some plants and some eukaryotic and prokaryotic microorganisms (for review see,[1,2] and references therein). In mammals the MTs form a polymorphous family of proteins subdividable in several structurally distinct and in part tissue specifically expressed subclasses, i.e. MT-1, MT-2, MT-3 and MT-4.[3,4] MT-1 and MT-2 are the most abundant forms and subsume in some mammalian genera a number of genetically distinct isoforms. All have a molecular weight of approximately 6500 and contain seven equivalents of bivalent d^{10} metal ions, usually Zn^{2+}, bound through thiolate coordination to 20 cysteine residues of the polypeptide chain. The best supported biological functions of the mammalian MTs are in attenuating the harmful influences of toxic metal ions and of various stress conditions.[5]

The abundance of MTs in tissues and cultured cells varies widely as its biosynthesis can be greatly stimulated by exposure to heavy metal ions, certain hormones, cytokines, tumor promoters and toxic electrophiles as well as by a number of stress conditions.[2] Thus, addition of Zn^{2+} or of dexamethasone has resulted in massive cell- and inducer-specific isoMT accumulation.[6] Naturally enhanced expression of some of the MT-genes has also been found in fetal and perinatal development.[7,8]

In the absence of such biosynthesis promoting influences the cellular iso-MT concentrations range at much lower basal levels which are at the limit of detection by the commonly used chromatographic methods of analysis.[9,10] Thus, for exploring potential physiological variations of iso-MT's under basal conditions sensitive and specific analytical methods are needed.

In previous studies, we have successfully developed and employed a method which allowed the separation and accurate quantification of partially purified samples of induced human and rabbit MT's.[6,11]

In this report we describe an adaptation of this reversed phase HPLC method for a more direct quantification of human MT isoforms in the cytosol fraction of cultured Chang liver cells. The new method allows the detection of as little as 5 pmol of MT isoforms and provides thus an adequate and highly convenient means to assess variations in basal iso-MT concentrations under a variety of experimental conditions.

MATERIALS

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, gentamycine, amphotericin B (Fungizone), trypsin-EDTA solution (10x) and cell culture dishes were purchased from Gibco and fetal-calf serum (FCS) from Amimed. Ultrafree-CL ultrafiltration tubes (exclusion size 30kDa) were obtained from Millipore and Tris (Trizma base) from Sigma. All other chemicals were of analytical grade and obtained from either Merck or Fluka. All water used was obtained from an ultra pure water system (Milli-Q Plus, Millipore).

METHODS

Cell Culture

Chang liver cells were obtained from Flow Laboratories. DMEM cell culture media was supplemented with 2 mM glutamine, 50 mg/L gentamycine, 2.5 mg/L fungizone and 10% (v/v) FCS. Cells were grown at 37°C, 100% humidity and 8% CO_2. Confluent cultures were harvested by trypsinisation and cell numbers were determined with a haemocytometer.

Sample Preparation

Cells were harvested in DMEM at 4°C, suspensions of known cell count were centrifuged at 250 x g for 10 min and resuspended in 2.5 mL 25 mM Tris/HCl, pH 7.5. The cells were disrupted by ultrasonication (Sonifier cell disruptor B15, Branson) with 25 bursts of 1 sec duration at 10-20 W. The resulting homogenate was centrifuged at 100000 x g for 60 min. The supernatant was incubated for 4 min at 80°C, cooled on ice and centrifuged at 10000 x g for 10 min. The supernatant was diluted to a constant protein concentration equivalent to that obtainable from 5.5 x 10^6 cells/mL and passed through a Ultrafree-CL ultrafiltration tube. 1 mL of filtrate was loaded onto the HPLC column.

