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Analysis of 5-methyltetrahydrofolate in human blood, serum and urine by on-line coupling of capillary isotachophoresis and zone electrophoresis

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Abbreviations:

CITP-CZE, on-line combination of capillary isotachophoresis with capillary zone electrophoresis; **EACA**, ε-aminocaproic acid; **FA**, folic acid, **FEP**, fluorinated ethylene-propylene copolymer; **HPC**, hydroxypropylcellulose; MFA, methylfolate; **5-MTHF**, 5-methyltetrahydrofolate; **LE**, leading electrolyte; **TE**, terminating electrolyte; **ZED**, zone existence diagram

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Abstract

The predominant circulating folate coenzyme in plasma/serum, 5-methyltetrahydrofolate (5-MTHF) was determined in human blood, serum and urine using a method based on hyphenation of capillary isotachophoresis and zone electrophoresis. Measurements were done with a commercially available instrument for capillary isotachophoresis equipped with a column-switching system. The choice of electrolytes was limited by the instability of 5-MTHF and volatility of electrolytes for the potential coupling of the instrumentation with MS detector. To get an insight into the separability of individual sample components in an isotachophoretic analysis we constructed zone existence diagrams for isotachophoretic electrolyte systems having a leading electrolyte composed of acetate and ammonium of pH 4.5 and 7.0, hydrocarbonate and ammonium, pH 7.8, chloride and ammonium, pH 5.6, and chloride and creatinine, pH 5.0, with hydroxide ion as the terminator. For isotachophoretic preseparation, the non-volatile leading electrolyte with good buffering capacity composed of 1×10^{-2} M HCl and 2.5×10^{-2} M creatinine, pH 5.0, and terminating electrolyte composed of 1×10^{-2} M MES were selected as the most suitable. The optimum background electrolyte for CZE analysis from the standpoint of analyte stability, separability and volatility for MS coupling was 1×10^{-2} M acetate with 3.5×10^{-2} M ammonium, pH 4.5. Using this combination of electrolytes, LODs reached with optical detection at 220 nm were 1.6×10^{-7} M in human blood, 1.1×10^{-7} M in human serum, and 4.7×10^{-6} M in human urine. Estimated content of 5-MTHF in blood and serum samples of women following oral daily administration of 0.8 mg of folic acid was 1.2×10^{-5} M and 5.8×10^{-6} M, resp.

1 Introduction

Folic acid, (FA, vitamin B9, pteroylmonoglutamic acid), is essential in many bodily functions ranging from nucleotide synthesis to the remethylation of homocysteine. It affects rapidly dividing cells, particularly those of the bone marrow, and its deficiency can lead to megaloblastic erythropoiesis and anemia [1, 2]. Women are advised to take supplements of FA prior to conception and for the first 12 weeks of pregnancy to prevent neural tube defects to the fetus such as spina bifida and Down syndrome. [3-5]. Folate deficiency can lead to raised homocysteine level, which has now been identified as a risk factor for cardiovascular disease [6, 7]. Furthermore, low folate levels have been associated with certain cancers (notably colorectal and colon) [8, 9], with Alzheimer's disease in the elderly [10], and also with all types of dementia [7]. 5-MTHF is the predominant circulating folate coenzyme in plasma/serum [11-16] and its quantitative determination is one of several factors used in the diagnosis of folate deficiency.

Knowledge of the 5-MTHF chemical stability is necessary when its determination is intended. For prevention and treatment, FA is the form used in tablets and fortified foods because it is the most stable form of this vitamin. After ingestion, various kinds of folate were found in serum and plasma, e.g.. 5-MTHF, 5-formyltetrahydrofolate, 4-α-hydroxy-5-methyltetrahydrofolate, p- aminobenzoylglutamate, 5-methyl-5,6-dihydrofolate, and even the free form of FA [17, 18]. 5-MTHF has been directly utilized, e. g., in patients with coronary artery disease [6, 19], familial hypercholesterolemia [20], end-stage renal disease [21, 22], in uremic patients on convective hemodyalysis [23], and also suggested in the treatment of colorectal cancer [24, 25]. The determination of 5-MTHF in plasma is also an important method for the assessment of the systemic availability of the active folates after leucovorin administration [26].

The concentration of folates in biological fluids is usually very low (around 1–2 x 10^{-8} mol/L of plasma in a healthy adult). Moreover, natural folates are sensitive to heat, light, low and high pH, and have a tendency to interconvert so these facts make quantification difficult [11]. Owing to high sensitivity, microbiological assay or immunoassay (radioassay) still is the method of choice for the determination of food folates despite the fact that it is extremely laborious and provides only a sum total of folate forms [27, 28]. Folate extraction must precede the assays [29-31]. Moreover, neither of these techniques is specific for 5-MTHF and, in fact, both assays estimate "total folate", which is a nonspecific folate measurement. A number of liquid chromatography methods with different types of detection for determination of 5-MTHF and other individual folate forms have been reported [14, 15, 27, 32-44]. LC/MS was used for determination of 5-MTHF in several works [11-13, 16, 45, 46] with sufficient level of LOD. A preseparation step by, e.g., affinity extraction [11, 13, 39], extraction [42], SPE [12, 14, 15, 45], extraction and deconjugation [41], ultrafiltration [46] or deproteinization [16, 37, 38, 44] was always involved. Using extraction and SPE sample pretreatment, as low LODs as 0.07 ng/mL [27] or even 2 pg per injection of food extract [40] have been reported. Analysis of 5-MTHF in human urine by LC methods has been published in [11, 15]. Capillary electrophoresis with cyclodextrins as chiral selectors for 5-MTHF and leucovorin determination in human plasma was reported by Shibukawa et al. [47] but the sensitivity of the detection method was not adequate for its routine use.

