Capillary electrophoresis of metallothionein

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Mammalian metallothioneins (MTs) are a group of low molecular weight (6-7 kDa) proteins. They consist of a single polypeptide chain of 61-68 amino acid residues, including 20 cysteinyl residues. In the human, there are four isoforms: MT-1, MT-2, MT-3, MT-4. Heterogeneity of isoforms results also from post-translational acetylation and/or variations in metal compositions. MTs are involved in the metabolism of heavy metals and constitute an intracellular reservoir of trace elements. Furthermore, they neutralize electrophilic compounds, have a protective function against DNA damage and an ability to inhibit apoptosis. Due to the characteristics, such as low molecular weight and unique primary structure, elaborating the isolation, separation and determination conditions of MTs analysis poses many problems, especially in achieving a satisfactory sensitivity and specificity. In addition to traditional analytical techniques, such as gel electrophoresis, liquid chromatography, and size exclusion chromatography, capillary electrophoresis (CE) has proven to be a powerful separation technique for MT analysis. Numerous studies devoted to the optimization of CE conditions as capillary surface, electrolyte background or modifiers or kind of detectors. Coupling CE with mass spectrometry creates a powerful analytical tool for the characterization of MT.

Keywords: capillary electrophoresis; metallothionein

1. Introduction

Metallothionein (MT) was first isolated in 1957 from the horse kidney by Margoshes and Vallee [1], subsequently MT presence was demonstrated in other animals, in higher plants, eukaryotic microorganisms, and in some prokaryotes [2]. Mammalian MT belongs to a group of low molecular weight proteins (6000-7000 Da) with 30% content of cysteine. A characteristic feature of all MTs is a tripeptide sequence Cys-Xaa-Cys, where Xaa is an aminoacid other than cysteine [3]. The sulfhydryl groups of cysteine form with metals closely packed spatial structure in which metals are within the molecule [2,4]. It serves as the reservoir of metals for the body (mainly Zn and Cu) which are the part of many enzymes and proteins involved in the removal of DNA damage, replication or transcription. MT protects the cells against the toxicity of heavy metals (Cd, Pb, Hg) by binding them. MT is composed of two domains: α and β linked by lysine dimer. Domain α (C-terminal) is more stable and is able to bind four atoms of Zn or Cd, or five or six atoms of Cu. Beta domain (N terminal) is more reactive, it can bind tri- Zn or Cd, or six atoms of Cu [4].

Slight differences in amino acid composition, hydrophobicity and isoelectric point allowed to separate the four major isoforms: MT-1, MT-2, MT-3 (also known as, growth inhibitory factor GIF), and MT-4. Despite the mutual similarity of MT-1 and MT-2, their roles and presence in tissues differ significantly [5]. While MT-2 isoform in humans is encoded by a single gene MT-2A, MT-1 consists of multiple subtypes encoded by a gene set of MT-1 (MT-1A, 1B-MT, MT-1E, MT-1F, MT-1G, 1H and MT-MT-1X), which determine the micro-heterogeneity of the protein (Figure 1).
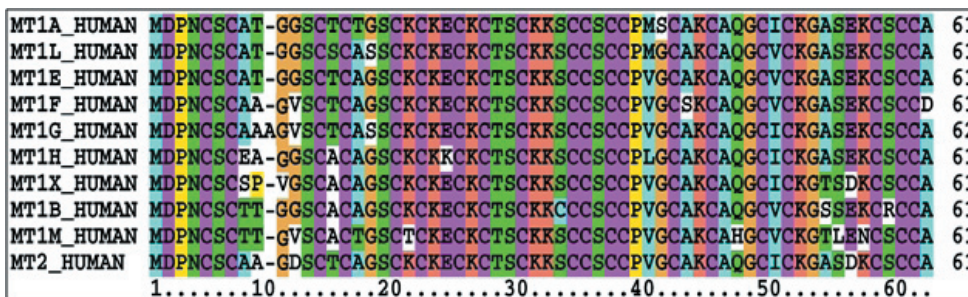
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MT1A_HUMAN MDPNCSCAT-GGSCTCTGSCKCKECKCTSCKKSCCSCCPMSCAKCAQGCICKGASEKCSCCA 61
MT1L_HUMAN MDPNCSCAT-GGSCSCASSCKCKECKCTSCKKSCCSCCPMGCAKCAQGCVCKGASEKCSCCA 61
MT1E_HUMAN MDPNCSCAT-GGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKGASEKCSCCA 61
MT1F_HUMAN MDPNCSCAA-GVSCTCAGSCKCKECKCTSCKKSCCSCCPVGCSKCAQGCVCKGASEKCSCCD 61
MT1G_HUMAN MDPNCSCAAAGVSCTCASSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASEKCSCCA 62
MT1H_HUMAN MDPNCSCEA-GGSCACAGSCKCKKCKCTSCKKSCCSCCPLGCAKCAQGCICKGASEKCSCCA 61
MT1X_HUMAN MDPNCSCSP-VGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGTSDKCSCCA 61
MT1B_HUMAN MDPNCSCTT-GGSCACAGSCKCKECKCTSCKKCCCSCCPVGCAKCAQGCVCKGSSEKCRCCA 61
MT1M_HUMAN MDPNCSCTT-GVSCACTGSCTCKECKCTSCKKSCCSCCPVGCAKCAHGCVCKGTLENCSCCA 61
MT2_HUMAN  MDPNCSCAA-GDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASDKCSCCA 61
           1......10........20........30........40........50........60..
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Figure 1: Comparison of the amino acid sequences of MT 1/2 human isoforms using the ClustalX program 2.0.11 [6].

