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Review

Determination of uremic toxins in biofluids: creatinine, creatine, uric acid and xanthines

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Abstract

Rapid and accurate determination of small molecule metabolic end-products is vital for clinical diagnosis and study of many metabolic disorders and medical abnormalities. Chromatographic and electrophoretic techniques are attractive for clinical analyses because of the inherent ability to analyze multiple component biofluids and determine the analytes of interest with minimal interference from other species. This manuscript reviews recent (1990–present) developments in chromatography and electrophoresis methodology for the determination of creatinine, creatine, uric acid and xanthines in biofluids.

Keywords: Reviews; Creatinine; Creatine; Uric acid; Xanthine

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1. Introduction

The use of chromatography and electrophoresis in the clinical setting has become increasingly attractive for drug and protein monitoring as well as diagnosis of abnormalities, particularly in the area of metabolic disorders. The ability to analyze multiple component biological fluids and to determine the compounds of interest while eliminating interfering species gives these separation-based methods an advantage over enzymatic and spectrochemical methods. Indeed, there have been several fine articles and reviews that highlight the numerous applications of chromatography [1,2] and electrophoresis [2–7] for clinical analyses.

This manuscript will focus on determination of uremic toxins including creatinine, creatine, uric acid, xanthines and their metabolites in biological fluids. Analyses for such substances are crucial for diagnosis and monitoring of renal disease, metabolic disorders and various types of tumorigenic activity. One can find in the literature other review articles describing analyses for these toxins [8–11]. This article will focus on recent advances (1990 to present) in analyses for these toxins using chromatographic and electrophoretic methods of analysis.

2. General considerations for sample pretreatment

Molecules that serve as effective biomarkers in clinical fluids are often low-molecular-mass metabolic end products. Moreover, most pharmaceutical agents (and their metabolites) are relatively small molecules. These small molecules can often be directly determined in urine; however, determination of low-molecular-mass compounds in plasma or sera often requires some form of protein removal. The removal of protein from clinical specimens is often the most time consuming step in the analysis, dictating the time required for the entire assay procedure. For chromatographic separations, the presence of protein in the injected samples can cause modification of the column and bias in the analytical results due to protein binding. In capillary electrophoresis (CE), proteins may adsorb onto the inner walls of untreated capillaries resulting in fluctuation of the electroosmotic flow-rate and irreproducible data [12]. Protein removal can be accomplished by several techniques such as solvent, solid-phase, or Soxhlet extractions, protein precipitation, ultrafiltration, centrifugation and column-switching or pre-column methodology [13,14]. In addition, the effect of proteins on CE separations can also be minimized with on-column techniques such as stacking methods [15], the use of high ionic strength buffers, the addition of sodium dodecyl sulfate (SDS) to solubilize proteins, and isotachophoretic techniques [16].

3. Creatine and creatinine

Creatine and the energy reserve form, creatine monophosphate are present in muscle, brain and blood. In recent years, ingestion of phosphocreatine has become extremely popular with many athletes for muscle building and performance enhancement [17]. Because of this, analyses for creatine has become more important in the clinical setting.

Creatinine results from the irreversible, non-enzymatic dehydration and loss of phosphate from phosphocreatine [18]. The determination of creatinine remains one of the most important routinely performed clinical assays. Creatinine is the most widely used clinical marker to assess renal function [19–21]. The Jaffe method is one widely accepted method for creatinine determination and involves a colorimetric procedure by way of picrate [22]. Over the years, enzymatic assays that have enhanced specificity for creatinine have also been developed [23–28]. However, these methods are not all free of interference. Chromatographic and more recently, electrophoretic techniques have also been developed for use in the clinical setting and these are described below. Huang and Huang [8] and Spencer [9] have previously reviewed some of the earlier separation-based techniques for determination of creatinine and other guanidino compounds. We present here some of the more recent advances.

3.1. Chromatography

Chromatographic techniques for creatinine determination have included simple reversed-phase and ion-exchange methods as well as more complicated

column-switching and tandem methods. Generally, the complexity of the method depends partly on the method of sample pretreatment and the number of compounds being determined.

