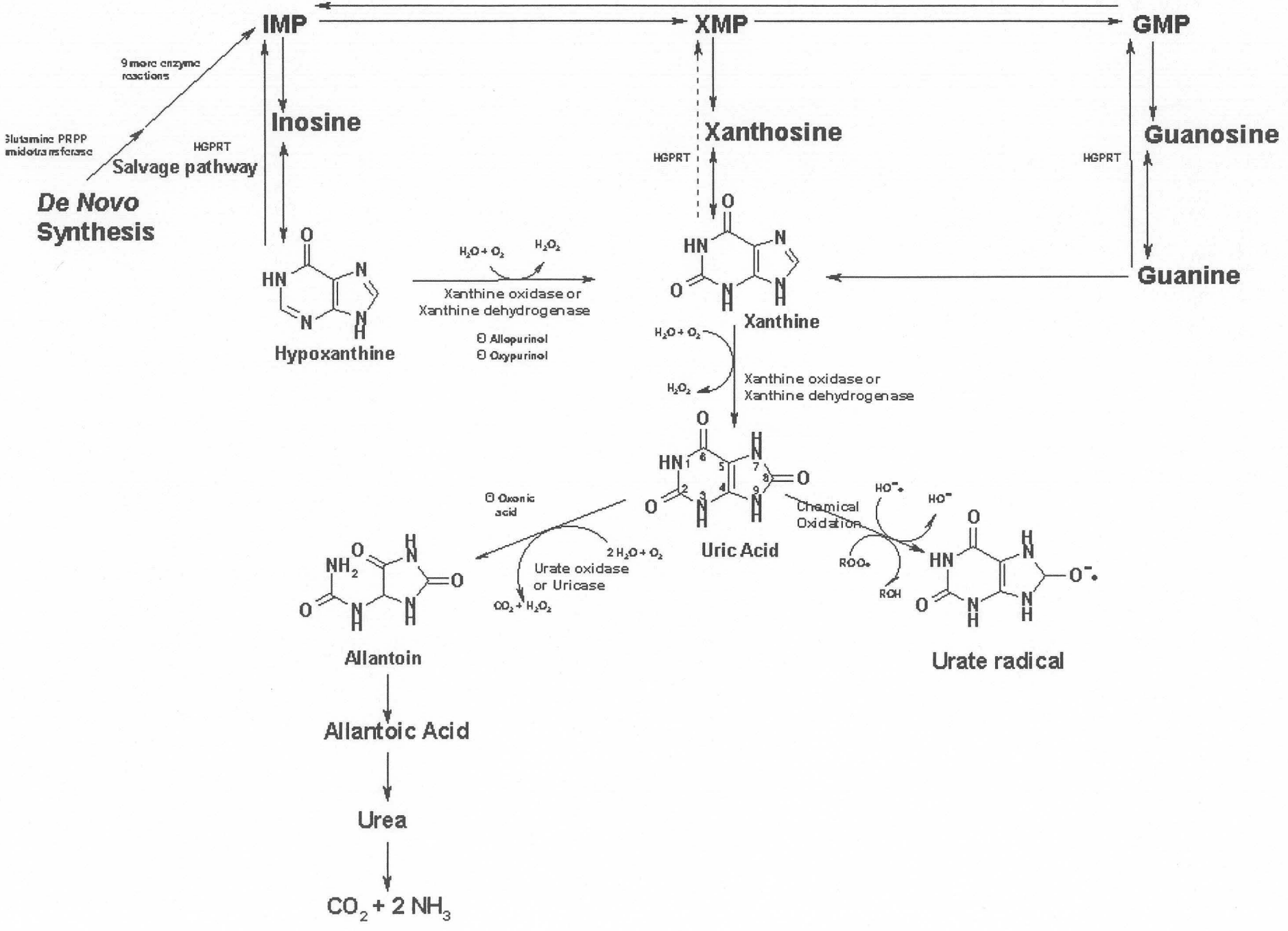
Chapter 4

In vivo analysis of Xanthine Oxidase substrates and products with CE

1 Introduction

Uric acid is derived solely from adenine and guanine–based purine compounds. Purines are essential compounds in animal cells and continuous metabolic turnover of these compounds occurs. In humans and higher primates, enzymatic catabolism of purines proceeds only as far as uric acid. Most mammals however excrete allantoin or urea as the major nitrogen containing enzymatic purine degradation products.

The concentration of uric acid levels in extracellular fluids is determined by: (1) normal metabolic turnover and obligatory nitrogen loss, (2) purine intake and (3) efficiency of excretion of uric acid. There are three important enzymes in the biochemical pathway of uric acid production: (1) pyrophosphate-ribose-P-amidotransferase (PPRP amidotransferase), (2) hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and (3) xanthine oxidase (Fig. 4.1). PPRP amidotransferase catalyses the first reaction in the purine biosynthetic pathway and is the primary rate-limiting step in the pathway. It is stimulated by increased levels of PPRP and inhibited by purine ribonucleotides. HGPRT is the enzyme that catalyses the reutilization of hypoxanthine ("salvage pathway") to inosine, a product that exerts negative feedback inhibition on PPRP amidotransferase. Xanthine oxidase is the third enzyme involved in the regulation of the uric acid levels. Physiologically the enzyme uses NAD^+ as electron acceptor and is known as xanthine dehydrogenase (XDH), but under stress conditions it is converted to xanthine oxidase (XO). XDH occurs mainly in the heart muscle and intestines while XO occurs mainly in the liver. The conver-


IMP
XMP
GMP
9 more enzyme reactions
Glutamine PRPP amidotransferase
De Novo Synthesis
Salvage pathway
HGPRT
Inosine
Xanthosine
Guanosine
Guanine
Hypoxanthine
Xanthine
Xanthine oxidase or Xanthine dehydrogenase
Allopurinol
Oxypurinol
Uric Acid
Oxonic acid
Urate oxidase or Uricase
Allantoin
Allantoic Acid
Urea
CO2 + 2 NH3
Chemical Oxidation
Urate radical

sion process was investigated by (Corte, 1972) and it was concluded that two factors were of essence. Proteolysis and changes in the enzyme cysteine thiol groups converted the enzyme from a NAD^+ reducing dehydrogenase to an oxygen reducing oxidase. The thiol groups are in close proximity to the FAD and may be essential for coupling with the NAD^+ or binding of NAD^+. Oxidation of the thiol groups may result in the loss of the binding site or a weakening in the coupling. This could be caused by increased steric hindrances that result from conformational changes in the protein structure. Proteolysis alters the protein conformation, moving the thiol away from the vicinity of the FAD. Isolated XO treated with dithioerythritol is restored to XDH. XO pre-treated with proteolytic enzymes before reduction with dithioerythritol did not regain the XDH activity. Only XO and XDH can produce uric acid.