HPLC

An HPLC system Model 215 (LKB/Pharmacia) was used. Samples were injected with an autosampler Model 231 (Gilson) equipped with a 1 mL sample loop. The column (Spheri 5 RP-18, 2.1 mm (i.d.) x 220 mm, 5 μm particle size, Brownlee) was thermostated to 28°C. Chromatography was performed at a flow rate of 0.4 mL/min using a linear gradient formed between buffer A (25 mM

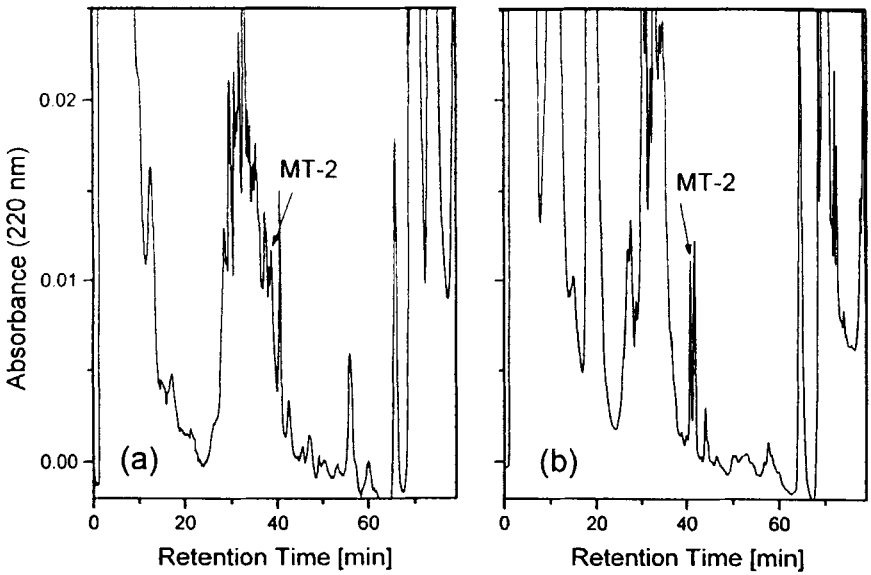

Figure 1. HPLC of cytosols obtained from proliferating Chang liver cells by (a) ultracentrifugation and heat denaturation and (b) additional ultrafiltration (30 kDa exclusion molecular weight).
Samples of 1 mL were injected on a Spheri-5 RP-18 column (2.1 (i.d.) x 220 mm) and eluted with a gradient formed between buffer A (25 mM Tris/HCl, pH 7.5) and buffer B (same as A, containing 60% (v/v) acetonitrile) at a flow-rate of 0.4 mL/min. The gradient used was as follows: 0% buffer B for 15 min.; from 0 to 23% buffer B during 45 min. The effluent was monitored at 220 nm

Tris/HCl, pH 7.5) and buffer B (same as buffer A containing 60% (v/v) acetonitrile). The gradient used was as follows: 0% buffer B for 15 min; from 0 to 23% buffer B during 45 min. The effluent was monitored at 220 nm and peak integration was performed on a Data System 450 (Kontron Instruments).

Calibration

The concentration of a stock solution of chromatographically pure human MT-2 (27.2 nmol/mL) was determined by quantitative amino acid analysis (Model 420A/H, Applied Biosystems). The concentrations (pmol/mL) used for calibration were prepared by diluting the stock solution either with 25 mM Tris/HCl, pH 7.5 or with cytosol prepared from Chang liver cells. For each calibration point, 1mL of the