3.1.1. Reversed-phase (RP)

Werner et al. have developed a method for direct injection of serum that employs either an NH_2 or a C_{18} pre-column for sample clean up prior to the analytical separation [29]. The isocratic reversed-phase separation employs photodiode array detection to allow simultaneous detection of creatinine, creatine and uric acid over a wide linear range. The method was shown to be consistent with isotope dilution gas chromatography–mass spectrometry (GC–MS) method originally described by Siekman [30]. Typical data obtained with the NH_2 pre-column are shown in Fig. 1, with linearity for creatinine, creatine and uric acid being 0.2–40 mg/dl, 0.2–20 mg/dl and 0.2–20 mg/dl, respectively. An earlier method developed by Yang et al. used methanol to deproteinize serum samples before injection onto an ODS column [31]. UV detection at 235 nm allowed simultaneous determination of creatinine and uric acid with detection limits of 0.083 mg/dl and 0.0075 mg/dl, respectively. Yokoyma et al. investigated the use of RP-high-performance liquid chromatography (HPLC) for simultaneous determination of creatinine, amino acids and organic acids in urine using UV detection at 210 nm [32] allowing for even lower detection limits but with linearity up to 11.5 mg/dl. In this work, a preliminary step of cation-exchange was performed before the reversed-phase separation was applied to separate the organic acid fraction from the amino acid fraction. Another method for urine analysis, this by Yue-dong [33], reported simultaneous determination of creatinine, creatine, uric acid and hippuric acid at 220 nm with results being quite comparable to those reported by Werner et al. For these methods of urine analysis, only dilution of the specimens was needed for sample preparation.

Taking a different approach, Yasuda et al. performed analyses of both serum and urine using liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS). They determined creatinine, creatine and guanidinoacetic acid with a linearity of 200–3000 μg/ml, 20–500 μg/ml and 20–500 μg/ml, respectively [34]. An extensively prepared sample having been passed through two different ion-exchange columns, was separated on an ODS-2 analytical column and analyzed by APCI-MS with the quasi-molecular ion $[M+H]^+$ being recorded.

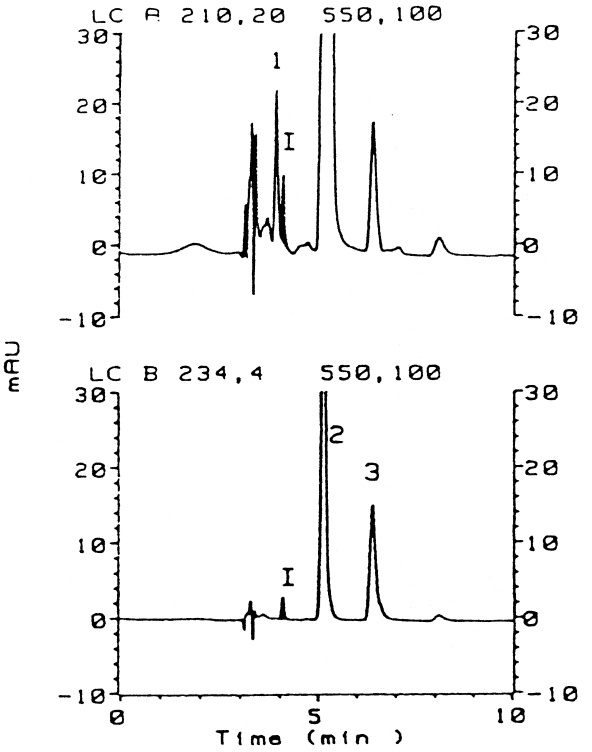

Fig. 1. Chromatogram of a serum sample containing an unknown interfering substance obtained with RP-HPLC and a NH_2 pre-column. Separation conditions: NH_2 pre-column, 40 mm×4.6 mm I.D., 5 μm particle size; ODS analytical column, 250 mm×4.6 mm I.D., 5 μm particle size; elution with a 0.03 *M* dipotassium hydrogenphosphate–methanol (99.5:0.5, v/v) buffer, pH 7.5 for 8 min followed by a gradient to 20% methanol from 8 to 10 min; UV detection at 210 and 234 nm. Peaks: 1=creatine (162 μmol/l), 2=uric acid (5.2 mg/dl), 3=creatinine (1.55 mg/dl), I=unknown interference. Reproduced with permission from Ref. [29].