In clinical studies, hyperuricemia is defined as uric acid levels of 2 S.D. above the population mean (40-50 µg/ml) (Beck, 1981). In pathophysiological terms hyperuricemia occurs when the uric acid concentration reaches saturation levels in the serum (60-70 µg/ml). There are three main causes of hyperuricemia (Wyngaarden, 1974). 1) A partial deficiency in the enzyme HGPRT results in insufficient salvaging of hypoxanthine so that more hypoxanthine is converted to uric acid. 2) An increase in the activity of the enzyme PPRP-synthetase leading to increased purine biosynthesis. 3) The renal tubulars are defective in handling uric acid, resulting in increased reabsorption or decreased excretion of uric acid. Potent diuretics and drugs such as salicylates and antituberculous drugs may also cause hyperuricemia.

Hyperuricemia can be treated with uricosuric agents or with xanthine oxidase inhibitors. Uricosuric agents increase the excretion of uric acid lowering the serum

urate levels. Allopurinol is the drug of first choice for treatment of gout. Allopurinol and its major metabolite oxypurinol (Fig. 4.2A) are potent inhibitors of xanthine oxidase, thereby decreasing the uric acid production from hypoxanthine and xanthine. The accumulated hypoxanthine is "salvaged" by HGPRT forming inosine mono-phosphate (IMP). IMP is a potent inhibitor of PPRP amidotransferase and causes the entire *de novo* purine synthesis pathway to slow down.

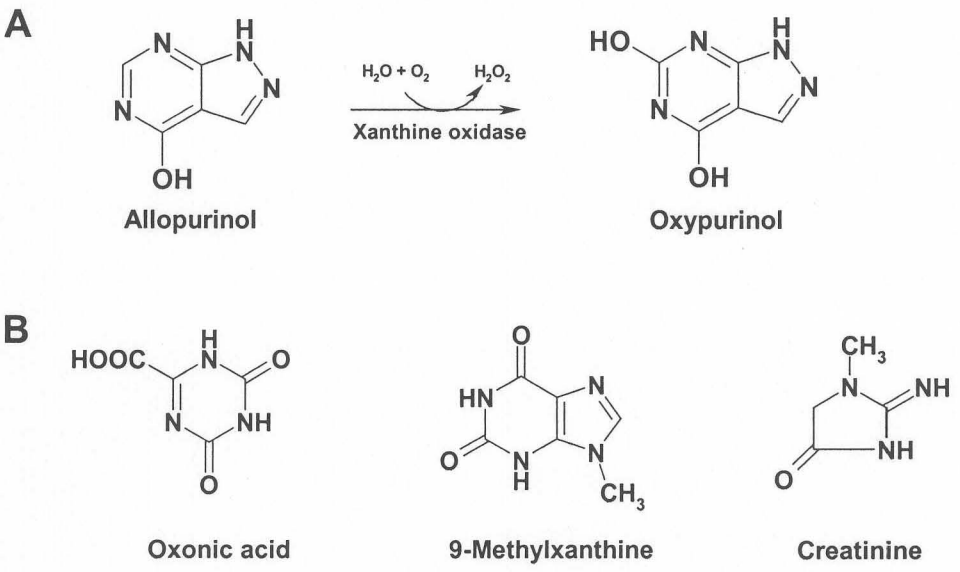


Figure 4.2 A: Xanthine oxidase catalyzes the conversion of allopurinol to oxypurinol. Both compounds serve as inhibitors of XO. B: The chemical structures of other compounds of interest in the analysis of rat serum.

The levels of serum uric acid in most mammals are 10% of that found in humans. The distinction arises from the active uricase (urate oxidase) enzyme responsible for trans-forming uric acid to allantoin. The gene required for producing this enzyme is apparently still present in the human genome but is no longer expressed (Lee, 1988), (Motojima, 1988), (Yeldandi, 1992). There is no certainty whether uric acid offers humans any physiological advantage or if evolutionary processes merely designated uric acid as the waste product of purine catabolism in higher primates. Serum uric

acid is freely filtered at the glomerules but undergoes extensive reabsorption (up to 98%). Uric acid secretion in the kidneys takes place through an anionic transport system and also the secreted uric acid undergoes extensive reabsorption (Diamond, 1973).

It would seem as if the body goes to great lengths to reabsorb this "waste product" which is notoriously known for causing gout and nephrolithiasis. Once hypoxanthine is converted to xanthine and uric acid it cannot be salvaged for reuse as purines. Purines have to be synthesized *de novo* at great energy expenditure to maintain the purine levels. Despite this huge energy wastage, all the purines taken up in the diet are converted to uric acid in the gastrointestinal tract by the mucosal XDH. Only uric acid appears in the circulatory system after ingestion of purines.

