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ISOTACHOPHORETIC DETERMINATION OF BIOLOGICALLY ACTIVE COMPOUNDS – ANALYSIS OF PHARMACEUTICALS AND DIETARY SUPPLEMENTS

THESES OF THE DOCTORAL DISSERTATION

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REFERENCES

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ABSTRACT

The brief summary of chosen bioactive compounds together with possibillities of their isotachophoretic analysis obtained from scientific literature are mentioned in theoretical part of this thesis. Experimental part is focused on development of isotachophoretic methods, which could be suitable for analyses of various compounds (amino acids, vitamins of B complex, ethanolamines, antidiabetics, formaldehyde) in pharmaceuticals and dietary supplements. Method validation and real sample analyses are very important parts of method development.

ABSTRAKT

V teoretické části práce je uveden stručný přehled vybraných biologicky aktivních látek společně s možnostmi jejich izotachoforetické analýzy získanými z odborné literatury. Experimentální část práce je zaměřena na vývoj metodik izotachoforetického stanovení několika různých skupin látek (aminokyseliny, vitamíny sk. B, ethanolaminy, biguanidová antidiabetika, formaldehyd) ve vzorcích léčivých přípravků a doplňků stravy. Důležitou součástí vývoje je i následná validace metod zahrnující také analýzu reálných vzorků.

KEY WORDS

Isotachophoresis, Analysis, Pharmaceuticals, Dietary Supplements, Biologically Active Compounds

KLÍČOVÁ SLOVA

Izotachoforéza, analýza, léčiva, doplňky stravy, biologicky aktivní látky

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1 INTRODUCTION

Capillary isotachophoresis (ITP) is one of the electromigration separation technique, which could be used for analysis of pharmaceuticals and dietary supplements.

In comparison with chromatographic techniques, isotachophoresis is inexpensive in terms of instrumentation and analysis costs as well. ITP has minimal requirements for sample preparation and sample purity. Only dilution is often used in case of liquid samples, dissolution in water and dilution in case of solid samples or simple derivatization when nonionic analytes need to be determined. In most cases, it is not necessary to purify sample, but when undissolved particles are present, sample could be cleaned by disc filter placed in front of dosing valve. Absence of any organic solvents is the next advantage of ITP, thanks to which the health risks for analytical chemist and environmental destruction are reduced.

On the other hand, the higher detection limit and lower separation capacity are the main disadvantages of ITP in contrast to chromatographic techniques. Nevertheless, when samples with relatively simple matrix and higher concentration of analytes, as pharmaceuticals or dietary supplements, are analyzed, these disadvantages become insignificant.

Optimized ITP methods for analysis of various compounds in pharmaceuticals and dietary supplements are present in this thesis. These methods were developed during the doctoral study, but some of them unfortunately hasn’t been published yet.

2 BIOLOGICALLY ACTIVE COMPOUNDS

Interests about biologically active compounds are increased across various fields of science and application, as medicine, pharmacology, agriculture, cosmetics and chemistry as well. Because of that reason, it should be possible to say which compounds can be classified as bioactive substances. Different sources bring different definitions, but when we focus on the word "bioactive", it can be said, that it is composed of two words – bio- (from Greek *"bios"* which means life) and –active (from Latin *"activus"* means effective or curative). The term bioactive compound in science can be explain as compound with biological activity, i.e. compound which has some effect on living organism. This effect can be positive or negative. Nevertheless, in most cases, when we talk about bioactive compounds we limit ourselves only to that, which have positive effects [1,2].

Opletal [3] created detailed overview of biologically active compounds, which he divided into three main groups with several subgroups. The main groups are:

- compounds coming from primary metabolism
- compounds of structured biological systems
- compounds with character of secondary metabolites

In this thesis, the main attention is focused on the first group, because these substances are mentioned in literature in connection with ITP most often. Into this group, amino acids with its derivatives, peptides, proteins, fatty acids with its derivatives, lipids, carbohydrates with its derivatives, organic acids of primary metabolism, nucleotides, nucleic acids, vitamins and ubiquinones are included [3].

Biogenic amines are compounds formed by decarboxylation of amino acids. In low concentrations, they are necessary for human health because they act mainly as neurotransmitters and hormones, nevertheless in high concentrations they are considered dangerous and can cause health problems as headache, migraines, problems with blood pressure, nausea, etc [4]. Amino acids are main components of peptides and proteins, moreover they serve as building material of hormones, neuromediators, nucleic acids and other important biologically active compounds. Some of amino acids play roles during regulation of translation and transcription, affect the immune response, hormones secretion and membrane transport [5,6]. Into group of peptides and proteins, many hormones, antioxidants, antibiotics, ion carriers, building components and enzymes can be included [5,7].

Fatty acids have several functions; they act as energy source and basic components of lipids. Some of them are important for healthy neural and cardiovascular system [3,5]. Lipids are formed by reaction of fatty acids with trihydric alcohol (mainly glycerol). They play important roles as source and storage of energy, main components of cell membranes, mechanical and thermal barrier of body and have a number of other no less important functions [5,8,9].

Carbohydrates are group of compounds, which provide source of energy, energy storage or construction function [5,10].

Organic acids of primary metabolism are involved in biosynthesis of important metabolites or are direct part of metabolic cycles [3].

Nucleotides and nucleic acids are important for storage of genetic information, regulation of protein synthesis, enzyme activity and signal transmission [10].