3.1.2. Ion-exchange

Though ion-exchange methods are often used in a preparatory manor, they have also been used for the main analytical separation. The preliminary step described by Yokoyma et al. [32] for the fractionation of organic and amino acids, has also been used

for analytical determination of creatinine and aromatic amino acids, in urine [35]. Kågedal and Olsson compared three ion-exchange methods for creatinine determination in serum, previously deproteinized using trichloroacetic acid (TCA) [36]. They compared a strong cation-exchange method with a weak cation-exchange method and a reversed-phase method given strong cation-exchange properties by a mobile phase containing *N*-methyloleoyl taurate. They preferred the weak cation-exchange method due to the minimal interference form other compounds, higher throughput, and the simplistic nature of the method. Harmoinen et al. developed a method for analysis of both serum and urine for the determination of creatinine over a wide linear range from 1 to 2000 μmol/l [37]. This was accomplished by employing a silica-based cation exchanger with sulfate as the functional group. Deproteinization of serum was carried out using TCA, and the urine was diluted and directly injected.

3.1.3. Ion-pair

Separation in ion-pair chromatography is accomplished via interactions between ions present in the mobile phase and ions from the sample. Kock et al. have employed octanesulfonic acid as an ion-pair agent for cationic serum components allowing simultaneous determination of creatinine and uric acid [38]. Others have simultaneously determined creatinine and pseudouridine in urine of leukemia patients using that same ion-pairing agent [39]. Yokoyama et al. [40], in addition to their work with exchange methods [35], have described a technique they termed dual-mode gradient ion-pair chromatography. The method involved the use of a butyl silica column and a mobile phase with some combination of acetonitrile and a solution of SDS–perchloric acid, where SDS served as the ion-pair reagent. This technique was used for separation and determination of creatinine, amino acids, bioactive amines and nucleic acid bases. Detection was accomplished using fluorescence and UV absorbance, depending on the separation being performed.

3.1.4. Column-switching techniques

With column-switching, a variety of chromatographic approaches can be used to efficiently separate the components of a complex sample. These techniques are often used for automated sample pretreatment or clean up of serum samples prior to creatinine determination [29,41]. Column-switching has also been used for determination of creatinine, uric acid [42,43], and hypoxanthine [42]. Seki et al. have described two different techniques for simultaneous determination of these three compounds [42] as well as a method for creatinine and uric acid [43]. They used as many as six different columns for separations by size-exclusion, cation-exchange and anion-exchange chromatography, with the only sample preparation being dilution and filtration through membrane filters.

3.2. Capillary electrophoresis

Because creatinine has rich acid–base chemistry with pK_a values at 4.8 and 9.2 [44], one can employ capillary zone electrophoresis (CZE) at pH below 4.8 (cationic form) or above pH 9.2 (anionic form). The former has been reported for urine analysis using a background electrolyte of pyridine, tartaric acid and 18-crown-6 [45]. This method is useful for creatinine determination but results in coelution of many acidic metabolites. The latter has been reported for urine analysis and appears to be free of most metabolites; however, it was found that if the CZE separation was performed at a pH below about 7.0, other UV-absorbing substances migrate with creatinine [46]. In our laboratory, creatinine was not separable from other neutral species over the pH range of 6 to 9 using CZE [47]. In order to separate creatinine from other UV absorbing neutrals, we ([48], Fig. 2) and others [49–51] have employed the use of micellar electrokinetic capillary chromatography (MEKC).

The most common psuedo-stationary phase in MEKC is formed by the addition of SDS to the buffer. Miyake et al. reported such a technique for simultaneous determination of creatinine and uric acid in plasma and urine using antipyrene as an internal standard [49]. Buffer systems consisting of phosphate, SDS and isopropanol at pH 9 and also at pH 6 were used to separate directly injected serum samples, without protein removal. SDS has the ability to solubilize plasma proteins and reduce unwanted adsorption to the capillary inner walls, as well as separate neutral species by partitioning in and