Uric acid has the ability to undergo non-enzymatic oxidation. One-electron oxidation by strong oxidants such as hydroxyl or peroxyl radicals produces an urate radical anion in neutral solution (Simic, 1989) (Fig. 4.1). An interspecies comparison of primates yields a positive correlation between plasma uric acid concentrations and longevity, with man coming out on the forefront (Cutler, 1984). Because cellular aging in general, and life-limiting diseases such as cancer and cardiovascular disorders may well be caused in part by oxidants and radicals, the positive relationship has been ascribed to the antioxidant capacity of uric acid. It has been estimated that uric acid comprises 30-65% of the peroxyl-radical (Wayner, 1987) and 10-15% of the hydroxyl scavenging capacity of blood plasma (Thomas, 1992). The concentration of uric acid is generally high in comparison to other non-enzymatic antioxidants such as ascorbate (6 µg/ml), tocopherols (9-19 µg/ml), methionine (3-6

µg/ml) (Cole, 1998), (van Guldner, 1999) and glutathione (7-10 µg/ml) (Orter, 1982), (Halliwell, 1989). Uric acid has the capacity to interrupt radical chain reactions and prevent the formation of peroxides. It also plays a role in the attenuation of lipid auto-oxidation in plasma. Uric acid has access to all extracellular fluid compartments and is not compartmentalized as is the case with the enzymatic scavengers superoxide dismutase, catalase and glutathione peroxidase (Halliwell, 1989).

Ironically the production of one mole uric acid by XO from xanthine results in the production of two moles of peroxide or superoxide radicals. The production of uric acid from hypoxanthine produces double the amount of oxygen radicals when compared to xanthine. This contributes to the serum radical contents that may result in the onset of carcinogenesis, arteriosclerosis and other diseases linked to radicals.

The *in vivo* inhibition of XO activity is discussed in Chapter 5. For now suffice it to say that the inhibition of XO *in vivo* results in a decrease in uric acid levels and an increase in xanthine levels in both serum and urine samples. Inhibition of XO and therefore uric acid production with polyphenols may not compromise the total anti-oxidant status of the serum. From a clinical point of view there are many reasons for the determination of uric acid and its precursors as can be deduced from the occur-rence of metabolic disorders and the possible physiological effects.

The aim was to identify and implement an appropriate method to analyze the purines, allopurinol, oxypurinol and allantoin with the internal standard 9-methylxanthine. Several CZE and MEKC methods existed for similar applications. A CZE method was developed by (Shihabi, 1995) to analyze hypoxanthine, xanthine, uric acid, allo-

purinol and oxypurinol in serum samples. This method required that acetonitrile be added to the serum sample for deprotonation and for on-column stacking purposes. A CZE method developed by (Xu, 1997) used amperometric detection for high sensitivity to quantify uric acid in human urine and serum. A MEKC method was developed by (Atamna, 1991) to separate several different methylxanthines and methyluric acid derivatives. Another MEKC method was developed by (Thormann, 1992) to analyze methyl-substituted xanthines in body fluids. The samples were either injected directly onto the column without any pre-treatment taking place, or solid-phase or liquid-liquid extraction was done. Direct injection made this method an attractive prospect, since time would be saved if no sample pre-treatment was required.

2 Materials and Methods

2.1 Experimental strategy

It was decided that the CZE method of (Shihabi, 1995) and the MEKC method of (Thormann, 1992) will be compared with each other using standards. The best method will then be used to analyze rat serum and urine samples to determine its applicability. The method of Shihabi was selected because it already separates the required metabolites in serum. The method of Thormann was selected because direct injection would be less laborious. Both methods were set up and the LOD's and column efficiencies obtained with each method were compared.

2.2 Instrumentation

Electrophoresis was carried out using the HP 3^{D}CE instrument (Hewlett Packard, Waldbronn, Germany) with on-column detection. For the MEKC method a 50 μm ID

uncoated fused silica capillary column with an extended optical window from HP was used. The total length was 64.5 cm and the effective separation length was 56 cm. The detection window was a bubble window with a pathlength of 150 μm. All samples were injected pneumatically (50 mbar) for 2 sec. The operating temperature was 40°C. For the CZE method an uncoated fused silica capillary column from HP with an internal diameter of 50 μm and total length of 64.5 cm was used. The effective separation length was 56 cm and the detection window had a pathlength of 50 μm. All samples were injected pneumatically (50 mbar) for 20 sec. The operating temperature was 35°C. Detection for both methods was effected by measurement of UV absorbency at 200 and 280 nm. Analyte identification was done based on migration times and UV spectrum analysis.

2.3 Reagents

The creatinine, allopurinol, allantoin, oxypurinol, xanthine, hypoxanthine and uric acid were obtained from Sigma Chemical Company (St Louis, MO, USA). The internal standard 9-methyl-xanthine was obtained from ICN Pharmaceutical (CA). Sodium dodecyl sulfate (SDS) was obtained from E. Merck (Darmstadt, Germany). The SDS was of analytical grade suitable for electrophoresis. All buffer salts were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore Corp., Bedford, MA. USA).

2.4 Analytical conditions

Standards and samples were analyzed with the anode on the inlet side and a running buffer of 75 mM SDS, 6 mM disodium tetraborate and 10 mM disodium phosphate

for the MEKC method. The buffer pH was adjusted to 9.5 by 0.1M NaOH. A few serum and urine samples were analyzed with an applied voltage of 30 kV. For the CZE method a running buffer with 0.7% (w/v) boric acid and 0.7% (w/v) sodium carbonate was used. The pH was adjusted to 9.5 with 0.1N NaOH. Samples were analyzed with an applied voltage of 27 kV.

At the beginning of each day the capillary was regenerated by rinsing for 5 min with H_2O, 10 min with 0.1M HCl, 5 min with H_2O, 5 min with 1.0M NaOH, 5 min with H_2O, 10 min with 0.1M NaOH and finally 2 min with H_2O. Before each analysis the capillary was rinsed with running buffer for 2 min. After each analysis the capillary was rinsed for 30 sec with H_2O, 2 min with 0.1 M NaOH and 2 min H_2O.

2.5 Preparation of samples and standard

MEKC:

Serum samples were filtered with 0.2 µm syringe filters before they were injected into the capillary (Thormann, 1992). All standards were prepared by dissolving individually in 0.01 N NaOH. Creatinine and allantoin were prepared at 2.0 mg/ml, hypoxanthine at 1.5 mg/ml, uric acid at 0.6 mg/ml, allopurinol at 0.35 mg/ml and oxypurinol and xanthine at 0.1 mg/ml.