Vitamins and ubiquinones can be characterized as biocatalysts; they serve predominantly as antioxidants and cofactors of enzymes. They play role in metabolism of proteins, lipids and carbohydrates, reduce degenerative processes of organism and strengthen immune reactions [3,11–13].

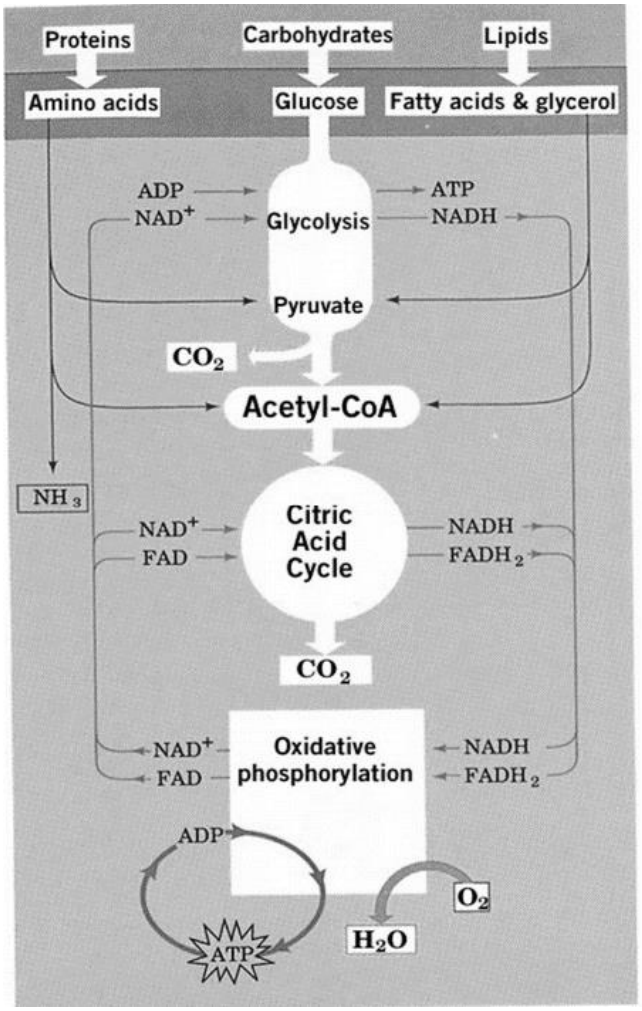

Figure 1: Basic scheme of primary metabolism [14]

3 ITP ANALYSIS OF AMINO ACIDS IN DRINKS AND DIETARY SUPPLEMENTS

Amino acids are ionized in water, but their molecules have amphoteric character. For that reason, their mobility in electric field is rather low within the pH range from 3 to 10 which is recommended for isotachophoretic analysis. These conditions bring problems in direct ITP separation of most amino acids. Nevertheless, there are some exceptions such as amino acids of acidic character (glutamic and aspartic acid) and basic amino acids (histidine, lysine, and arginine), which can be easily separated by isotachophoresis without derivatization, hence their mobilities in electric field is satisfactory.

One of the possibilities of amino acids determination in foodstuffs and food supplements is use of isotachophoretic analysis, which has been demonstrated by Jastrzebska et al. [15]. They optimized direct isotachophoretic separation for determination of free amino acids in cheese samples by using both modes - anionic mode in combination with alkaline electrolyte systems for determination of tyrosine and phenylalanine and cationic mode for lysine, arginine, histidine, and ornithine. Moreover, cationic mode was also used for analysis of histidine derivates in meat samples [16]. Another works dealing with ITP analyses of amino acids using anionic mode and alkaline electrolyte systems were performed by Kvasnička et al. [17], Everaerts et al. [18], Hirokawa et al. [19], and Prest et al. [20]. However, ITP analysis using alkaline electrolyte brings a problem connected to migration of carbonate (originated from dissolved atmospheric carbon dioxide) which cause prolongation of analysis time together with decrease of column separation capacity. The main problem of carbonate is creation of mixed zones, which have negative influence on separation process. The content of carbonate is usually decreased by addition of barium hydroxide, hence formation of insoluble barium carbonate. However, carbonate can't be completely removed [21].

More often acidic or neutral conditions are used in ITP analyses but in case of amino acids analyses only glutamic and aspartic acid can be separated directly under acidic conditions. As an example, work of Zgola-Grześkowiak [22] can be mentioned, they dealt with determination of these two acids in tomato juice using anionic mode.

One of the possibilities how to determine other amino acids under acidic conditions is using non-aqueous solvents, for example dimethylsulfoxide [23], and propionaldehyde [18]. The other possibility is their derivatization, hence conversion of amino acid to other compounds, which are not ampholytes. There are several ways of derivatization suitable for ITP analysis e.g. reaction with formaldehyde in alkaline conditions [24] or esterification [25]. The other option, which has not yet been used in ITP analysis, is reaction with nitrous acid [26,27].

Therefore, we focused on development of isotachophoretic method for determination of amino acids based on reaction with nitrous acid, i.e. conversion of amino group to diazo group, which is subsequently hydrolyzed to form hydroxy acid.

We optimized several parameters of conversion, as type and time of reaction support, type and concentration of acid, amount of amino acid and concentration of nitrite. We found out that the best composition of reaction mixture is 100 mM nitrite, 100 mM acetic acid, 1 – 20 mM amino acid together with mechanical stirring in boiling water bath for 10 minutes.