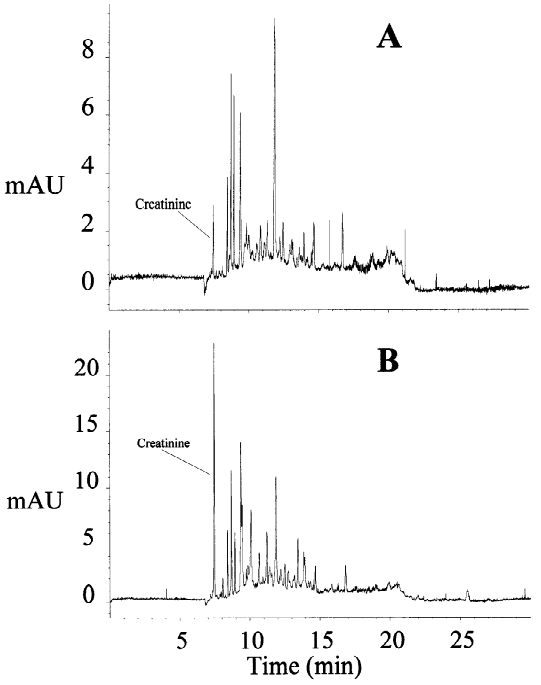

Fig. 2. MEKC separations of deproteinized sera obtained from (A) a healthy individual and (B) a patient with chronic renal failure. MEKC separation conditions: 20 m*M* borate–80 m*M* SDS, pH 9.0; 80.5 cm (72.0 cm injector to detector)×50 μm I.D. capillary; 22 kV (21 μA); 5 s, 5 kPa (50 mbar) injection; UV detection at 210 nm. Creatinine concentrations in these samples were determined to be 0.14 and 1.60 m*M*, respectively. Reproduced with permission from Ref. [48].

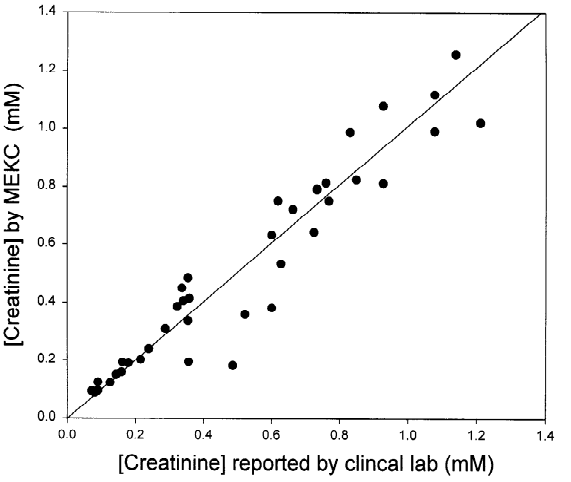

Fig. 3. Illustration of the ability of the MEKC method shown in Fig. 2 to provide quantitative data that matches that obtained with accepted methodology (enzymatic assay for 29 of the specimens, Jaffe method for 11 of the specimens). Each data point represents one serum sample.

out of the micelles. Schmutz and Thormann have also employed a direct injection technique with a phosphate–borate–SDS buffer for studying drugs and endogenous low-molecular-mass compounds in serum [50]. These compounds migrate ahead of the high-molecular-mass proteins. The use of a direct injection technique is effective but flushing of the capillary with NaOH between runs is necessary.

Shirao et al. performed MEKC with a phosphate–SDS buffer and found that a pH of 7.0 was needed in order to resolve creatinine, as well as uric, homovanillic and vanilmandelic acids from other compounds in urine [51]. They used centrifugation in order to clarify the urine samples and also a somewhat extensive flushing system involving methanol, distilled water and the buffer to ensure reproducibility. Tran et al. have reported creatinine determination in deproteinized sera using MEKC with a borate–SDS buffer ([48], Fig. 2). This allows for less interference from proteins coeluting with small molecules and eliminates the extensive flushing of the capillary needed for reproducible results. For quantification of creatinine, theobromine was used as an internal standard, and has been shown to produce reliable results with respect to accepted clinical assays (Fig. 3).

Recently, we have been exploring the use of electrophoretic mediated microanalysis (EMMA) to adapt one of the widely used assays, the Jaffe method, to a CE-based system. The Jaffe reaction is carried out on-column by electrophoretically mixing creatinine, which is net neutral, and picrate, which is anionic, to form a red Janovski complex, which is detected at 485 nm. Preliminary results with this method are promising.