CZE:

The serum sample (50 µl) was first spiked with 3 µl of 1 mg/ml 9-methylxanthine before it was deproteinated with 97 µl cold (-10°C) acetonitrile. The final concentration of 9-methylxanthine was 20 µg/ml. All standards were prepared by dissolving

individually in 0.01 N NaOH. Allantoin was prepared at 2.0 mg/ml, hypoxanthine, uric acid, allopurinol, oxypurinol and xanthine at 1.0 mg/ml and 9-methylxanthine at 0.88 mg/ml.

2.6 Preliminary inhibition experiments.

Allopurinol is the classical inhibitor of xanthine oxidase and potassium oxonate is a known inhibitor of the uricase enzyme (Fig. 4.2A). Preliminary studies were done to determine whether changes in the levels of the xanthine and uric acid could be detected in rat urine and serum upon inhibition of these enzymes. This is important to evaluate the applicability of the analytical method. This was only done with the CZE since it was found to be the better of the two methods.

Blood and urine were collected from rats at t=0h before any treatment. The blood was obtained by making an incision in the tail and collecting from the abdominal tail vein. Three rats were then injected 250 mg/kg potassium oxonate subcutaneously and three were orally fed 20 mg/kg allopurinol. Blood and urine were again collected two hours later at t=2h. Urine samples were marked and immediately snap-freezed in liquid nitrogen. Blood samples were centrifuged at 11000g for five minutes and the serum collected. The serum was then snap-freezed. All samples were stored at –20°C for later analysis.

3 Results and Discussion

All the compounds of interest are shown in Fig. 4.1, 4.2A and 4.2B. The level of creatinine is an indication of the metabolic size of individual animals and was therefore included as an analyte that could potentially be detected. In the end however it

was not possible to quantify this metabolite with capillary zone electrophoresis, since it had no charge in the working pH range.

3.1 Comparison of the two methods

In both methods the migration sequence of the analytes were the same (Fig. 4.3A & 4.3B). This is an indication that the surfactant did not add selectivity to the analysis in the MEKC method. The method was originally intended for methylxanthines where the surfactant might have exploited the hydrophobicity changes better than with hydroxypurines. The MEKC method still provided good resolution of all the metabolites of interest. The sequence of elution can easily be explained when the migration times (from the CZE method) are compared to the mass to charge ratios of the analytes at pH 9.5 (Table 4.1, Fig. 4.4). The ionic charges on the analytes were calculated according to Equation 1 with the pKa values shown in Table 4.1. When a pKa value is two or more units below the pH value, the specific group is seen as fully dissociated and will have a charge of –1 (providing dissociation takes place as for a weak acid). The apparent electrophoretic mobility (μ_{app}) is the vectorial sum of the electrophoretic mobility (μ_{ep}) and the electro-osmotic mobility (μ_{eo}) (Equation 2).

$$\alpha = \frac{1}{10^{(pKa-pH)}+1} \qquad (1)$$

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad (2)$$

The mobility is calculated with Equation 3.

$$\mu = \frac{L_d / t_M}{V / L_t} \qquad (3)$$

Where L_d is the column length to the detector, L_t is the total length of the column and

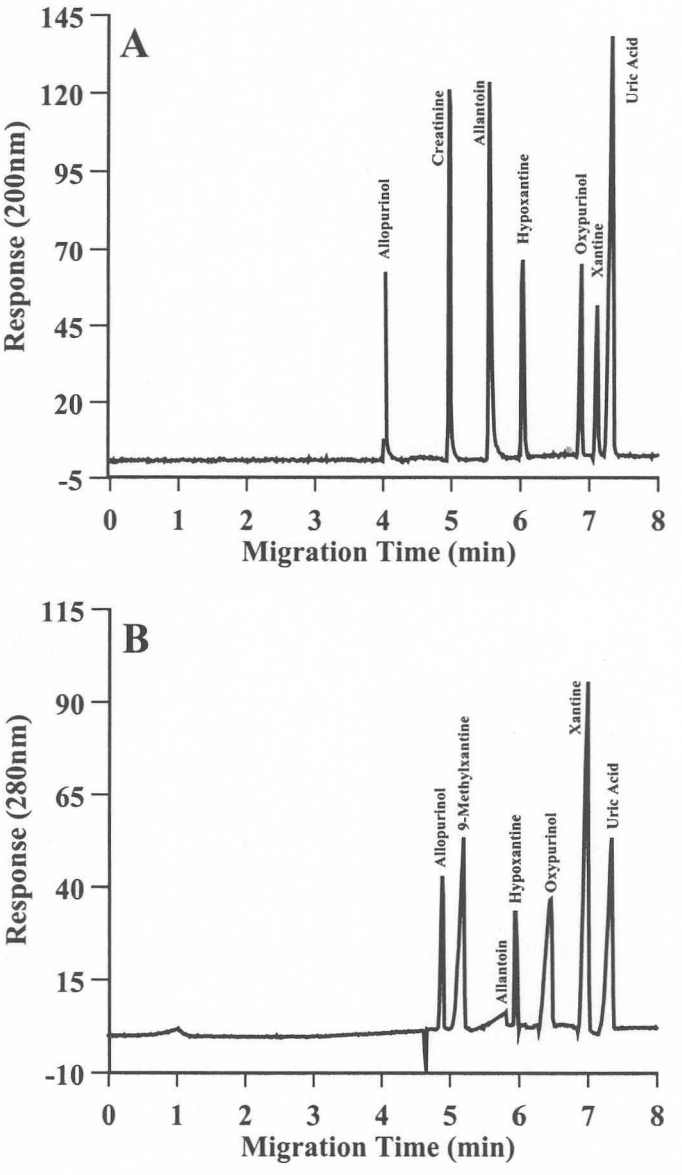


Figure 4.3 The separation of XO substrates, products and inhibitors. A: The MEKC method Conditions: 75 mM SDS, 6 mM $Na_2B_2O_7$, 10 mM Na_2HPO_4 pH 9.5. Separation with 30 kV. B: The CZE method Conditions: 0.7% (w/v) H_3BO_4, 0.7% (w/v) $NaHCO_3$ pH 9.5. Separation with 27 kV.