ISOFORM	OCCURRENCE
MT-1/MT-2	Nucleated cells of peripheral tissues, interstitial fluids, blood plasma. Particularly high levels in the liver and kidneys. Also present in the cells of the CNS serving as nourishing and supporting cells (glia, astrocytes, cells of the choroid plexus and tires).
MT-3	Neurons (in the brain mainly in cells of the neocortex, hippocampus, cells of the olfactory bulb and dentate gyrus), renal tubular cells, male reproductive tract.
MT-4	Squamous cells (mouth, esophagus, stomach, appendages, skin, feet, hands).

Table 1: Places of expression of particular isoforms of human MT [Based on 11].

MT-1 and MT-2 genes expression have been demonstrated in many tissues: in the parenchymal cells of the kidney, liver, lung, intestines, pancreas [7], in cells of the sweat glands of the skin, in germ cells, and in epithelial cells of the thymus [8]. Izofom MT-3 is a protein mainly present in brain tissue [9]. The expression of MT-4 is limited to squamous skin and upper gastrointestinal [10]. Sites of presence of particular isoforms of human MT are listed in Table 1 [11].

MT cysteine residues also play a critical role in the removal of free radicals. MT has antitumor activity as it neutralizes toxic electrophiles, reactive oxygen and nitrogen species. MT is also capable of inhibiting apoptosis and developing resistance to radiotherapy and chemotherapy. Higher expression of this protein has been observed in many tumor tissues: lung, kidney, pancreas, bladder, urinary tract. Lower concentration levels have been observed in gastrointestinal tumors tract, including liver cancer or colon cancer.

To identify MT, sensitive and selective analysis techniques are applied. Low molecular weight of protein, and the heterogeneity of the biological material to be analyzed (serum, erythrocyte lysate, urine, different tissues), also causes a variety of methods used for its quantitative determination.

Electrochemical methods such as differential pulse and cathodic stripping voltammetry, saturation analysis methods based on Cd,

Ag and Hg, spectrophotometric methods as well as chromatographic and electrophoretic techniques are used [12]. Among immunological techniques used in MT analyses are: enzyme-linked immunosorbent assay, immunofluorescence assay and radioimmunoassay. These methods are highly sensitive and can detect even small amounts of MT in tested material [13,14]. One of the most widely used techniques in the analysis of proteins including MT is two-dimensional polyacrylamide gel electrophoresis or capillary electrophoresis (CE) often coupled with mass spectrometry (MS). CE allows analysis of both MT as MT in complexes with metals. As the determination of various isoforms is concerned, there must be very sophisticated detection system connected with separation one.

Today, medicine has a lot of interest in research on this protein, and thanks to modern analytical methods with high sensitivity and specificity, the determination of MT concentrations in the biological material is possible, wherein its amount in relation to other proteins is small.

2. Capillary electrophoresis

Electrophoresis was developed by Swedish chemist Arne Tiselius in the 30s of the twentieth century, in order to test serum proteins. For this achievement he obtained the Nobel Prize. The basis of this analytical method is the ability to move of charged species, placed in an electric field to the corresponding pole of the power source.