4. Uric acid

Uric acid is the final product of catabolization of the purine nucleosides; adenosine and guanosine; hypoxanthine and xanthine are intermediates along this pathway. As a consequence, uric acid and xanthines are markers for metabolic disorders such as gout, Lesch–Nyhan syndrome and xanthinuria, to

name a few. Thus, uric acid is often determined simultaneously with xanthine and hypoxanthine by chromatographic [42,52] and electrophoretic [47,48,50,53,54] means. Uric acid, like creatinine, is also a marker for renal failure, as well as toxicity. Many of the chromatographic [29,31–33,39,42,43] and electrophoretic [47–51] methods presented earlier for creatinine have also been or could be applied for the determination of uric acid.

4.1. Chromatography

One method developed by Chen et al. for determination of uric acid in urine preserved by thymol and/or thimersoal was also used for creatinine determination [55]. Uric acid and creatinine eluted with retention times of 10 min and 5 min, respectively, from a C_{18} column in a acetate buffer at pH 4.5. Though uric acid elutes at 10 min, total analysis time was 45 min due to extensive washing and reequilibration of the column. Kock et al. developed a chromatographic method for the determination of uric acid, hypoxanthine, xanthine and allantoin in sera [52]. The separation of these compounds required 20 min by isocratic reversed-phase chromatography, employing an aqueous–phosphate buffer mobile phase at pH 4.60. The serum preparation was minimal because a guard column was used, eliminating any need for ultrafiltration or protein extraction. It was necessary, however, to replace the guard column after every 30 injections in order to maintain good reproducibility. Another method for uric acid and allantoin determination, though in urine, was by ^{15}N isotopic enrichment of the analytes and using GC–MS for separation and detection [56]. Sample preparation was required due to the high salt content of the urine. Levels of uric acid in sera have been assessed by Ellerbe et al., who also used ^{15}N isotopic enrichment GC–MS after a sample preparation step by ion-exchange [57].

4.2. Capillary electrophoresis

Uric acid determination by CZE is straightforward as uric acid is negatively charged above about pH 3.9. Atamna et al. used both CZE and MEKC for separation of uric acid and xanthine derivatives [54]. The CZE separation was sufficient for determination of uric acid and some xanthines using a sodium phosphate buffer (pH 7.0). However MEKC was required to resolve the methylated xanthines [54]. Grune et al. used CZE with UV detection to separate purine bases and nucleosides, including uric acid in human serum (Fig. 4) [53]. They chose to use a sample preparation step with perchloric acid prior to injection, and a borate buffer system at pH 9.4.

Xu et al. also employed CZE, however with amperometric detection for uric acid determination in urine and plasma [58]. A tricine buffer was used for separation in a 25 μm I.D. fused-silica capillary. Detection was accomplished with a carbon fiber bundle working electrode held at a potential of 0.80 V (vs. SCE) to oxidize the uric acid. For identification of sample constituents, they attempted stopped flow-linear sweep voltammetry (SF-LSV) by sweeping at a rate of 50 mV/s from 0.0 to 1.0 V (vs. SCE). They have also employed CZE with amperometric detection at a 200 μm copper disk electrode held at 0.65 V (vs. SCE) for determination of purine bases, ribonucleosides and ribonucleotides (Fig. 5) [59]. Quantification of uric acid and xanthine were linear over ranges of 1–1000 μmol/l and 0.5–500 μmol/l, respectively.

5. Xanthines

Xanthines are the intermediates of the metabolism of adenine and guanine to uric acid and, are therefore important analytes for diagnosis of certain types of metabolic disease. In addition to xanthine and hypoxanthine, notable members of the xanthine class include caffeine, theophylline and theobromine. Like uric acid and creatinine, xanthine and hypoxanthine are often simultaneously determined with other compounds chromatographically [42,52] or electrophoretically [47,48,50,53,54]. However there have also been several publications of methods designed specifically for the determination of xanthines. Harkness has reviewed some of the pre-1988 analyses for xanthines [10]; we will focus on some of the more recent developments.