V the applied voltage. When calculating μ_{app}, the t_M equals the migration time of the analyte and for μ_{eo}, the t_M is equal to t_0 which is the migration time of the EOF. A larger negative charge on the analyte will result in a larger negative electrophoretic mobility and a smaller apparent electrophoretic mobility. The smaller the apparent electrophoretic mobility the longer the analyte needs to pass the detection window resulting in a longer migration time.

Table 4.1 The calculated charges and mass:charge ratios from pKa values as well as the calculated electrophoretic mobilities for the analytes using the CZE method.

Analyte	pKa	Charge at pH 9.5	Mass g/mol	Charge/Mass Ratio	Mobility $m^2.V^{-1}.s^{-1}$
Allopurinol	10.20	-0.166	136.11	-0.00122	-0.00966
9-MX	6.30	-1.000	165.14	-0.00606	-0.02388
Allantoin	8.96	-0.776	157.12	-0.00494	-0.04054
Hypoxanthine	1.98, 8.94, 12.10	-0.784	135.11	-0.00580	-0.04414
Oxypurinol	7.74	-0.983	151.11	-0.00651	-0.05651
Xanthine	0.80, 7.44, 11.12	-1.017	151.11	-0.00673	-0.06009
Uric acid	5.40, 10.30	-1.137	167.11	-0.00680	-0.06773
Creatinine	3.55	0	0	0	0

With the MEKC method creatinine could be separated in addition to all the other compounds for it separated solely on hydrophobic interactions at pH 9.5 where it is neutral. Unfortunately the LOD's were very poor and the MEKC method could not be used. Even with solid phase extraction the detection limits remained poor. Only uric acid could be detected in urine and the baseline level in serum was too low to be detected.

With the CZE method the LOD was improved with a factor of between two and ten. The enhanced sensitivity was achieved by means of stacking. With stacking the

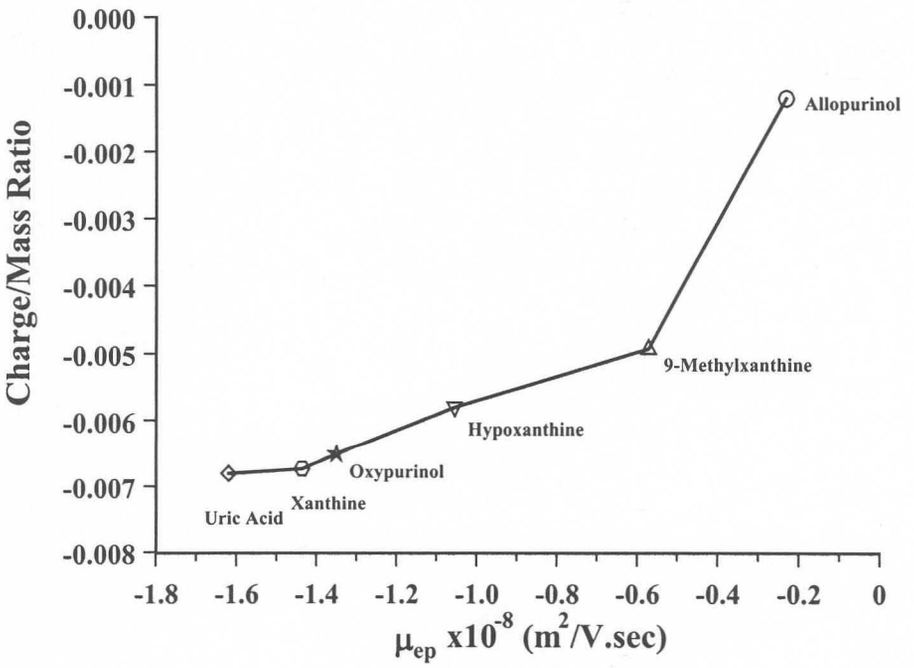


Figure 4.4 The linear relationship between the mobilities and charge/mass ratios of the different analytes in CZE mode.

sample solution has a lower conductivity than the running buffer. This is achieved by preparing the sample in water or adding a high concentration organic solvent. A long plug of sample is introduced pneumatically into the capillary and a separation voltage applied across the column. Because the solution in the sample plug has a low concentration of ions, the resistivity in the sample plug region will be higher than the resistivity in the rest of the column. Consequently, a high electric field is set up in this region. As a result, the ions will migrate rapidly under this high field toward the steady-state boundary between the lower concentration plug and the running buffer. Once the ions pass the concentration boundary between the sample plug and the rest of the column, they immediately experience a lower electric field and slow down, thus causing a narrow zone of analyte to be formed in the running buffer region (Burgi,

1991). For optimum stacking the conductivity difference between the sample solution and the running buffer must be between 10 (Vinther, 1991); (Burgi, 1991) and 100 fold (Mikkers 1979). The sample plug should not fill more than 10% of the column (Roder, 1995). These restrictions are necessary to limit the occurrence of laminar flow that causes peak broadening. The laminar low is generated due to mismatch EOF between the sample solution and running buffer. The EOF pressure difference is alleviated by laminar flow that occurs as the sample plug pushes the running buffer forwards (Chien, 1992). The field-amplified sample injection (FASI) method could increase the loading efficiency even further but the loading is performed electrokinetically resulting in bias loading, for analytes with a large negative charges are loaded preferentially due to higher mobilities.