CE is one of the methods of electrophoresis, which is characterized by high efficiency, substantially higher than the efficiency achieved by high-performance liquid chromatography (HPLC). In CE, capillaries made of synthetic quartz not absorbing ultraviolet radiation are used. The capillaries are immersed in a suitable buffer solution and when cross potential gradient is applied, hydrated cations are attracted toward the cathode, causing a flow in the whole volume of liquid. This phenomenon is known as electroosmotic flow (EOF). In CE, this effect is responsible for the movement of all particles (cationic, anionic or neutral) along the capillary

toward the detector. EOF in capillaries filled with a buffer is formed by the ionization of silane groups (SiOH) on the inner surface of the walls at pH above 4. Cations accumulate near the negatively charged surfaces and form electrical double layer - the potential is formed. Applying a voltage across the capillary initiates EOF, whose speed is typically about one order of magnitude greater than the flow rate of solute. So the total migration rate of the substance (μ total) is μsubst + μEOF. As the result of EOF, all substances are transferred into the cathodic end of the capillary, where they pass through the chamber of the detector. Most commonly used detectors are UV-VIS, diode-array detector, fluorescent, electrochemical and mass spectrometer [12,15].

2.1 Types of capillary electrophoresis

There are several methods of separation of different substances by capillary electrophoresis.

Capillary zone electrophoresis (CZE) is the simplest from CE methods. Separation of substances occurs within the capillary filled with a buffer solution of appropriate pH and ionic strength. Upon application of the electric field substances are transferred toward the cathode end of the capillary in a strong flow - EOF. The particles reach the detector in order of decreasing total mobility, which depends on the size and charge of individual substances. CZE cannot be used for the separation of neutral substances. The main means influencing EOF include electric field, pH, ionic strength, composition and additives of electrolyte, as well as capillary wall coating and separation temperature [12].

Micellar electrokinetic capillary chromatography (MECC) is used for distribution of both ions and neutral compounds. It uses the addition to buffer of surfactant solution which forms micelles with a hydrophobic inside and positively or negatively charged surface. The micelles divide neutral substances according to their partition coefficient. More hydrophobic substances arrange inside the micelles, and hydrophilic in buffer solution [16]. Then micelles migrate towards the detector, complexes with a positive charge appear first, then a neutral and at the

end negative ones. The most commonly used surface-active agent is sodium dodecyl sulfate (SDS), which exhibits the best resolution [17]. The MECC basis for separation of substances is their division between the hydrophilic aqueous electrolyte environment and the hydrophobic interior of the negatively charged SDS micelles. Separation is based on differences in hydrophobicity and charge. Separation achieved by MECC is generally lower than in other CE techniques. However, MECC has much higher efficiency of separation and reproducibility of peaks migration times and their surfaces.

Capillary gel electrophoresis is used for separation polymers, proteins and DNA sequencing. The capillary tube is filled with a polyacrylamide or agarose gel. The sample is applied electrokinetically to the capillary, and prior to the analysis it is necessary to remove impurities from sample. Separation of analyte particles takes place under the influence of differences in particle size and speed of their passage through crosslinked gel bed. The larger particles migrate slower through the gel [15,18].

Capillary isoelectric focusing is a technique with a high degree of proteins separation and other amphoteric compounds. The analysis is carried out in a capillary, wherein pH gradient is formed using ampholytes carrier. The isoelectric point values are typically in the range from 3 to 10. Upon application of an electric field, components of the sample migrate and become embedded in such places of capillary, which correspond to their pI. After termination of the separation, all components are analyzed by a detector through which they pass under the pressure applied to the anode end of the capillary [15].

Capillary isotachophoresis is characterized by the use of two different buffering sets, leading buffer with a higher mobility than the mobility of the sample and buffer ending with a lower mobility. In them, the particles of analyte migrate depending on their electrophoretic mobility, to form a chain of adjacent zones, moving at the same speed between the two solutions. This method uses capillaries with inner diameters 500 μm, and the primary detection system is conductometry [18].

Anhydrous capillary electrophoresis is a method that has better solubility and stability for hydrophobic molecules. Separation takes place in a medium consisting of organic solvents in place of aqueous buffer. Acetonitrile or methanol are usually used. The viscosity and dielectric constant of the solvent affect the mobility of analyzed ions and the level of electroosmotic flow [19, 20].

Capillary electrochromatography is a method that combines the advantages of HPLC and CE. It is characterized by a strong EOF with flat profile and a higher selectivity than other CE methods. Separation of charged analytes is carried out on the basis of their electrophoretic migration and adsorption chromatography of neutral particles [19].