5.1. Chromatography

Sumi et al. used a column-switching technique for direct injection of filtered urine for determination of hypoxanthine and xanthine [60]. The diluted urine

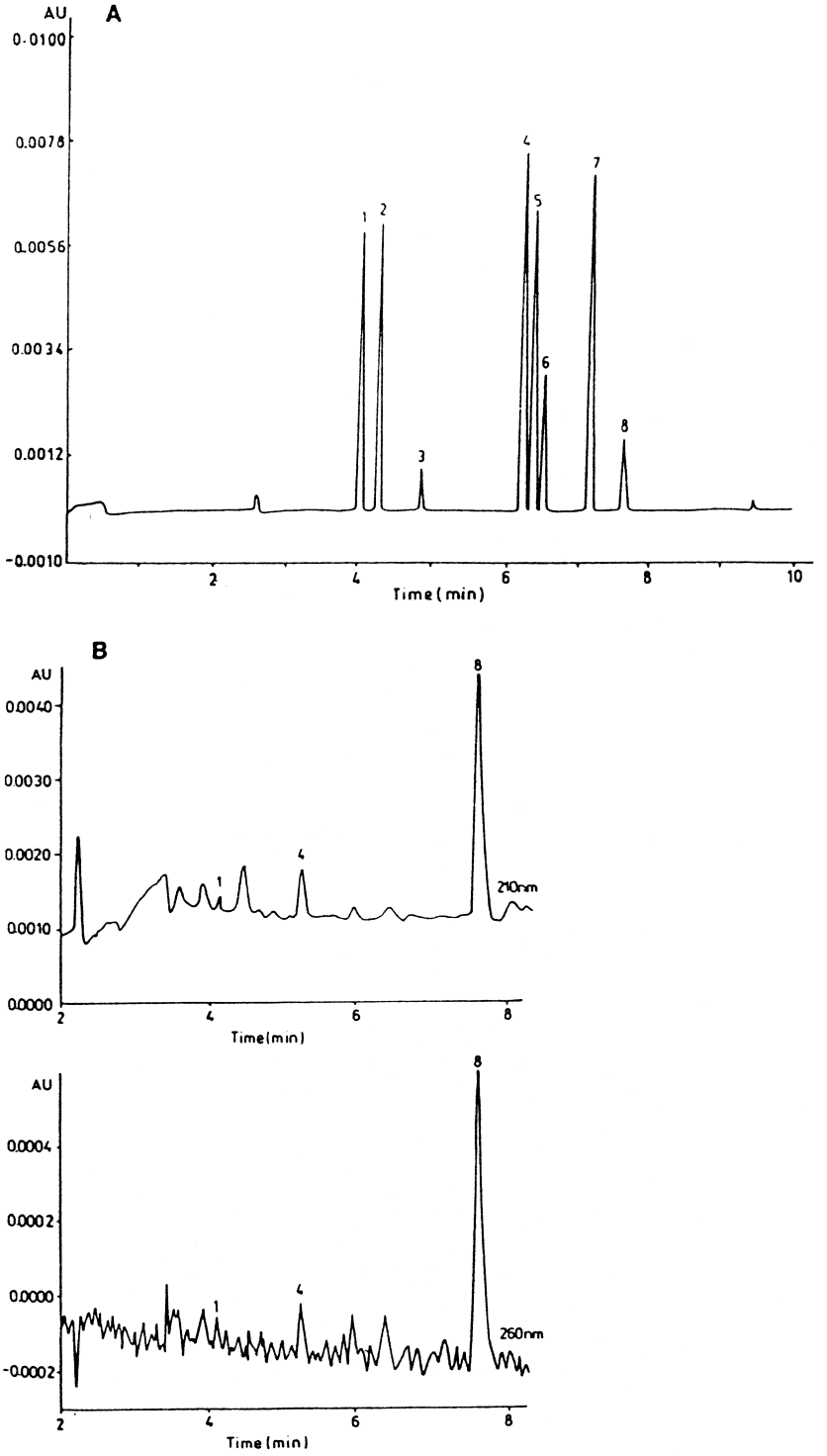

Fig. 4. Electropherograms of (A) a standard solution of purines and (B) a serum extract. Separation conditions: uncoated silica capillary, 44 cm (37 cm injector to detector)×75 μm I.D.; 20 m*M* borate, pH 9.4; 20 kV; 9 s hydrodynamic load; UV detection at 260 nm for (A) and the lower (B) chromatogram, 210 nm for the upper (B) chromatogram. Peaks: 1=adenine, 2=adenosine, 3=guanine, 4=hypoxanthine, 5=guanosine, 6=xanthine, 7=inosine, 8=uric acid. Reproduced with permission from Ref. [53].