For separation commonly used electrolyte system which is composed of 10 mM HCl and β-alanine for setting pH value to 3.6 as leading electrolyte and 10 mM acetic acid as terminating electrolyte was chosen. Methods for determination of branched chain amino acids (BCAA, i.e.

valine, leucine, isoleucine), proline, theanine and taurine were validated (results of validation are listed in Table 1) and some parameters was modify for selected amino acid. For example, during separation of BCAA 20 mM α-cyclodextrine was added to leading electrolyte for separation of zones belonging to leucine and isoleucine, proline was separated in leading electrolyte with pH 2.4 and theanine conversion time was reduced to 8 minutes. More details are given in full text of dissertation. All validated methods were tested on real samples (dietary supplements, tea, energy drinks and energy gels) and were found to be suitable for routine analyses used to quality control of these products.

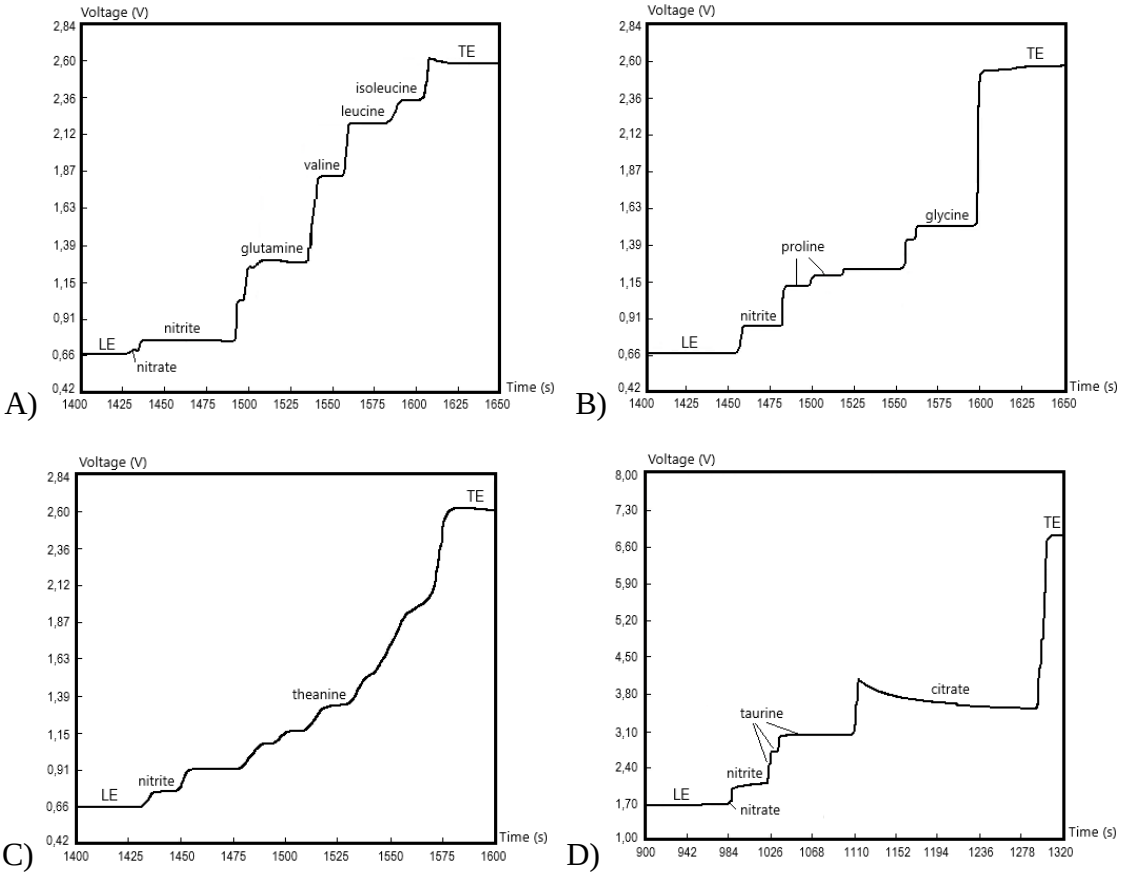

Figure 2: Isotachophoreograms of analysed samples – isotachophoreogram of dietary supplement BCAA Liquid Orange Flavour – leading electrolyte: 10 mM HCl + β-alanine + 20 mM α-cyclodextrine (pH 3.6); terminating electrolyte: 10 mM acetic acid; analytical column (A); isotachophoreogram of dietary supplement Flexit Liquid – leading electrolyte: 10 mM HCl + β-alanine + 0,05% HEC (pH 2.4); terminating electrolyte: 10 mM acetic acid; analytical column (B); isotachophoreogram of tea Yunnan – leading electrolyte: 10 mM HCl + β-alanine + 0,05% HEC (pH 3.6); terminating electrolyte: 10 mM acetic acid; analytical column (C); isotachophoreogram of energy dring Tiger – leading electrolyte: 10 mM HCl + β-alanine (pH 3.6); terminating electrolyte: 10 mM acetic acid; analytical column (D).

4 ITP ANALYSIS OF B-COMPLEX VITAMINS IN MULTIVITAMIN PREPARATIONS

Vitamins of B group are compounds with relatively various structures, which belongs to group of vitamins soluble in water. All these compounds are essential and necessary for normal function of organism. Deficiency of some vitamin causes the state called hypovitaminosis, which can be dangerous for human health, especially when it is not cured. B vitamins play important roles in metabolic pathways; they serve predominantly as cofactors of enzymes, regulators of metabolism and antioxidants [13].