The aim of this work was to develop a method based on electrophoretic techniques that could be combined with MS detection. Tandem arrangement of isotachophoresis with isotachophoresis or zone electrophoresis makes it possible to clean-up the sample and to enhance the concentration of the analyte. A crucial parameter for successful analysis by CITP-CITP or CITP-CZE combination is the selection of the electrolyte system that affects both separability of analytes and sensitivity of the method. The electrolyte system applied for

determination of 5-MTHF has to ensure stability of the analyte, its separation from serum components and sensitive and unambiguous detection. Above and beyond these demands, and for purposes of combining with MS, the electrolyte system has to be volatile, which limits the supply of electrolytes to free formic, acetic and carbonic acids and their volatile salts.

2 Materials and methods

2.1 Chemicals, reagents, and origin of samples

All chemicals used were of the highest analytical purity. Ammonium hydroxide, ascorbic acid, 5-methyltetrahydrofolic acid disodium salt (5-MTHF), Good`s buffers [48]: MES, HEPES, TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), MOPS, BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), HEPPSO (N-2-[hydroxyethyl]piperazine-N`-[hydroxypropanesulfonic acid]), MOPSO (3-[N-morpholino]-2-hydroxypropanesulfonic acid), ADA (N-[2-acetamido]-2-iminodiacetic acid), ammonium acetate, creatinine and histidine were from Sigma (St. Louis, MO, USA). Hydroxypropylcellulose (HPC) was from Ega (Steinheim/Albuch, Germany), 2-(4-sulfophenylazo)chromotropic acid trisodium salt (SPADNS), and all other chemicals were from Lachema Chemapol (Brno, Czech Republic). Deionized water prepared by trapping ions in a mixed-bed ion exchanger by an aqua purificator G 7749 (Miele, Gütersloh, Germany) was used for the preparation of all solutions. A standard 2 x 10^{-2} M solution of 5-MTHF was prepared from powder added to water with/or without an antioxidant immediately prior to use. The concentration of hydrazine and ascorbic acid in the stock solution of 5-MTHF was 5 x 10^{-3} M. The stock solutions were divided into 50 µL aliquots and stored in 1-mL Eppendorf sample tubes at -20 °C until use. Electrolytes for the CZE experiment were prepared by mixing 1 M acetic acid with 0.1 M ammonium

acetate stock solution. Electrolytes for CITP were prepared every day from freshly boiled water and were protected from the atmosphere. To suppress electroosmotic flow (EOF), HPC was added to electrolyte solutions [49]. Carbon dioxide dissolved in terminator was precipitated with 0.1 M barium hydroxide that was used to adjust its pH value. All electrolyte systems used in this study are summarized in Table 1.

The standard lyophilized serum (Sigma, St. Louis, MO, USA) was reconstituted with 1 mL of deionized water. Human blood samples of healthy volunteers and of women following oral administration of 0.8 mg of FA daily (as recommended for pregnant women or those planning pregnancy) were collected and diluted according to need immediately with the anticoagulant agent EDTA to a final concentration of 2.0 mg/mL of blood. No EDTA was added when the blood samples were left to start clotting for serum preparation. Urine samples were diluted with deionized water and analyzed directly without any pretreatment. To prevent 5-MTHF decomposition, all body fluid samples were diluted with deionized water containing hydrazine to reach the final 5 x 10^{-3} M hydrazine concentration, and with regard to blood samples also containing EDTA, see above.

2.2 Instrumentation

The CS Isotachophoretic Analyzer EA 100 Villa Labeco (Spišská Nová Ves, Slovakia) equipped with a column switching system was used for CITP and CITP-CZE measurements. The capillary used for CITP separations was made of fluorinated ethylene-propylene copolymer (FEP) with the internal diameter (id) of 0.8 mm; its total length was 90 mm, while the effective length to the conductivity detector was 52 mm and the distance between the detector and the bifurcation point was 38 mm. Id. of the CZE capillary (made of FEP,

equipped with both conductivity and spectrophotometric detectors) was 0.3 mm; its total length was 160 mm, the effective length to the detection window was 140 mm. For UV detection, a spectrophotometric detector LCD 2083 ECOM (Prague, Czech Republic) was used and set at 220 nm.

Samples were injected with a 10 μL microsyringe (Hamilton, Bonaduz, Switzerland). Constant current 100-450 μA and 50-100 μA was used for the CITP and CZE separation steps, respectively. Voltage range was 1500-3600 V for the CITP step and 2500-3500 V for the CZE step. After the front boundary of the sample cut selected for further analysis in CZE mode reached the conductivity detector in the CITP step, migration continued towards the auxiliary electrode for an additional 250-270 seconds (precise timing was checked every day with a red dye SPADNS). This time corresponds to the moment when the part of sample zones containing 5-MTHF is located in the bifurcation point and the front part of the sample zones had moved toward the auxiliary electrode. After switching the current through terminating and BGE electrode in the CZE capillary, the selected sample cut followed by the terminator continues in migration into the second capillary filled with BGE. Conditions for CZE migration in BGE-S-BGE system [50] are created and the analysis continues for next 300 seconds with the current decreased to 40 μA. At this time, the rear boundary of the sample cut enters the CZE capillary, the current is switched off and the terminating electrolyte (TE) in the CITP capillary is substituted with BGE. Afterwards, the current of the proper value 50-100 μA is switched on again over both capillaries and the analysis continues in the BGE-S-BGE system [50].