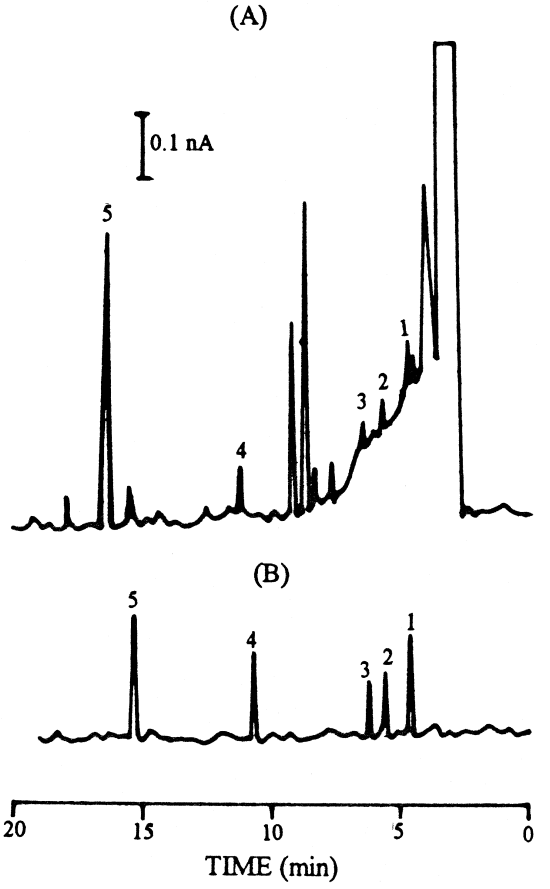

Fig. 5. Electropherograms of (A) human plasma sample and (B) purine standards. Separation conditions: uncoated silica capillary, 38 cm×25 μm I.D.; 40 m*M* NaOH buffer; 12.5 kV separation potential; 3 s electrokinetic injection. Electrochemical detection with a 200 μm copper disk electrode held at a potential of 0.65 V (vs. SCE). Peaks: 1=guanosine, 2=adenine, 3=5′-AMP, 4=5′-GMP, 5=uric acid. Reproduced with permission from Ref. [59].

was first injected onto a RP column. When the xanthine fraction eluted, it was then passed isocratically through a anion-exchange column for detection at wavelengths of 245 and 265 nm. Total analysis time was 30 min, and the automated system allowed for 60 samples to be analyzed consecutively. Papadoyannis et al. developed a simpler and shorter analysis procedure but this method required extensive solid-phase extraction and deproteinization steps prior to the chromatographic analysis [61]. Nonetheless, the separation of nine xanthines, including xanthine, caffeine, theobromine and theophylline was carried out with in 20 min using a gradient RP elution with ammonium acetate–methanol mobile phase.

5.2. Capillary electrophoresis

One of the advantages of CE is the relative short analysis time when compared with chromatography. In one example of this, Bory et al. compared the analyses of xanthine and hypoxanthine by CE [62] and LC [63] and found the quantitative results similar but analysis time was three times shorter with CE. Another rapid analysis was reported by Shihabi et al.; it involved the determination of xanthine in biological fluid and required less than 10 min for the separation (Fig. 6) [64]. In this work, acetonitrile was used for deproteinization; this lead to a stacking effect [15], which increased the sensitivity.

6. Closing comments

Chromatographic methods find numerous applications in the clinical setting and are often employed in conjunction with enzymatic and spectrochemical methods. The ability to address specificity for the analyte by a physical separation of the analyte from potential interfents makes separation-based assays attractive, and fuels the activity in this area. The major drawbacks with LC are the relatively long analysis times, and large consumption of solvents, as well as an inability to effectively maintain the quality of the column in some cases. With CE, analysis times are sometimes reduced by factors of 2 to 4 relative to LC and resolution is often increased; however, detection limits with CE are often inferior (when using UV absorbance as the detector), and reproducibility often becomes an issue as well. Sample stacking techniques [15] and Z-shaped detection cells [65,66] have begun to address the former, and time consuming sample preparation and/or column flushing must be employed to minimize the latter.