3. The use of capillary electrophoresis in metallothionein analysis

For the separation of MT isoforms, different CE techniques in combination with appropriate detectors are used. It provides a wide range of possibilities to optimize the degree of resolution by selecting the pH, temperature, buffer, electrolyte and the type of capillary.

3.1 Types of capillaries

Two types of capillaries have been used: uncoated or coated capillary. The use of uncoated capillaries gives advantages in terms of their stability and the shorter time of analysis, while the use of surface-modified capillaries increases the resolution of MT isoforms separation [21].

3.1.1 Uncoated capillaries

As a result of using uncoated capillaries often occurs undesirable phenomenon of adsorption of proteins on the inner wall of the capillary tube. The reason is that the silane groups at pH 3 or more are ionized. This can be avoided by: (1) the addition of SDS or methanol that limits the proteins ability to interact with the wall; (2) analysis of MTs at low pH but it has limitations because MTs are unstable at pH below 3 and they dissociate metals; (3) the use of buffers of high ionic strength. These buffers generate current which causes excessive heating of the sample and the quantitative loss of resolution.

Therefore, effective cooling of the capillaries is essential; (4) increase or decrease the length of the capillary diameter. As a result, the electrical resistance is reduced and there is an increase of dissipation of excessive heat, what reduces the current flow and heating effects [22].

At low pH MTs are unstable. They dissociate metals and the level of apolipoprotein arise. The release of metal causes a reduction in UV absorbance and MTs sample more concentrated should be used for their determination. Separation of MTs complexes with Cd and Zn in the pH range of 3-5 is not reproducible due to the partial dissociation of the metal. Often, in the electropherogram it is difficult to distinguish the peaks of MT-1 and MT-2 from each other. There is no such problem with MT-3, because it is highly charged, migrates faster and it is completely separated from the MT-1 and MT-2 in neutral and low pH. In order to improve resolution of MT-1 and MT-2 isoforms, ions of surfactants with the electrolyte are added to samples of MT. Use of MECC technique enables the separation of all MT isoforms, which is not possible using only phosphate buffer. The best MECC recommended conditions include using of a 50 cm x 75 μm uncoated capillary, 300 mM borate buffer pH 8.4 containing 85 mM SDS. Furthermore MECC at 10 kV gives a better separation by changing pH from 8.4 to 10.4 or SDS concentration from 60 to 120 mM, when compared with the use of higher voltage [17,21].

3.1.2 Modified capillaries

The inner surface of a fused silica capillary is modified or coated with various substances or ligands in order to eliminate EOF and interaction of proteins with walls. This affects its charge, hydrophobicity and surface chemistry. Neutral capillaries are used for the separation and qualitative assessment of proteins, among others MT isoforms. They enable to use a wide range of different electrolytes and buffers. Both low and neutral pH can be used for analysis by changing the polarity. Good resolution is observed in organic buffers and phosphate. Capillaries coated with amino groups give better results for the separation of proteins positively charged (at pH below the pI of the protein), because the wall has a positive charge. However,

some of capillaries are not suitable for use in pH below 3.5. MT isoforms with pI 4-4.5 and charge from 1 to 13 at neutral pH, should not be separated in capillaries coated with amino groups in neutral or alkaline pH. However, using high ionic strength and phosphate buffer at pH above 8 as the electrolyte, a good separation of all MTs is obtained [22].

3.2 Sample preparation for metallothionein analysis

Quantitative determination of MT isoforms in tissues requires adequate sample preparation technique. Radioimmunoassays and some analytical techniques do not require sample purification. They can be used directly to MT measurements. In contrast, samples for chromatography and electrophoresis should be wholly or partially purified. Purification always reduces the amount of tested substance in the sample, so finding optimal solution is desired. MTs in relation to other proteins are heat-stable and they can be purified by heat treatment at the temperature of 60ºC for 10 min. or to 100ºC for 1 min. The temperature and heating time are depending on the size of the sample [23].

Another method of sample purification is to acidification of tissue fragments prior to analysis, MTs are difficult to denaturize at a low pH, but they lose metals. They are also stable in 50% aqueous solutions of a mixture of solvents: ethanol, acetone and acetonitrile. Many other proteins in these solutions are deposited on the vessel walls.

These procedures cause significant dilution of samples, and from analytical point of view, the use of concentrated samples is required. Therefore, the most used technique for MTs preparation from tissue is by a two-stage solvent extraction having a concentration of 50% and 80-90%. It gives partially clean and concentrated extract, which is analyzed using CZE. In this technique is important to choose a suitable solvent. Using of a mixture of acetonitrile-ethanol as a solvent gives a reproducible and highly efficient procedure. The purification yield can be increased by adding 0.5 mM of sodium chloride to the homogenisation buffer 100 mM Tris-HCl pH 8.0 [22,24].