For determination of vitamins in various matrices many procedures including different analytical techniques was described. The most used are chromatographic techniques, especially HPLC, and electromigration techniques, between which capillary zone electrophoresis and micellar electrokinetic chromatography are dominant. In last few years, HPLC methods for analysis of B vitamins in foodstuffs [28–42], infant formulas [43,44], pharmaceutical preparations [45–48], breast milk [49], urine [34,50], serum [51], plasma [33,34,52], and tissues [53] were published. Micellar electrokinetic chromatography was used predominantly for determination of B vitamins in multivitamin tablets [54–56]. The use of capillary zone electrophoresis was in the analyses of foodstuffs [57–59], livestock feed [60], pharmaceutical preparations [61], urine [62], plasma [62], and saliva [62]. In the past several ITP methods was published [63–71] as well, nevertheless the most of them deals with determination of only one or two vitamins in one analysis. When we focus on other analytical techniques for vitamin analysis spectrophotometry [72–74], fluorimetry [75–78], infrared spectrometry [79], Raman spectrometry [79], FIA [80–87], voltammetry [88,89], and amperometry [90,91] were used.

ITP method for simultaneous analysis of as many vitamins as possible in one analysis was optimized. Because of various structures, vitamins was divided into two groups, first group with anionic structure and second with cationic structure. Into first group nicotinic acid, calcium pantothenate and folic acid were included. Electrolyte system containing 10 mM histidine-chloride with histidine (pH 6.2) as leading and 10 mM MES as terminating electrolyte appeared to be the best for separation of these compounds. For second group of vitamins included thiamine, pyridoxine, pyridoxal and pyridoxamine, electrolyte system consisted of 10 mM potassium acetate and acetic acid (pH 5.0) as leading electrolyte and 10 mM acetic acid as terminating electrolyte was chosen.

Except standard conductometric, spectrophotometric detection was used. It was found out that pantothenate cannot be determined by spectrophotometry under chosen conditions, so its detection was only conductometric. Ideal detection wavelengths were 250 nm for thiamine, 259 nm for nicotinic acid, 281 nm for folic acid, 289 nm for pyridoxine and pyridoxal, and 324 nm for pyridoxamine.

Method was validated (results of validation are given in Tables 2 and 3) and tested on real samples of multivitamin tablets. Measured contents were in good agreement with declared. It can be assumed, that this method is suitable for routine quality control of these types of samples.

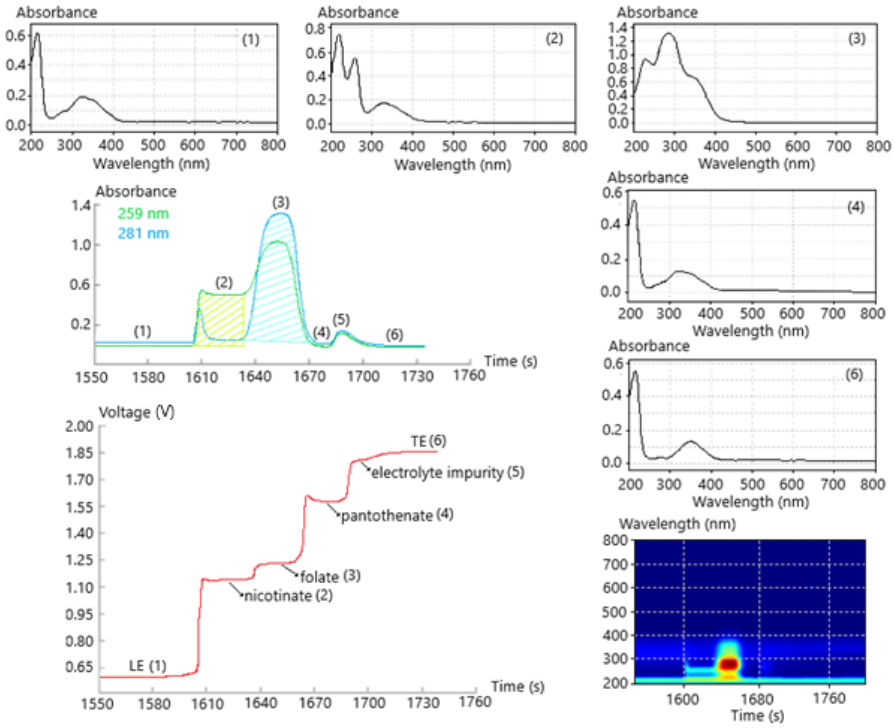

Figure 3: Conductometric and spectrophotometric detection of vitamins with anionic structure – concentrations of standard solution about 20 mg/l; leading electrolyte: 10 mM histidine-chloride + histidine (pH 6.2); terminating electrolyte: 10 mM MES; analytical column.