High concentrations of organic solvent also precipitate the serum proteins resulting in sample clean-up. In a study conducted, the protein precipitation efficacy of acetonitrile, acetone, ethanol and methanol was determined (Blanchard, 1981). It was found that when two volumes of acetonitrile at room temperature were added to one volume serum 99.7% of the proteins precipitated out. For the other organic solvents the efficacy ratings were acetone (99.4%), ethanol (98.3%) and methanol (98.7%). In this method two volumes acetonitrile were added to one volume sample resulting the precipitation of serum proteins and a reduction in the conductivity of the sample solvent. It had an added advantage in that it had the highest boiling point of the four organic solvents, resulting in less evaporation during automation and more reliable results. The creatinine co-eluted with the EOF and could not be analyzed with this method. The efficiency of the stacking that occurred is underlined by the column efficiency that was obtained. The initial peak width (upon injection) for the CZE method

was ten times more than for the MEKC method, yet the column efficiency obtained with CZE compared well with that of the MEKC method (Table 4.2).

Table 4.2 Comparison of the MEKC and CZE methods, focusing on the column efficiency, peak symmetries and limits of detection for the respective methods.

Analyte	MEKC			CZE		
	Plate-number	Peak symmetry	LOD (200nm) µg/ml	Plate-number	Peak symmetry	LOD (280 nm) µg/ml
Creatinine	263508	0.656	100.0			-
Allopurinol	216132	0.786	17.5	132184	3.979	5
9-Methyxanthine				81070	7.784	<5
Hypoxanthine	121887	0.232	75.0	153906	0.886	5
Allantoin	165893	0.333	125.0	9228	10.034	8.33
Oxypurinol	215077	1.766	5.0	41478	2.865	2.5
Xanthine	162243	0.983	5	142823	2.514	0.5
Uric acid	92554	3.46	30	81031	6.628	2

The pH condition of 9.2 used by (Shihabi, 1995) did not resolve the xanthine and uric acid well in our system. The charge difference between xanthine (-1.01) and uric acid (-1.07) is a mere 0.06 at pH9.2 compared to a difference of 0.114 at pH 9.5. A pH optimum study was done to determine conditions that resolve the analytes completely. (Fig. 4.4) It was found that a pH of 9.5-9.6 resolved the peaks completely with a slight increase in the analysis time. We selected to work at pH 9.5 even though allopurinol and 9-methylxanthine migrated close together. Both the allopurinol and 9-methylxanthine peaks were very sharp, resulting in good resolution even when migrating near each other. The total analysis time was slightly shorter when working at pH 9.5 rather than pH 9.6. The MEKC method also required a pH of 9.5 to separate the peaks properly, instead of the pH 9.0 used by (Thormann, 1992). The pH curve indicates an

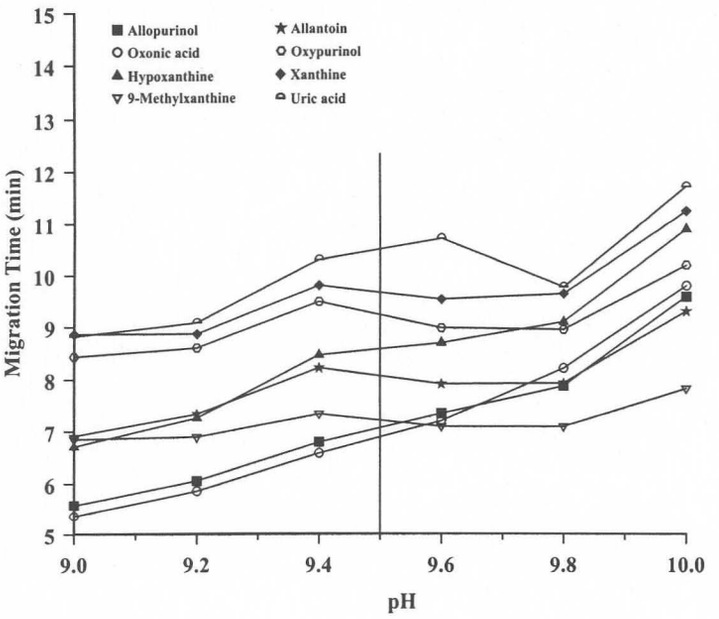


Figure 4.5 The influence of pH on the migration times of the analytes in CZE mode.

Table 4.3 A summary of the calibration curve data for the analytes with the CZE method.

Analyte	Linear range µg/ml	r	Linear equation for calibration curve	%RSD
Allopurinol	5-1000	0.999	y = 3.07e-3*x	2.40
Hypoxanthine	5-1000	0.999	y = 1.68e-3*x	1.29
Allantoin	8.33-2000	0.999	y = 1.10e-2*x	9.58
Xanthine	0.5-500	1.000	y= 9.62e-3*x	0.56
Uric Acid	2-1000	0.999	y = 8.30e-3*x	3.39
Oxypurinol	5-100	0.998	y = 5.91e-3*x	1.76
9-Methylanthine	5-500	0.996	y = 2.41e-3*x	6.58

increase in the anionic properties of the metabolites with an increase in the pH. As the analytes became more anionic their negative electrophoretic mobility increased and therefore the migration times became longer. The fact that the compounds separate solely on their charge to mass ratio's can be seen in the peak symmetry values that were obtained. Most of the analytes had either leading fronts or lagging tails which is

an indication of differences between the charge to mass ratio's of the analytes and that of the surrounding running buffer ions. Both with CZE and MEKC the peak symmetry was poor. Calibration curves were setup for the analytes in the CZE method and all correlation coefficients were better than 0.995 (Table 4.3)

3.2 Serum and Urine Samples

The suitability of the CZE was determined by its ability to detect changes in the levels of hypoxanthine, xanthine and uric acid in serum and urine samples of rats. Rats were treated with allopurinol or potassium oxonate. Upon treatment with allopurinol the levels of xanthine and oxypurinol increased in both urine (Fig. 4.6A&B) and serum (Fig. 4.7A&B) samples. There was a concurrent decreased in the levels of uric acid, as was expected. The levels of uric acid increased significantly in serum (Fig. 4.8A&B) and urine (Fig. 4.9A&B) samples taken from animals treated with potassium oxonate. No accumulation of hypoxanthine or xanthine occurred. The reverse reaction where xanthine is produced from uric acid is to slow to produce any xanthine. Although uric acid is capable of inhibiting XO, the K_i is very high (250 μM) and the serum uric acid levels are probably still to low to have a significant inhibitory effect on XO. In the urine samples a lowering in the allantoin level at 280 nm was difficult to detect. The LOD for allantoin at 280 nm is very poor. Detection at 230 or 200 nm was also very difficult due to noise and the presence of other peaks that are not well resolved from allantoin.