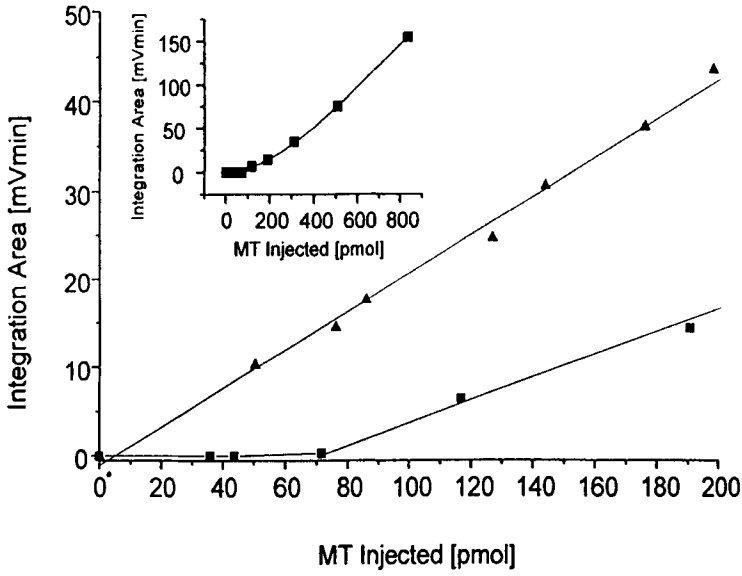

Figure 2. Calibration plots for MT-2 solutions in protein-free 25 mM Tris/HCl, pH 7.5 (■) and in 25 mM Tris/HCl, pH 7.5, containing cytosolic supernatant of Chang liver cells (▲_). Standard quality MT-2 was quantified by amino acid analysis before preparing the calibration solution. ■: 35 to 190 pmol of MT-2 were diluted in buffer A (25 mM Tris/HCl pH 7.5) and 1 mL was injected onto the HPLC column. The complete range examined under these conditions (35 to 800 pmol of MT-2) is shown in the insert. ▲: 50 to 200 pmol of MT-2 were diluted in 1 mL of cytosol prepared from a suspension of 5.5 x 10^6 cells/mL and injected onto the HPLC column. Cytosol was prepared by ultracentrifugation, heat denaturation and ultrafiltration. Chromatography was performed as described in Fig. 1. The effluent was monitored at 220 nm and the peak area of MT-2 was integrated using a Data System 450 (Kontron). In presence of cytosol the area of MT-2 was corrected for the contribution of endogenous MT-2 (0.49 ± 0.01 mVmin). Correlation coefficients (r^2) for linear and second order polynomial regression were 0.980 and 0.999, respectively, in the absence of cytosol (■). In the presence of cytosol (▲) $r^2 = 0.994$ for linear regression.

appropriate calibration solution was loaded onto the HPLC column. The influence of the concentration of cytosolic proteins on the recovery of MT-2 was monitored by diluting increasing amounts of cytosol containing approximately 7 pmol endogenous MT-2/10^6 cells with 25 mM Tris/HCl, pH 7.5 to a total volume of 1 mL. Samples corresponding to 0.25 x 10^6 to 14 x 10^6 cells were analyzed by HPLC and MT-2 was quantified by peak integration.

RESULTS

Figure 1 compares the profiles of cytosols of Chang liver cells obtained by two different preparation protocols which differ by an ultrafiltration step designed to eliminate interfering proteins from the cytosolic fraction. While MT-2 was hardly recoverable from cytosol prepared by ultracentrifugation of a cell homogenate and a heat denaturation step (Fig. 1(a)) this protein was easily resolved and quantified when an ultrafiltration step was included in the preparation of the sample (Fig. 1(b)). With the extended purification scheme a clear separation and a reproducible retention time of MT-2 with a relative standard deviation of ± 0.5% were achieved.

The HPLC system was calibrated using purified human MT-2 as external standard protein. Two different calibration curves which correlate the amount injected onto the HPLC column and the peak integration area of the effluent are shown in Fig. 2. The relative recovery of the aqueous standard samples of MT-2 from HPLC decreased progressively with decreasing load. The plot is not linear (Fig. 2, insert) and at sample quantities below 70 pmol no MT-2 was recovered from the column. This effect was avoided when ultrafiltrated cytosol from 5.5×10^6 cells was included in the MT-2 calibration solution. After subtracting the endogenous amount of MT-2 present in the cytosol preparation, i.e. 0.49 ± 0.01 mVmin, from the integration area the correlation between the injected amount of MT-2 and the integrated area of the peak recovered between 45 and 200 pmol was linear. The extrapolated detection limit under these conditions was approximately 5 pmol.