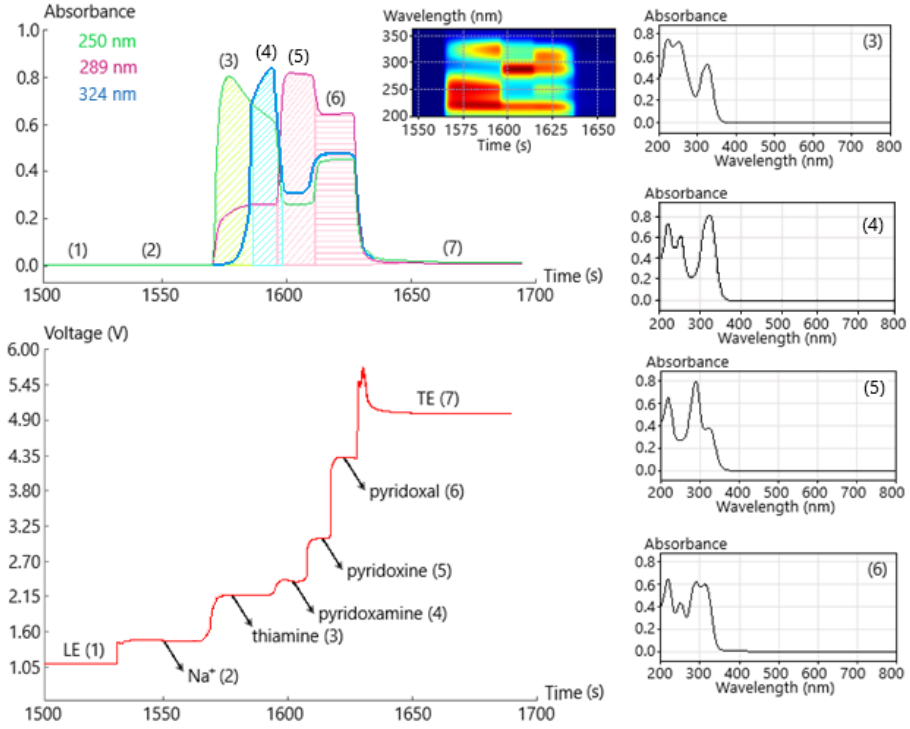

Figure 4: Conductometric and spectrophotometric detection of vitamins with cationic structure – concentrations of standard solution about 20 mg/l; leading electrolyte: 10 mM potassium acetate + acetic acid (pH 5.0); terminating electrolyte: 10 mM acetic acid; analytical column.

5 ITP ANALYSIS OF ETHANOLAMINES IN PHARMACEUTICAL AND COSMETIC PRODUCTS

Ethanolamines (monoethanolamine – MEA; diethanolamine – DEA; triethanolamine – TEA) are pure, viscous, colorless or slightly yellowish liquids with ammonia odor, miscible with water and several organic solvents. Due to presence of amino and hydroxyl group in their structure, they have similar chemical properties as amines and alcohols – they create salts with free acids and their hydroxyl group allows formation of esters [92].

Monoethanolamine, $HOCH_2CH_2NH_2$, is used for production of textile and agricultural agents, as component of adhesives and metalworking fluids. In cosmetic and pharmaceutical industry MEA is predominantly used for pH adjusting and its salts (salicylate and benzoate) work as preservatives. Content of MEA in cosmetics is not limited, nevertheless EU orders that its purity has to be minimally 99 % with maximally 0,5 % of secondary amines and 50 μg/kg of nitrosamine [93]. Acute and chronical toxicity of MEA is mild – oral lethal dose is from 0,7 to 15 g/kg, after the dermal application it can irritate skin and cause skin redness. When high doses of MEA (about 200 mg/kg) are used during dermal application the formation of skin necrosis, scabs or scars can occurs [94].

Diethanolamine, $(HOCH_2CH_2)_2NH$, is used for production of emulsifying and dispersant reagents for textile industry and agriculture, for production of waxes, mineral and vegetable oils, paraffins, polishes and other industrial products. It is used as lubricant, leather softener, alkalizing agent and surfactant in pharmaceutical industry and absorbent of acidic gases as well. Using of DEA in cosmetics is prohibited due to formation of carcinogenic nitrosamines [95]; it can be present only as MEA or TEA contaminant. Currently, DEA is classified as potential carcinogen for human [96].

Triethanolamine, $(HOCH_2CH_2)_3N$, plays a role in broad spectrum of applications, for example in cement production, cosmetics, medicine, and textile industry. It is also acts as an additive to metalworking and cooling fluids, herbicides and insecticides [92]. In cosmetic industry, TEA is usually added to products because of its emulsifying, neutralizing, and detergent properties, hence it can be found in most of cosmetic products, mainly in body creams, shampoos, soaps, face cleaners, make-ups, hair colors, and shaving creams [97,98]. EU regulation stipulates maximal content, i.e. 2.5 % (m/m) of TEA in cosmetic products. Additionally, the purity of used TEA must be at least 99 % with maximal concentration 0.5 % of DEA) [95]. TEA is slightly toxic for human; oral lethal dose is from 5 to 15 g/kg. Moreover, TEA can cause contact dermatitis and eye irritation, other effect such as carcinogenicity was not proved, but some study found out that in case of female mice fed with TEA was higher risk of liver tumor genesis [99].

Requirements for determination of ethanolamines in cosmetics are mainly because of EU regulation. In the past, several methods for their determination in various matrices were published, the most commonly used are chromatographic [100–120], electromigration [121,122] and electrochemical [123–125] techniques.

Isotachophoresis is one of the analytical methods, which allows determination of ethanolamines in cosmetics. Advantages of ITP in comparison to HPLC are lower costs, and sample pretreatment, as well as no derivatization is needed. On the other hand, the disadvantage

of ITP is higher limit of determination, but relatively high content of TEA in cosmetics breaks this hindrance.

ITP method for determination of ethanolamines in pharmaceutical and cosmetic products was optimized. The optimal electrolyte system for separation of ethanolamines was consisted of 10 mM sodium acetate with acetic acid for adjusting pH to 4.6 as leading electrolyte and 10 mM acetic acid as terminating electrolyte. At this pH differences between signals are sufficient and buffering capacity of acetic buffer is maximal.