For single CZE experiments, an automated capillary electrophoresis instrument P/ACE 5010 (Beckman Instruments, Fullerton, CA, USA) equipped with either UV detector (214 nm) or diode array detector (DAD, wavelength range 190-600 nm) was used. With the DAD detector the absorption spectrum of 5-MTHF as well as FA was measured within the range of 200-360

nm. Absorption maxima of 5-MTHF are at 200, 220 and 290 nm while FA has maxima at 200 and 240 nm. Electrophoretic separations were performed in a fused-silica capillary of 27/20 cm total/effective length, 0.1 mm id (Composite Metal Services, The Chase, Hallow, UK). Prior to the first use, the bare capillary was treated as described in [51]. Each day before measurements, the capillary was washed only with BGE for 0.5 min at 20 psi (137.89 kPa). For measurements of 5-MTHF in anionic form the analyses were performed with cathode at the injection side. Termostating temperature was 25°C. Samples were injected for 5 s with pressure of 0.5 psi (3.45 kPa). All measurements were performed at a constant voltage. EOF was measured with mesityl oxide (dilution 1:500, detection at 254 nm) with anode at the injection side and obtained values were within the range 2.4 - 3.6 x 10^{-9} $m^2V^{-1}s^{-1}$. Data were processed using software supplied by the producer of the instrument or by ITP WIN Software. For construction of zone existence diagrams (ZED), pH and effective mobilities were calculated using RFQ function published by Beckers and Everaerts [52]. ZEDs were introduced and their plotting is described in detail in [53, 54]. For parametrically selected pK and ionic mobilities the adjusted pH in the isotachophoretic zone of such a defined component as well as its effective mobility is calculated and plotted for a given leading electrolyte. Values of pK and ionic mobilities used in calculations are given in Table 2. For 5-MTHF the pK and mobility values estimated for MFA [55] were used as they fitted well with experimentally reached results for 5-MTHF.

3 Results and discussion

3.1. Stability of 5-MTHF

To avoid loss of 5-MTHF due to its chemical degradation that could lead to false results, its chemical stability was checked in acidic and neutral pH range by both CITP and CZE methods. Preliminary tests revealed that 5-MTHF rapidly decomposes at slightly acidic pH starting at pH 3.6 (Table 1, electrolyte system No.2). By CITP, the pH range 4.5 - 6.5 (Table 1, electrolyte systems No.3-5), was tested in more detail. Tests at higher pH were not performed as above the pH 6.5 disturbing hydrocarbonate zone appeared in the system resulting from dissolved atmospheric CO_2. Measurements in the tested area were performed with 5- MTHF dissolved in water in the absence and presence of an antioxidant. We tested ascorbic acid that was frequently used in other analytical methods to determine 5-MTHF [11-14, 27, 33-35, 37-39, 41]. To get an observable effect, a relatively high concentration of ascorbic acid had to be added to the sample solution (5×10^{-3} M – 5.6×10^{-2} M), which resulted in a long zone migrating in the same direction as the analyte thereby decreasing the sample loadability. Therefore, we tested another agent, hydrazine, which is positively charged in the tested pH range and migrates to the cathode (pKa = 8.1 [56]). A 5×10^{-3} M hydrazine in the sample solution was proven to ensure satisfying prevention of 5-MTHF decomposition for several days. The preventive effect of hydrazine was tested also by CZE in BGEs containing 1×10^{-2} M acetate + ammonium + 0.005% HPC, pH 4.0 - 5.8. Degradation was observed at pH 4 and lower even in the presence of hydrazine, however, within pH 4.5 – 5.8 a constant 5-MTHF concentration was observed in the presence of 5×10^{-3} M hydrazine even when the solution was kept at room temperature (data not shown). In all the following measurements samples containing 5-MTHF were stabilized with hydrazine, prepared fresh every day, and optimum separation conditions were searched within the pH range 4.5 - 5.8.

3.2 Selection of electrolyte systems

The CITP-CZE hyphenated technique was described in more detail previously, e.g. in [57-67]. Using this method, the original injected sample is cleaned twice. First, only ionic components of mobilities between the mobilities of leading and terminating ions can migrate in the CITP step. Second, only a selected segment of isotachophoretically migrating zones is analyzed in the CZE step [67]. Success of the analysis depends both on a suitable electrolyte selection for CITP and CZE steps, and on proper current switching (sample cutting).

3.2.1 Selection of electrolyte systems for isotachophoresis

When selecting electrolyte systems for isotachophoresis, a suitable mobility window is searched for that ensures migration of analytes of interest between the fastest leading ion and slowest terminating ion. No care is taken of compounds that migrate either faster or slower outside this window. pK and ionic mobilities of components of analyzed matrices that are important from the point of analysis of 5-MTHF in blood, serum and urine are listed in Table 2. For ions of weak acids, pH of LE should ensure sufficient ionization as well as differences in effective mobilities of sample components. Disturbance resulting from the penetration of fast H^+ or OH^- ions should be eliminated by proper selection of the electrolyte system including its buffering capacity. Here, we put additional demand on the electrolyte system, i.e., its volatility, to enable potential hyphenation of CITP with MS.