The baseline levels of hypoxanthine and xanthine were not always detectable in serum. Significant increases, as expected with the inhibition studies, would however be easily detectable. The compounds were identified mainly on their UV spectrums,

since the migration times varied by up to 2 minutes. The UV spectrums of 9-methylxanthine, uric acid, oxypurinol and xanthine are compared to that of the pure

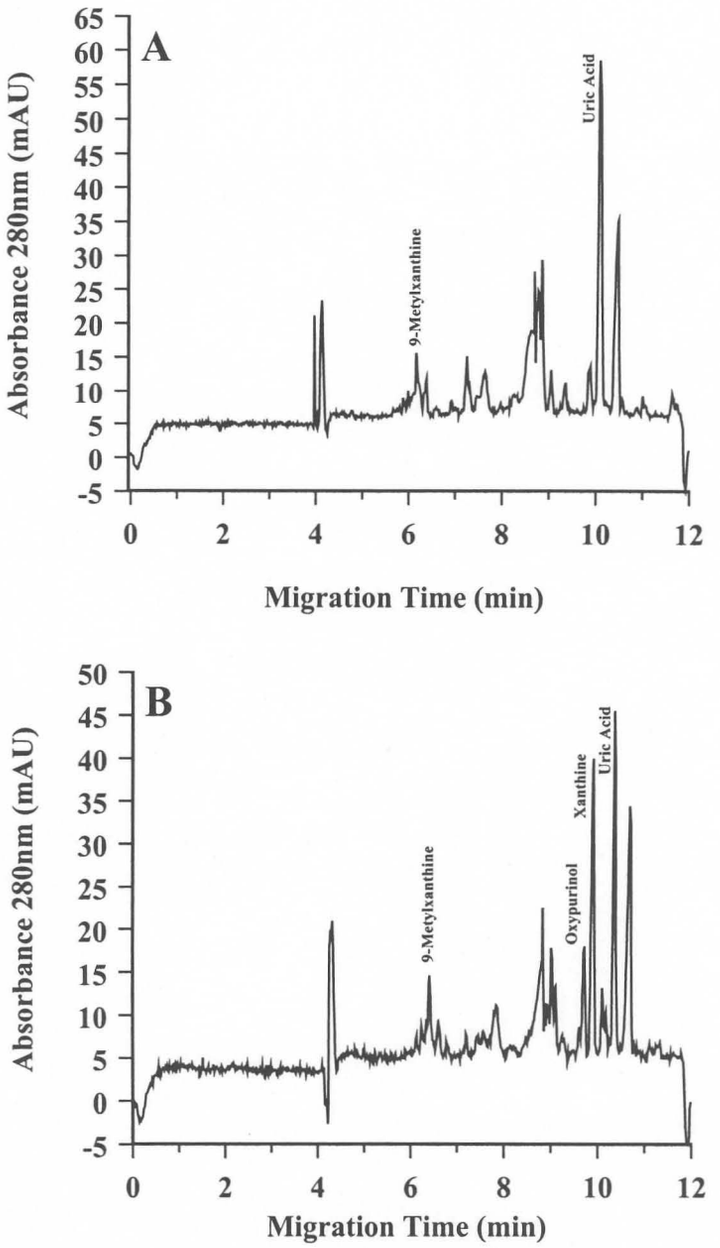


Figure 4.6 A: Analysis of control rat urine (untreated) separated with the CZE conditions as in Fig 4.3. **B**: Analysis of rat urine from rats orally dosed 20 mg/kg allopurinol with the same analytical conditions.

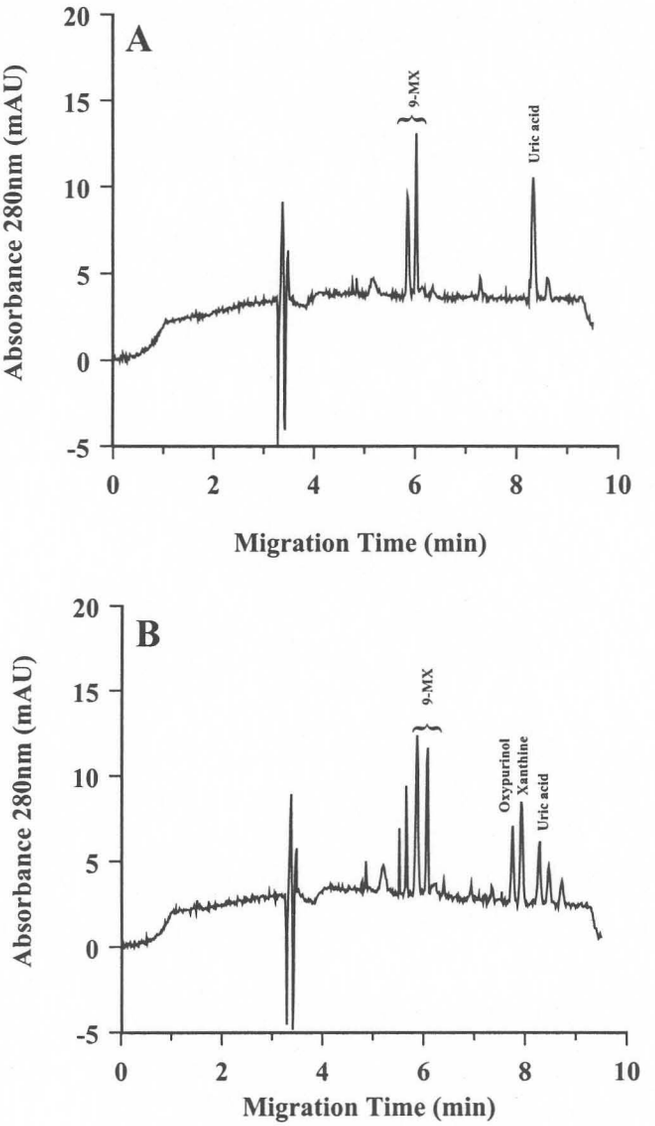


Figure 4.7 **A**: Analysis of control rat serum (untreated) with same conditions as in Fig. 4.6. **B**: Analysis of rat serum from rats orally dosed 20 mg/kg allopurinol with same analytical conditions.