The dependence on the amount of cell cytosol needed for the full recovery of MT-2 is shown in Fig. 3. The detection of MT-2 failed when the amount of cytosolic protein of less than 3.2×10^6 cells was present in the analyzed sample. Using cytosol derived from 3.7×10^6 cells the yield increased sharply to 86% and a quantitative recovery is reproducibly obtained in the presence of cytosolic proteins of more than 5.1×10^6 cells.

DISCUSSION

A method to quantify the basal accretion of MT in culture human cells by reversed phase HPLC was developed. Because MT-2 is the most abundant isoform in human cells,[6] all data reported here relate to this isoprotein although other isoMTs have also been reliably quantified by the same protocol.

For better separation from protein background in chromatography and accurate quantification of MT in cytosol an additional purification step was necessary (Fig. 1). Calibration of the system using buffered solutions of human

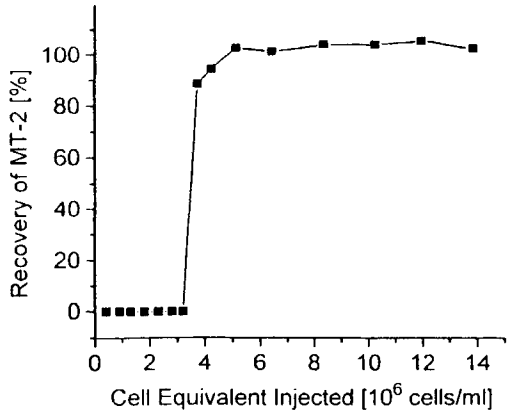

Figure 3. Influence of the Concentration of Cytosolic Proteins on the Recovery of MT-2. Cytosol of proliferating Chang liver cells was prepared by ultracentrifugation, heat denaturation and ultrafiltration and diluted with 25 mM Tris, pH 7.5, (HPLC buffer A). Samples of 1 mL corresponding to the different cell equivalents indicated were chromatographed as described in Fig. 1. and the recovery of endogenous MT-2 (7.2 ± 0.2 pmol/10^6 cells) was determined by using the linear calibration curve of Fig. 2.

MT-2 standard revealed a non-linear relation between the amount injected and the integrated area of the peak in the effluent (Fig. 2, insert). The skewed shape of the calibration curve and the overall lower recovery suggest that, under the conditions used, a constant amount of the MT standard is lost by unspecific binding to the column matrix. These losses are significantly reduced when the MT standard is injected together with prepurified cytosol. The cytosolic proteins apparently compete for the unspecific binding sites within the column matrix and thus allow for a good recovery of MT-2. The calibration curve becomes linear within the range examined and the detection limit is lowered approximately 14-fold (Fig. 2). However, this effect depends critically on the amount of protein in the cytosol, i. e. on the number of cells providing the cytosol. Below a minimal amount of cytosolic protein (1 mg/mL) in the sample the MTs are lost completely on the column (Fig. 3). Above a critical concentration the recovery of MT increases and reaches with a protein concentration of 1.3 mg/mL, derivable from 5.1 x 10^6 cells/mL, a value of 100%. At protein concentrations derived from more than 14 x 10^6 cells/mL the yield of MT decreases again and MT emerges, in part, with the void volume of the column indicating that the capacity factor of the column is reduced by saturating the stationary phase with cytosolic proteins (data not shown). This change in the capacity factor is irreversible necessitating replacement of the HPLC column.

In preliminary experiments conducted, the present method proved its usefulness as a reliable means to quantify changes in basal concentrations of iso-MT's during cell growth. As the protein content of non-synchronized growing Chang liver cells (260 $\mu g \pm 50\ \mu g/10^6$ cells) remains constant and is only slightly lower in density-arrested cells (220 $\mu g \pm 42\ \mu g/10^6$ cells), a standardisation of all samples to the same number of cells assures an adequate cytosol protein admixture for a proper analysis of MT. The optimal number of cells allowing for accurate measurement of basal MT concentrations lies in the range between 5 to 6 x 10^6.

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