For cationic analyses performed by CITP-MS, the most often used electrolyte system is composed of ammonium acetate as LE and acetic acid as TE [68]. Here, ammonium is the leading ion and H^+ is the real terminator, acetate being the buffering counter ion. This also applies to systems with formic or carbonic acid systems – formate or carbonate serving as

counter ions. We looked in detail into the systems where anions of these acids should serve as leading ions with ammonium acting as a counter ion. After our consideration we used calculations of pH and effective mobilities of both pure and mixed zones based on the RFQ function [52], and constructed ZEDs [53, 54] for individual electrolyte systems (see also Section 2.2). We used this approach for a practical reason. When pH of electrolytes was set by adding ammonium hydroxide, a significant zone of hydrocarbonate appeared in the system. Contrary to CZE where BGE can be composed of more than one coion, which is often used in analyses with indirect optical detection, in CITP from the principle of the separation process it follows that each component of the leading and terminating electrolytes creates its own zone. We discovered that presence of hydrocarbonate in LE or TE leads not only to formation of its own zone but also to formation of mixed zones. These zones cannot simply be recognized and false results can easily be obtained. We have constructed ZEDs for volatile leading electrolytes composed of acetate and ammonium, pH_{LE} 4.5 – 7.0, and hydrocarbonate and ammonium, pH 7.8, a semi-volatile leading electrolyte composed of chloride and ammonium, pH 5.6, and non-volatile leading electrolyte composed of chloride and EACA, creatinine, or histidine, pH 4.5 – 5.8. ZEDs for selected electrolyte systems are presented in Fig. 1. For any analyte migrating isotachophoretically between leading and terminating ions its effective mobility as well as pH in its zone can be found on axes y and x, respectively. Analytes that do not migrate isotachophoretically in the electrolyte system can easily be disclosed because points defined by their ionic mobility and p*K* are out of the ZED net. ZED also shows the migration order of analytes and for a selected analyte X the set of analytes Y that will create steady-state mixed zones with X can also be found by calculating two curves expressing equalities $\bar{u}_{Y,\,X} = \bar{u}_{X,X}$ and $\bar{u}_{X,\,Y} = \bar{u}_{Y,Y}$ where $\bar{u}_{X,\,Y}$ is the effective mobility of a substance X in the zone Y.

Fig. 1A shows ZED for pH 4.5 (LE composed of 1 x 10^{-2} M acetate + 3.5 x 10^{-3} M ammonium) where chloride and lactate do not migrate isotachophoretically, and the points corresponding to their zones are outside the margin contours of the ZED (see lactate point in Fig. 1A). The grey area shows the group of analytes that will create mixed zones with hydrocarbonate when it will be present either in the sample or in the electrolyte system. Hydrocarbonate formed a mixed zone also with MES that was aimed to be used as a potential terminator. The effective mobility of the mixed zone was -9.8 x $10^{-9}m^2V^{-1}s^{-1}$, i.e., even higher than that of the analyte. As hydrocarbonate appeared in the system always when electrolyte systems were prepared using ammonium hydroxide and its elimination by barium hydroxide was powerless, electrolyte systems should be prepared directly by dissolving solid salts in water.

By dissolving directly ammonium acetate in freshly prepared deionized water we get pH 7.0. At this pH value, however, carbon dioxide can easily be absorbed and acetate itself will create a mixed zone with hydrocarbonate. This holds also for some other sample components, e.g., lactate, and the system is very sensitive to the content of hydrocarbonate (see Fig. 1B). ZED for chloride-creatinine system, pH 5.0, is shown in Fig. 1C. Evidently, in the leading electrolyte with chloride as leader and creatinine as counter-ion there is only a very small set of analytes that can create mixed zones with hydrocarbonate which is not expected to appear in the slightly acidic system. This system was found to be optimal for 5-MTHF determination by CITP, where both analyte decomposition and disturbing mixed zones were minimized. This electrolyte system was used for isotachophoretic determination of 5-MTHF in serum diluted 1:5; LOD obtained was 4 x 10^{-5} M. However, we did not consider this analysis sensitive enough and decided to use CITP in the buffered non-volatile electrolyte system only for simplification and clean-up of the sample. We introduced CZE as further separation step.

3.2.2 Selection of electrolyte systems for CZE appended to CITP

For the pH range suitable for determination of 5-MTHF from the point of its stability (pH 4.5 –5.8), BGEs composed of acetate as the coion and varying concentrations of ammonium as the counter-ion were tested in CZE mode. When a BGE system totally differs from the composition of electrolytes used as LE or TE in CITP, very precise current switching between CITP and CZE capillaries is required. Such electrolyte combination was described as BGE-S-BGE [50, 67].

The precise time ensuring removal of the major front part of the sample bulk components by CITP in the first capillary had to be checked every day using a red dye SPADNS (see Section 2.2). With SPADNS, the time interval was found for the analyte to move from the detection window to the bifurcation point. Then, the time interval when the current was switched over both capillaries enabling the important sample cut to enter the second capillary filled with BGE had to be found. The time needed for the transfer of the whole sample cut into the second analytical capillary was estimated experimentally. After this, the voltage was switched off and TE filling now the first capillary was washed out and substituted with BGE identical with the BGE filling the second capillary. Finally, the proper current was switched on between electrodes in vessels containing originally TE and BGE and the analysis in CZE mode continued. The peak area of 5-MTHF was constant on condition that the whole sample cut containing 5-MTHF was transferred into the second capillary. If the time for the CITP step was set too long the part of the sample containing 5-MTHF had moved towards auxiliary electrode and the analyte peak area was lower. If this time was too short, 5-MTHF could be flushed out when the first capillary was cleaned and filled with BGE or its concentration could be decreased. If too long a time was set for the zone transfer, too long part of the

terminating zone was transferred into the analytical capillary with pronounced effect on migration time and peak shape as well. Also an impact on the sensitivity of MS detection caused by a long zone of nonvolatile TE moving to the MS detector would be undesirable.