After this, we tried to find out if some cations generally present in cosmetics do not interfere the analysis of ethanolamines. Therefore, standard solutions of calcium, magnesium and lithium were analyzed in optimized electrolyte system for this purpose. None of these ions influence the analysis, because all of them migrate much faster than all ethanolamines (calcium migrates even faster than leading ion).

After optimization of electrolyte system, method was validated (results of validation are listed in Table 4) and tested on real samples of pharmaceuticals and cosmetics. It was found that this method is suitable for this purpose and that all tested products are in accordance with EU regulation.

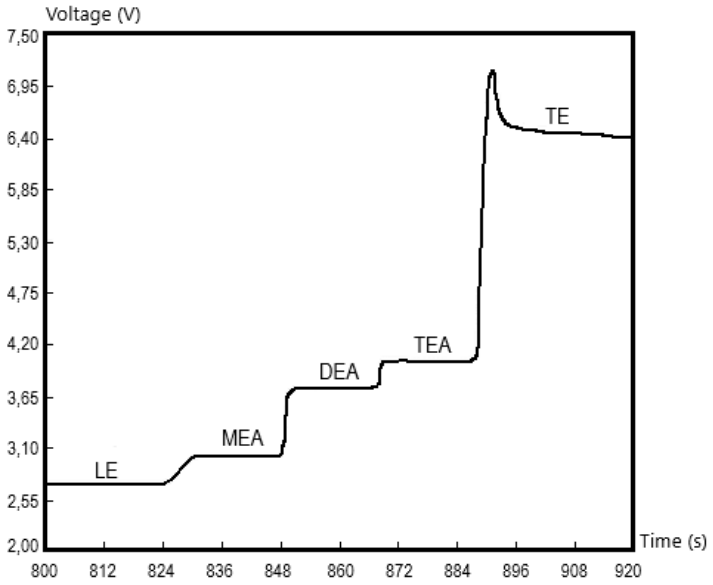

Figure 5: Isotachophoreogram of 0,5 mM ethanolamines standard mixture – leading electrolyte: 10 mM sodium acetate + acetic acid + 0,05% HEC (pH 4,6); terminating electrolyte: 10 mM acetic acid, pre-separation column.

6 ITP ANALYSIS OF BIGUANIDE ANTIDIABETICS

Biguanides are drugs of plant origin, which are commonly used for diabetes mellitus type 2 treatment since 50s of 20th century. Into this group of antidiabetics, metformin, buformin and phenformin can be included. Metformin is the first choice medicine and the most described pharmaceutical for treatment of diabetes mellitus type 2 worldwide. Moreover, it is used as additive drug for treatment of polycystic ovary syndrome and gestational diabetes. The other two mentioned biguanides are not clinically used because of high risk of lactate acidosis. Metformin was also withdrawn from American pharmaceutical market, but when its safety was proven, its sale was relaunched in 1995 [126].

Metformin acts predominantly in liver, where it inhibits gluconeogenesis by blocking of mitochondrial redox transport. Nevertheless, the whole mechanism of its action is not clear yet, because effects of metformin are various. After oral application, metformin is from 70 to 80 % absorbed in gastrointestinal tract, quickly distributed throughout the body and accumulated in esophagus, stomach, small intestine, salivary glands and kidneys. Metformin is probably not metabolized in human body; its leftover is excreted by faeces and urine [126,127].

Several methods for metformin analysis was published, HPLC predominantly with mass spectrometry detection is the most commonly used technique. With this technique, metformin was determined in human plasma [128–133], urine [131,134], surface water [135–139] and pharmaceutical preparations [140–143]. For metformin analysis in pharmaceuticals, capillary zone electrophoresis [144] and thin layer chromatography [145] were also used.

Use of isotachophoresis is the other possibility for analysis of metformin; nevertheless, no paper dealing with this problematics has been published yet. Because of that reason, ITP method for simultaneous determination of biguanides was developed and optimized.

Optimal electrolyte system was consisted of 10 mM potassium acetate with acetic acid for adjusting pH to 5.0 as leading electrolyte and 10 mM acetic acid as terminating electrolyte. Detection was conductometric and spectrophotometric (optimal detection wavelength was 230 nm for all three analytes). Method was validated (method parameters are given in Table 5) and tested on real samples of antidiabetic tablets. Obtained results was compared with declared contents. Optimized method provided lower contents in all cases, so it is necessary to analyze samples with other reference method; nevertheless, due to time reasons it has not been done yet.

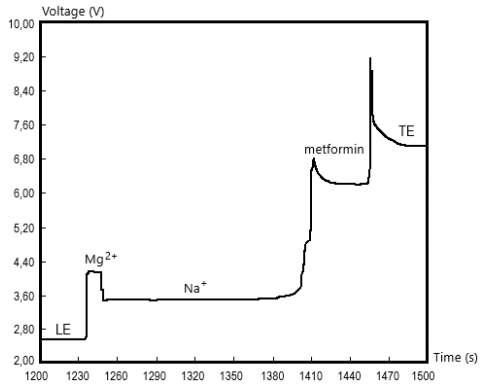

Figure 6: Isotachophoreogram of antidiabetic tablet Siofor 500 – leading electrolyte: 10 mM potassium acetate + acetic acid (pH 5.0); terminating electrolyte: 10 mM acetic acid; analytical column.