Finally, it should be noted that as devices aimed at the lab-on-a-chip concept become more widespread, one might expect more separation-based assays to become more prevalent, as they allow separations to be performed in seconds as opposed to minutes. The limiting factor of progress in this area is detection.

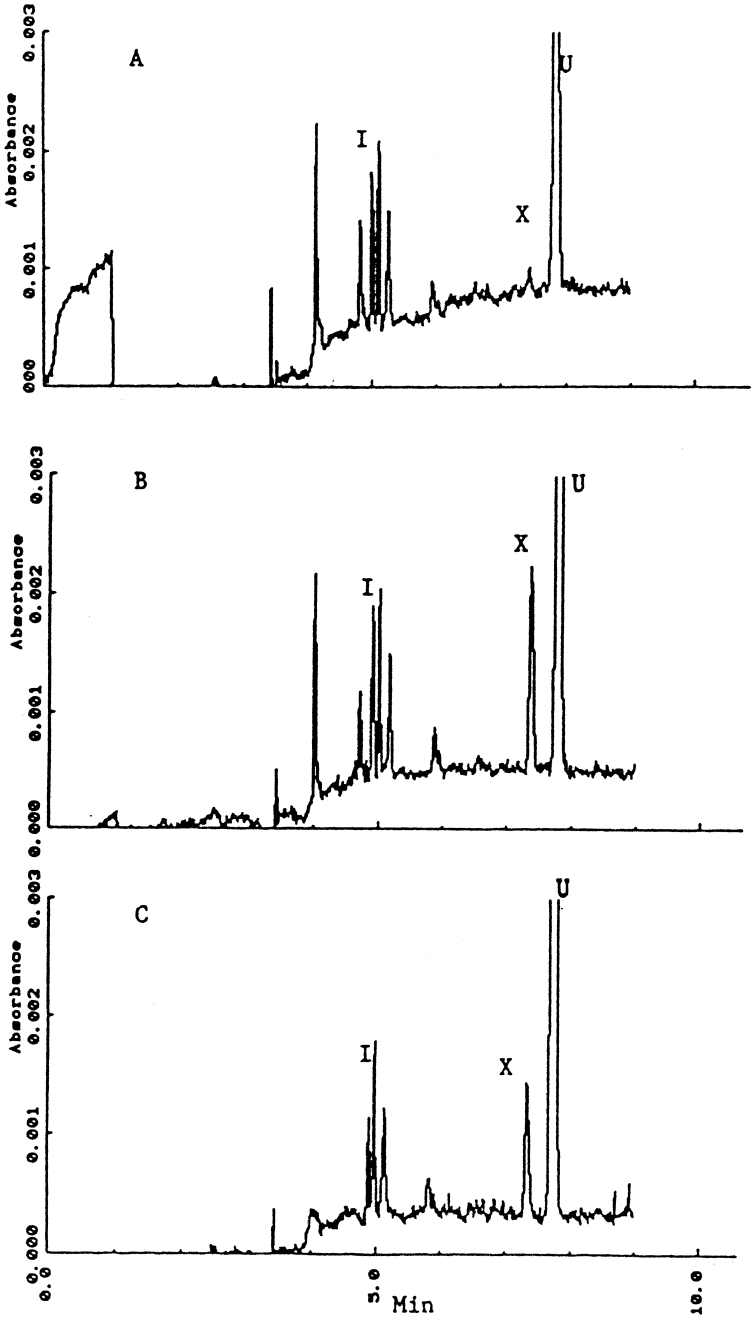

Fig. 6. Electropherograms of xanthine in serum: (A) patient with normal level (0.45 mg/l), (B) same patient spiked with 4.5 mg/l xanthine, and (C) patient with elevated xanthine (3.1 mg/l). Separation conditions: 500 mm×50 μm I.D. capillary; boric acid (7 g/l)–sodium carbonate (7 g/l) buffer, pH 9.2; UV detection at 280 nm. Peaks: X=xanthine, U=uric acid, I=internal standard (iothalamic acid, 100 mg/l). Reproduced with permission from Ref. [64].

Laser excited fluorescence has been applied successfully, but the extremely small channels makes the use of absorbance much more difficult. Liang et al. have attempted to overcome this problem with a ''U'' cell [67]. With more advances in this technology, reproducible analyses on a chip will likely be adapted for clinical usage.

Acknowledgements

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