After the precise time switching was set for each tested system, model as well as real serum samples were tested. The experiments with real serum samples revealed some problems that could not be deduced in model samples composed of a defined but not varying concentrations of serum macrocomponents such as phosphate, lactate, urate, and acetate. At pH 5.8, (close to pH of acetate zone adjusted in preceding CITP step), where promising results with model samples were reached, increased concentration of urate found in some serum samples resulted in comigration with 5-MTHF zone. Sufficient separation of 5-MTHF from urate was achieved at pH 5.0, however, resolution of 5-MTHF from another unknown serum component was lower than 1. The best results were obtained at pH 4.5, where reliable migration window for 5-MTHF was ensured. The most suitable electrolyte combination, LE composed of 1 x 10^{-2} M HCl + 2.5 x 10^{-2} M creatinine + 0.005% HPC, pH 5.0, TE composed of 1 x 10^{-2} M MES and BGE composed of 1 x 10^{-2} M acetate + 3.5 x 10^{-3} M ammonium + 0.005% HPC, pH 4.5, was used for determination of 5-MTHF in real samples.

3.3 Real sample analysis of human blood, serum and urine

The optimized electrolyte system of the BGE-S-BGE mode was used for qualitative and quantitative analyses of 5-MTHF in real samples. Body fluid samples were collected and handled as described in paragraph 2.1. Traces of analyses of human blood, serum and urine are presented in Figs. 2-4. The concentration of 5-MTHF in the samples of human blood, serum or urine from the healthy volunteers was under LOD. Human blood of a volunteer consuming daily 0.8 mg of FA was analyzed during one week and the blood was collected

always 2 hours after FA consumption. In Fig. 2, the estimated 5-MTHF concentration in the injected sample of 5times diluted blood was 2.4 x 10^{-6} M that corresponds to 1.2 x 10^{-5} M in the original blood sample. CZE record of human serum of a volunteer with the same dosage and collection of FA can be seen in Fig. 3. A 5-MTHF concentration in the 5times diluted sample was 1.2 x 10^{-6} M, which corresponds to 6.0 x 10^{-6} M in serum. In this matrix we also verified separation of 5-MTHF from FA. Mobilities of FA and 5-MTHF differ substantially and no comigration was imminent (data not shown). Identification of FA and 5-MTHF peaks was performed also by their absorption spectra in UV region, see Section 2.2.

We applied the elaborated method also for the analysis of human urine samples and obtained a good resolution of 5-MTHF also in this very complex matrix (Fig. 4). A 5-MTHF concentration in a sample of 5times diluted urine of a volunteer following the same dosage of FA as mentioned above was under LOD (Fig. 4, trace a). In trace b, Fig. 4, a well developed peak of 5-MTHF is seen when the same urine sample is spiked with 5x 10^{-5} M 5-MTHF.

Parameters for quantitative analysis of 5-MTHF standard and 5-MTHF in real samples are summarized in Table 3. Human blood, serum and urine were diluted 1:5 and analyzed directly without any pretreatment. Calibration curves for standard 5-MTHF analyte and for real samples were constructed for the concentration range of 1 x 10^{-5} M – 5 x 10^{-7} M. LOD values and intra-day and inter-day RSD for peak area are presented in this table as well.

4 Concluding remarks

A method for tandem arrangement of capillary ITP with zone electrophoresis and UV detection was elaborated for human body fluids: blood, serum and urine. CITP-CZE combination enabled an effective clean-up of the sample and lowering LOD. A detailed

insight into electrolyte systems including zone existence diagrams made it possible to choose conditions that are suitable also for MS detection. For the CZE step, a different BGE from that of LE and TE was used. A 5-MTHF decomposition was prevented by hydrazine added to the sample. Attained LODs were 1.6 x 10^{-7} M for human blood, 1.1 x 10^{-7} M for human serum, and 4.7 x 10^{-7} M for human urine. The method is now ready for coupling with an MS detector; a new interface is being constructed applicable for the commercial apparatus used in this study.

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Legends to the figures

Figure 1

Zone existence diagrams for LEs composed of (A) 1 x 10^{-2} M acetate + ammonium, pH = 4.5; (B) 1 x 10^{-2} M acetate + ammonium, pH = 7.0; (C) 1 x 10^{-2} M chloride + creatinine, pH = 5.0, with terminating OH^- ions. Effective mobilities ū (10^{-9} $m^2V^{-1}sec^{-1}$); The curves 0-12 correspond to set pK_{HX} and variable $u_{X;}$ the curves 10-80 correspond to set ionic mobilities u_X and variable pK_{HX}. MIX = area of mixed zones with hydrocarbonate. For further explanation, see text.