7 ITP ANALYSIS OF FORMALDEHYDE IN KUTVIRT'S GARGLE

Formaldehyde has disinfecting and cleaning effects, due to them it is used in medicine for sterilization of medical tools and also acts as component of Kutvirt's gargle, which is recommended for sore throat treatment or nasopharynx disinfection. In food industry formaldehyde is used as preservative (under the code E240), but its using is prohibited in Czech republic. Formaldehyde is commonly present in environment, it is formed by oxidation of hydrocarbons and decomposition of plant residues. Formaldehyde significantly affects human health because of its toxicity and carcinogenicity [146]. Due to that, it is necessary to control its content in various matrices.

For this purpose several analytical methods was developed. The most common are gas and liquid chromatography. HPLC methods for determination of formaldehyde in cow milk [147], drinking water [148], cork [149], aerosols for e-cigarettes [150], human tissues [151], foodstuffs [152], beer [153], juices [154] and GC methods for analysis of tattoo ink [155] and urine [156] were published. Several electrochemical sensors (impedance [157], amperometric [158]) was made as well. Using of spectrophotometric [159,160] or other electrochemical techniques [161] can be also way, how to analyze content of formaldehyde.

By using isotachophoresis formaldehyde can not be analyzed directly, because it is not ionic substance. Therefore, derivatization is inevitable. In this work, reaction with sulfite to form hydroxymethylsulfonate, which was subsequently detected, was chosen.

Firstly, optimization of reaction time was done. It was found that zone length is relatively stable from 10 to 230 minutes of reaction. For practical reasons 30 minutes was chosen because it is the time of one ITP analysis, during that next sample can be prepared.

For separation acidic electrolyte system consisted of 10 mM HCl with addition of β-alanine for pH adjusting to 3.2 as leading and 10 mM formic acid as terminating electrolyte was chosen, because it seems to be the best for this purpouse.

Under these conditions, method parameters was measured (they are given in Table 2) and content of formaldehyde in Kutvirt's gargle was analyzed. Measured content corresponds with declared.

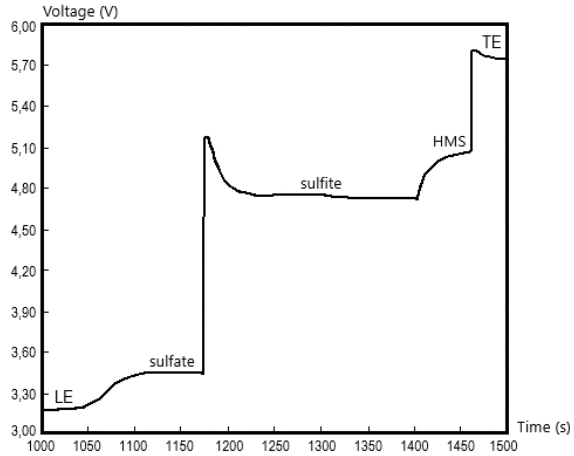

Figure 7: Izotachophoreogram of Kutvirt's gargle – leading electrolyte: 10 mM HCl + β-alanine (pH 3.2); terminating electrolyte: 10 mM formic acid; analytical column.

Table 1: Method parameters for determination of amino acids

	Valine	Leucine	Isoleucine	Proline	Theanine	Taurine
Mode	(-)	(-)	(-)	(-)	(-)	(-)
Leading ion	10 mM Cl^-	10 mM Cl^-	10 mM Cl^-	10 mM Cl^-	10 mM Cl^-	10 mM Cl^-
Counter ion	β-alanine	β-alanine	β-alanine	β-alanine	β-alanine	β-alanine
pH of LE	3.6	3.6	3.6	2.4	3.6	3.6
Aditives to LE	20 mM α-cyclodextrine	20 mM α-cyclodextrine	20 mM α-cyclodextrine	0.05 % HEC	0.05 % HEC	-
Terminating ion	10 mM Ac^-	10 mM Ac^-	10 mM Ac^-	10 mM Ac^-	10 mM Ac^-	10 mM Ac^-
RSH	0.599	0.791	0.875	0.300; 0.339	0.290	0.124; 0.170; 0.232
Calibration equation	L = 152.32 c + 4.27; L [s], c [mmol/L]	L = 181.07 c + 0.02; L [s], c [mmol/L]	L = 184.60 c + 0.04; L [s], c [mmol/L]	L = 302.06 c + 0.004; L [s], c [mmol/L]	L = 211.88 c + 0.005; L [s], c [mmol/L]	L = 123.68 c + 0.83; L [s], c [mmol/L]
Recovery	97.0 ± 2.5 %	97.8 ± 1.4 %	97.6 ± 1.7 %	96.2 ± 1.9 %	96.3 ± 0.9 %	92.6 ± 1.5 %
Repeatability	2.1 %	1.8 %	1.9 %	2.1 %	1.4 %	1.5 %
Limit of detection	5.3 µmol/L	5.0 µmol/L	5.0 µmol/L	0.9 µmol/L	1.2 µmol/L	2.7 µmol/L
Limit of quantification	16.0 µmol/L	15.0 µmol/L	15.0 µmol/L	2.6 µmol/L	3.6 µmol/L	8.0 µmol/L

Table 2: Method parameters for determination of B vitamins with anionic structure and formaldehyde