3.3 The choice of buffer and electrolyte

Increasing the resolution of MT isoforms is achieved by appropriate selection of buffers and electrolytes. Their selection should be guided by three main factors: UV absorbance, conductivity and pH buffering capacity. The most, sodium phosphate is used, because it has a very low absorbance in the UV wavelength range and has very good pH buffer capacity. The phosphate buffer gets a higher degree of resolution than the borate buffer, but generates a higher current because it has a higher ionic strength. Therefore, when using a sodium phosphate is recommended to use narrower (25 and 50 μm) and longer capillaries (50-100 cm). Borate buffer has low conductivity, lower ionic strength and resolution of phosphate buffer. However, the resolution is increased by using higher concentrations of buffer [21]. Good separation of MT is achieved with organic compounds for example: 2-amino-2-hydroxymethyl-propan-1,3--diol (Tris) and mixed buffer, eg. Tris-borate. Virtanen et al. presented a systematic study of the impact of buffer composition, concentration and pH as well as temperature and voltage on separation of MT isoforms [25]. Later, the same author introduced CE method using Tris-tricine buffer containing methanol as a background electrolyte [26]. The advantage of using bipolar organic buffers is their low conductivity and low ionic strength. When using UV detection below 220-230 nm, the absorption of some organic buffers is limited by their high concentrations [27]. From buffers discussed above, the best MT separation was obtained in uncoated capillaries in a phosphate buffer with high ionic strength and low pH [22].

3.4 Methods of metallothionein detection

Identification of separated MT isoforms from tissue extracts and in purified MT samples using CE is not so difficult in comparison with a further detection, especially because of the limited amount of material that can be obtained from tissues. The choice of method for determining MT isoforms in CE depends on the required degree of reproducibility, sensitivity and specifics. The techniques of detection which are used in the analysis of MT isoforms are UV detection, diode array detector and mass spectrometers.

3.4.1 Detection of metallothionein by UV and diode array detector

The absorbance detection is highly universal especially in the deep UV range of spectra, its sensitivity is dependent on the optical pathlength, which is given by the capillary diameter. MT absorbance detection is mostly carried out at 200 nm employing the light absorption by the peptide bond [12]. MT does not disclose the absorbance at 280 nm, as it does not contain aromatic amino acids. The most common method of MT isoforms identification is the use of diode-array detector. Simultaneous monitoring of spectra at different wavelengths: 200, 214, and 254 nm can be done to see: apoproteins at absorbance at 200 nm, Zn-MT - at 214 nm, Cd-MT - at 254 nm. Another problem is the type of buffer used. Organic buffers as HEPES, Tricine strongly absorbs UV light below 230 nm. This limits the use of lower wavelength. In contrast, buffers as phosphate and sodium borate do not absorb UV light and are suitable for the determination in wavelength lower than 230 nm. A mixture of alkaline borate and SDS shows a good separation and repeatability of migration times.

3.4.2 Metallothionein detection with mass spectrometry

A very good method for determining isoforms of MT is to use MS. The device separates the beam of charged particles according to a weight value of the particle charge by means of electric and magnetic fields. There are many types of mass spectrometers differing in the type and direction of fields, shape their area of operation and the distribution of the intensities.

With MS, monomers and polymers of MT isoforms can be identified. The spectrometer analyzes very small sample volumes, making it possible to obtain more detailed information about the fraction obtained after separation on CE, compared with UV or DAD detection. Furthermore, the combination of MS with various techniques for CE gained a lot of important

information on MT. CZE-MS shows the molecular structure of MT and inductively coupled plasma (ICP)-MS binding of MT with metal. The application of CE-ICP-MS has made significant progress in MT analysis in last few decades. The metals complexes of two major MT isoforms, MT-1 and MT-2, were separated and elements present in isoforms were selectively detected by CE-ICP-MS coupled via various interface designs [28,29].

4. Conclusions

MTs are considered as medical or environmental pollution biomarkers therefore methods of their effective determination are needed. The present paper shows advantages of capillary electrophoresis as a tool for MT determination. Despite many years of research on MT and the use of CE in its analysis, there is still lack of method that allows the simultaneous analysis of all isoforms of MT directly in the tissues.

Acknowledgments

Conflicts of Interest

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE „uniform reguirements" for biomedical papers.

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