Figure 2

CZE step record of CITP-CZE analysis of 5-MTHF in human blood. Sample: (a) human blood of a healthy volunteer prior to consumption of FA was diluted 1:5 with deionized water with the final concentration of hydrazine and EDTA of 5 x 10^{-3} M and 1 x 10^{-2} M, resp.; (b) human blood of a volunteer treated with FA (0.8 mg/day) was diluted as in (a). An estimated 5-MTHF concentration was 2.4 x 10^{-6} M; (c) as in (b) spiked with 5 x 10^{-6} M 5-MTHF; (d) as in (b) spiked with 1 x 10^{-5} M 5-MTHF. LE: 1 x 10^{-2} M HCl + creatinine + 0.005% HPC, pH 5.0; TE: 1 x 10^{-2} M MES; BGE: 1x 10^{-2} M acetate + ammonium + 0.005% HPC, pH 4.5. Injected sample volume 10 µL. Driving current during CITP step, zone transfer, and CZE step was 100, 40, and 60 µA, respectively. Detection at 220 nm. R, detector response. For other experimental conditions, see Section 2.

Figure 3

CITP-CZE analysis of 5-MTHF in human serum. (A) CITP step record; (B) CZE step record. Sample: (a) human serum of a healthy volunteer prior to consumption of FA was diluted 1:5 with deionized water with the final concentration of 5 x 10^{-3} M hydrazine; (b) human serum of volunteer treated with FA (0.8 mg/day) was diluted as in (a). An estimated 5-MTHF concentration was 1.2 x 10^{-6} M; (c) as in (b) spiked with 2.5 x 10^{-6} M 5-MTHF; (d) as in (b) spiked with 1 x 10^{-5} M 5-MTHF. LE: 1 x 10^{-2} M HCl + creatinine + 0.005% HPC, pH 5.0; TE: 1 x 10^{-2} M MES; BGE: 1 x 10^{-2} M acetate + ammonium + 0.005% HPC, pH 4.5. Injected sample volume 10 μL. Driving current during CITP step, zone transfer, and CZE step was 100, 40, and 60 μA, respectively. Detection at 220 nm. R, detector response. For other experimental conditions, see Section 2.

Figure 4

CZE step record of CITP-CZE analysis of 5-MTHF in human urine. Sample: (a) human urine of a volunteer treated with FA (0.8 mg/day) was diluted 1:5 with deionized water with the final concentration of 5 x 10^{-3} M hydrazine; (b) as in (a) spiked with 1 x 10^{-5} M 5-MTHF. LE: 1 x 10^{-2} M HCl + creatinine + 0.005% HPC, pH 5.0; TE: 1 x 10^{-2} M MES; BGE: 1 x 10^{-2} M acetate + ammonium + 0.005% HPC, pH 4.5. Injected sample volume 10 μL. Driving current during CITP step, zone transfer, and CZE step was 100, 40, and 60 μA, respectively. Detection at 220 nm. R, detector response. For other experimental conditions, see Section 2.

Table 1. Tested electrolyte systems for CITP

No.	LE	TE
1	1 x 10^{-2} M chloride + ammonium + 0.005 % HPC, pH 5.0-6.5	1 x 10^{-2} M HEPES +$Ba(OH)_2$, pH 5.0-7.5
2	1 x 10^{-2} M formate + β-alanine + 0.005% HPC, pH 3.6-4.0	1 x 10^{-2} M acetate + β-alanine, pH 4.1
3	1 x 10^{-2} M chloride + histidine + 0.005 % HPC, pH 5.5-6.5	1 x 10^{-2} M Good`s buffers, See 2.1.
4	1 x 10^{-2} M chloride + EACA + 0.005 % HPC, pH 4.0-4.8	1 x 10^{-2} M MES
5	1 x 10^{-2} M chloride + creatinine + 0.005 % HPC, pH 5.0-5.5	1 x 10^{-2} M MES

Table 2. Ionic mobility and p*K* values [69] (if not stated otherwise) of selected components

Analyte	p*K*	Ionic mobility (10^{-9} $m^2V^{-1}s^{-1}$)
Acetate	4.8	-42.4
β-alanine	2.3	34.0
Lactate	3.9	-36.5
Phosphate	2.2, 7.2, 12.7	-71.5, -61.4, -34.6
Carbonate	6.4, 10.3	-46.1, -71.8,
Urate	5.7[a]	-27.9[a]
HEPES	7.5	-23.5
Chloride	-2.0	-79.1
FA	2.4, 3.5, 5.0, 8.1[b]	-34.3, -25.8, -14.1, 11.5[b]
MFA	2.3, 3.4, 4.9, 7.9 [b]	-34.0, -25.5, -14.7, 12.2 [b]
Histidine	2.0, 6.0	44.7, 28.8
Ascorbate	10.5, 4.3	-51.0, -25.5
Ammonium	9.3	76.2
Hydrazine	8.1[c]	59.1[d]
MES	6.1	-28.0
EACA	4.4	28.8
Creatinine	4.8	37.2
Formate	3.8	-56.6

[a] from [5], [b] from [68], [c] from [51], [d] from [71].

Table 3. Parameters of quantitation

Sample	Equation of calibration curve for peak area	LOD (M)	RSD (%) for peak area)	
			Intra-day	Inter-day
5-MTHF in water	y(V.s) = 20820 x(M) – 27, R^2 = 0.999	9.9 x 10^{-8}	4.9 (n = 3)	3.5 (n = 2)
Human blood	y(V.s) = 16164 x(M) – 10, R^2 = 0.994	1.6 x 10^{-7}	4.7 (n = 2)	10.3 (n = 2)
Human serum	y(V.s) = 5719 x(M) + 21, R^2 = 0.990	1.1 x 10^{-7}	4.5 (n = 2)	8.1 (n = 2)
Human urine	y(V.s) = 14021 x(M) -186, R^2 = 0.976	4.7 x 10^{-6}	9.8 (n = 2)	11.4 (n = 2)