	Nicotinic acid	Folic acid	Calcium pantothenate	Formaldehyde
Mode	(-)	(-)	(-)	(-)
Leading ion	10 mM Cl^-	10 mM Cl^-	10 mM Cl^-	10 mM Cl^-
Counter ion	histidine	histidine	histidine	β-alanine
pH of LE	6.2	6.2	6.2	3.2
Aditives to LE	-	-	-	-
Terminating ion	10 mM MES^-	10 mM MES^-	10 mM MES^-	10 mM formate
RSH	0.57	0.644	0.818	0.796
Calibration equation	L = 0.37 c + 4.84; L [s], c [μmol/L]	L = 0.24 c + 1.46; L [s], c [μmol/L]	L = 0.27 c - 1.19; L [s], c [μmol/L]	L = 139.16 c + 0.89; L [s], c [mmol/L]
Recovery	99.3 ± 0.4 %	99.3 ± 0.4 %	99.8 ± 1.5 %	95.4 ± 0.5 %
Repeatability	0.3 %	2.5 %	4.8 %	1.6 %
Limit of detection	1.4 μmol/L	0.9 μmol/L	1.2 μmol/L	2.4 μmol/L
Limit of quantification	4.1 μmol/L	2.7 μmol/L	3.6 μmol/L	7.1 μmol/L

Table 3: Method parameters for determination of B vitamins with cationic structure

	Thiamine	Pyridoxine	Pyridoxal	Pyridoxamine
Mode	(+)	(+)	(+)	(+)
Leading ion	10 mM K^+	10 mM K^+	10 mM K^+	10 mM K^+
Counter ion	Ac^-	Ac^-	Ac^-	Ac^-
pH of LE	5.0	5.0	5.0	5.0
Aditives to LE	-	-	-	-
Terminating ion	10 mM H^+	10 mM H^+	10 mM H^+	10 mM H^+
RSH	0.245	0.508	0.858	0.318
Calibration equation	L = 0.13 c + 0.16; L [s], c [μmol/L]	L = 0.09 c + 0.27; L [s], c [μmol/L]	L = 0.10 c + 0.04; L [s], c [μmol/L]	L = 0.10 c + 0.65; L [s], c [μmol/L]
Recovery	106.9 ± 0.6 %	101.6 ± 0.2 %	108.9 ± 0.2 %	104.6 ± 0.2 %
Repeatability	0.9 %	0.4 %	3.7 %	0.3 %
Limit of detection	2.6 μmol/L	3.6 μmol/L	3.4 μmol/L	3.4 μmol/L
Limit of quantification	7.7 μmol/L	10.8 μmol/L	10.3 μmol/L	10.2 μmol/L

Table 4: Method parameters for determination of ethanolamines

	MEA	DEA	TEA
Mode	(+)	(+)	(+)
Leading ion	10 mM Na^+	10 mM Na^+	10 mM Na^+
Counter ion	Ac^-	Ac^-	Ac^-
pH of LE	4.6	4.6	4.6
Aditives to LE	0.05 % HEC	0.05 % HEC	0.05 % HEC
Terminating ion	10 mM H^+	10 mM H^+	10 mM H^+
RSH	0.125	0.392	0.462
Calibration equation	L = 0.233 c + 3.481; L [s], c [µmol/L]	L = 0.223 c + 2.438; L [s], c [µmol/L]	L = 0.245 c + 2.734; L [s], c [µmol/L]
Recovery	-	-	96.0 ± 3.6 %
Repeatability	-	-	3.5 %
Limit of detection	1.4 µmol/L	1.5 µmol/L	1.4 µmol/L
Limit of quantification	4.3 µmol/L	4.5 µmol/L	4.1 µmol/L

Table 5: Method parameters for determination of biguanides

	Metformin	Buformin	Phenformin
Mode	(+)	(+)	(+)
Leading ion	10 mM K^+	10 mM K^+	10 mM K^+
Counter ion	Ac^-	Ac^-	Ac^-
pH of LE	5.0	5.0	5.0
Aditives to LE	-	-	-
Terminating ion	10 mM H^+	10 mM H^+	10 mM H^+
RSH	0.419	0.512	0.575
Calibration equation	L = 1.027 c + 4.948; L [s], c [mg/L]	L = 0.940 c + 5.491; L [s], c [mg/L]	L = 0.667 c + 5.753; L [s], c [mg/L]
Recovery	100.1 ± 0.2 %	96.8 ± 0.7 %	103.8 ± 0.8 %
Repeatability	3.6 %	7.3 %	4.2 %
Limit of detection	2.3 µmol/L	2.1 µmol/L	2.3 µmol/L
Limit of quantification	6.9 µmol/L	6.3 µmol/L	6.9 µmol/L

8 CONCLUSION

This thesis deals with isotachophoretic analysis of biologically active compounds in pharmaceuticals and dietary supplements.

In theoretical part, biological effects of compounds connected with primary metabolism are summarized, because these kind of substances are mentioned in literature together with isotachophoresis most often.

Development of isotachophoretic methods for determination of chosen analytes, i.e. amino acids, vitamins of B-complex, ethanolamines, biguanides, and formaldehyde, in samples of pharmaceuticals and food supplements are described in the next part of dissertation thesis.

During development, all isotachophoretic methods were validated, limit of detection and quantification, repeatability, recovery, and linearity of calibration were chosen as method parameters, which characterized the method and can serve as indicators of method applicability. Obtained parameters met the requirements of routine analyses, so methods were tested on chosen real samples.

Optimized isotachophoretic methods have shown its suitability for routine analyses, especially for quality control of some products, so in some cases they can be less expensive alternatives to chromatographic methods.

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