Figure 1

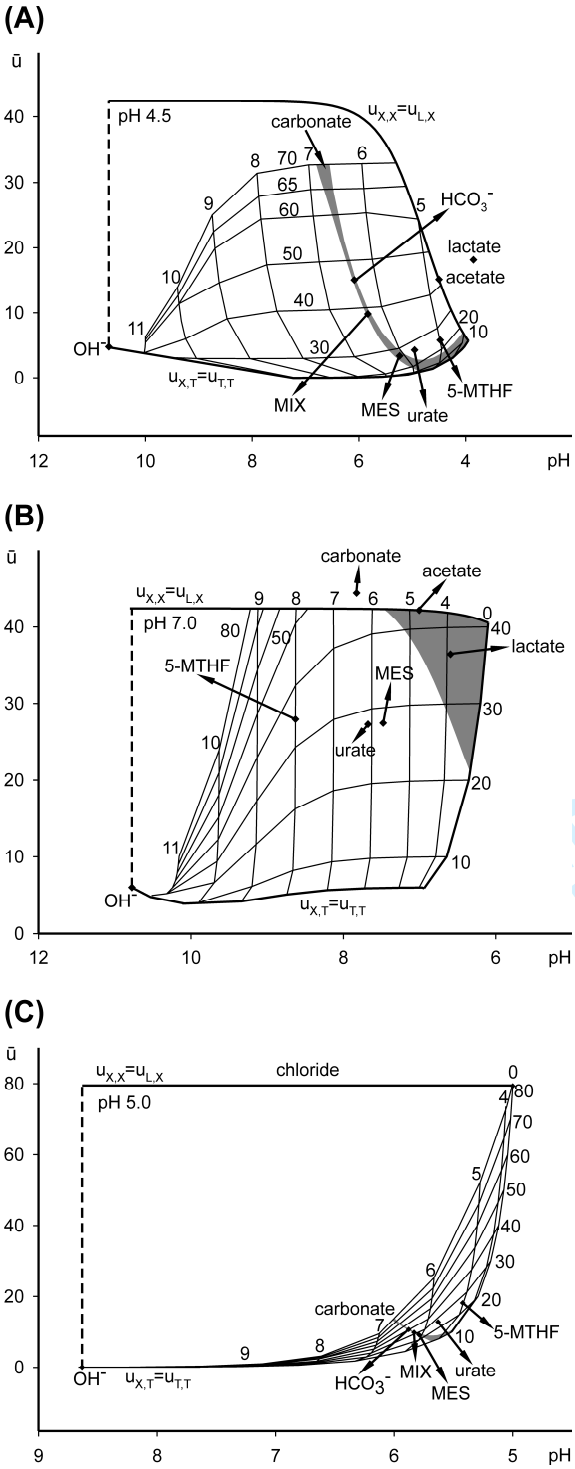

Figure 2

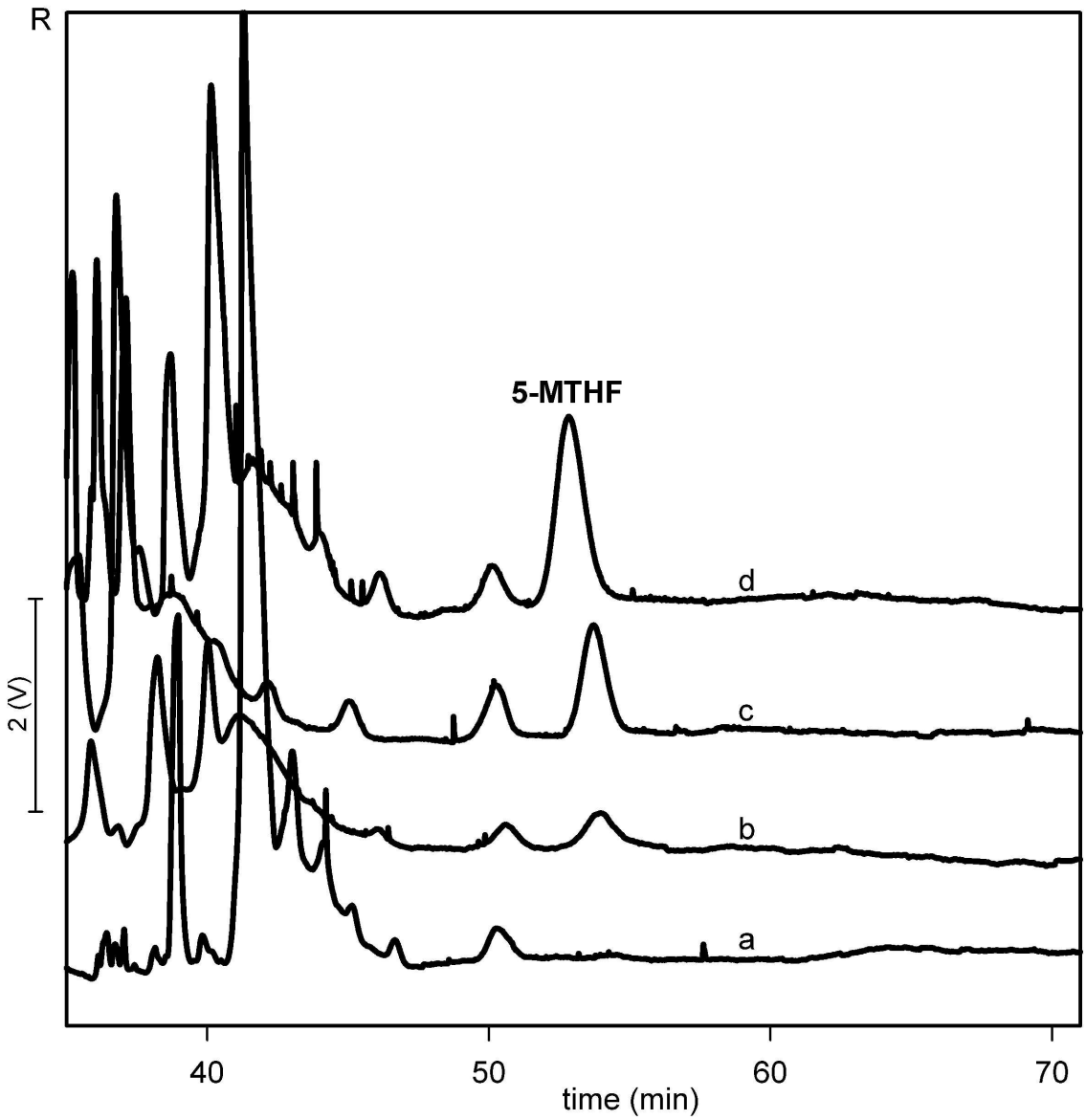

Figure 3

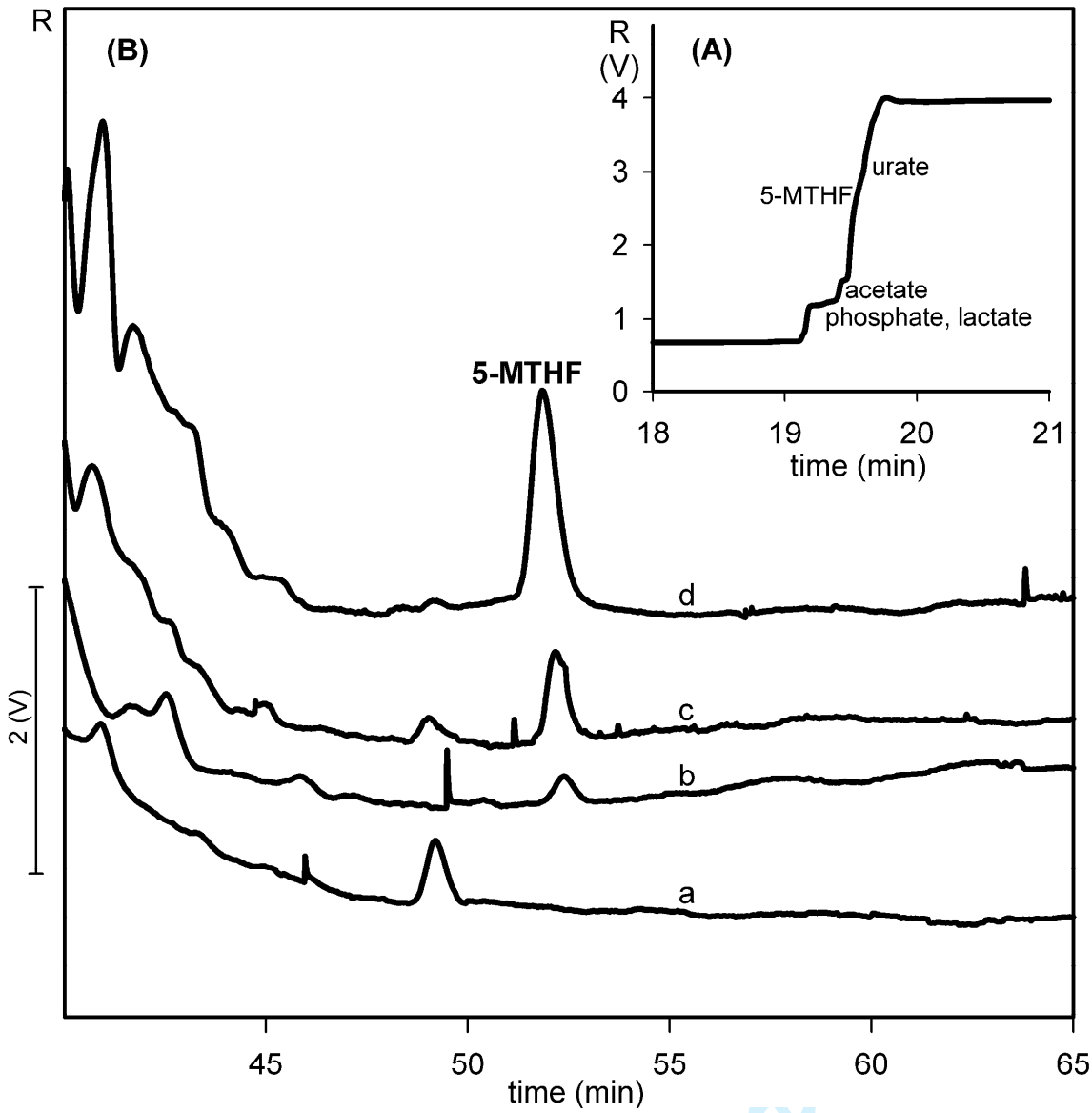
R
(B)
5-MTHF
d
c
b
a
2 (V)
45
50
55
60
65
time (min)
R
(V)
(A)
4
3
2
1
0
urate
5-MTHF
acetate
phosphate, lactate
18
19
20
21
time (min)

Figure 4