standards (Fig. 4.10A-D). It was found that the buffer composition had a huge influence in the repeatability of separations. When repeated runs are done without buffer renewal in the anodic buffer vial changes can be observed in the relative migration order and migration times. Continuous analysis with large buffer vials

(5ml) showed less variance than the same analyses with small buffer vials (2ml) (Schmutz, 1994). In this study the buffer vials have a volume capacity of only 0.8 ml. This caused small inter-run variation due to buffer property changes of the anodic vial. A significant deterioration in electropherograms were found if more than six runs were done without replenishing the anodic vial.

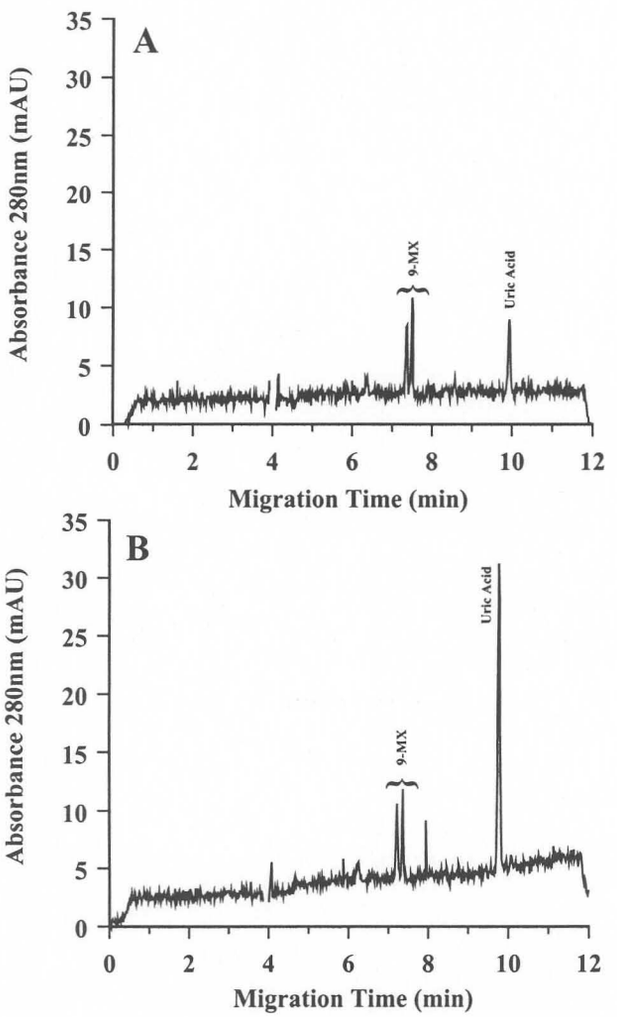


Figure 4.8 **A**: Analysis of control rat serum (untreated) with same conditions as in Fig 4.6. **B**: Analysis of rat serum from rats subcutaneously dosed 250 mg/kg potassium oxonate with same analytical conditions.

The sample matrix composition played a significant role on the migration time of the analytes. Analytes from urine or serum samples migrated slower than the standards of the same analyses. Analytes from urine samples migrate slower than the same analytes from serum samples. This variation might be the result of protein-analyte interactions or differences in the conductivites of the samples. Double peaks were

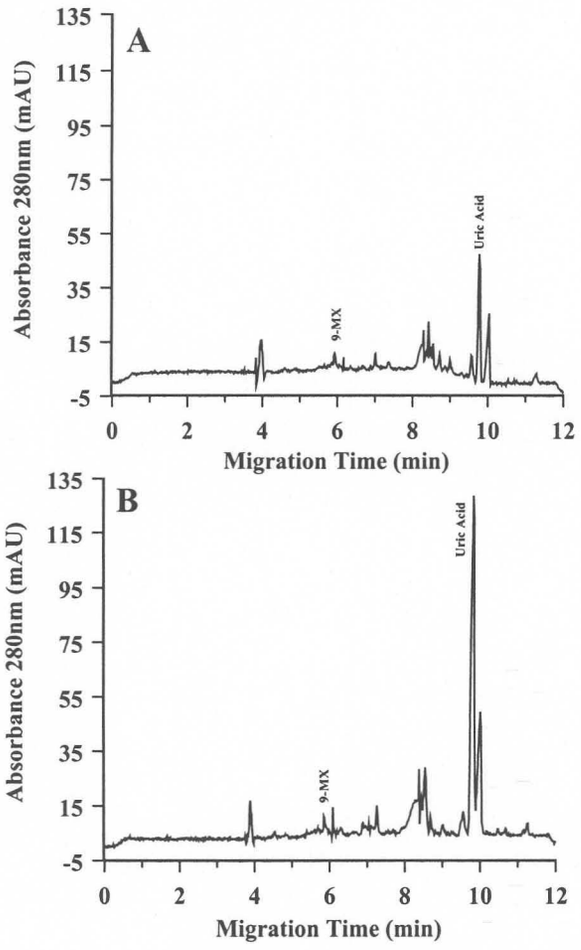


Figure 4.9 **A**: Analysis of control rat urine (untreated) separated with the CZE conditions as in Fig. 4.6. **B**: Analysis of rat urine from rats subcutaneously dosed 250 mg/kg potassium oxonate with the same analytical conditions.

observed for some of the analytes at low concentrations. The 9-methylxanthine displayed two well separated peaks in serum samples. Both peaks had the characteristic UV spectrum of 9-methylxanthine and co-integration of the two peaks added up to the amount that the samples were spiked with. This phenomena was previously reported by authors who tried direct serum injection with MEKC ((Schmutz, 1994)

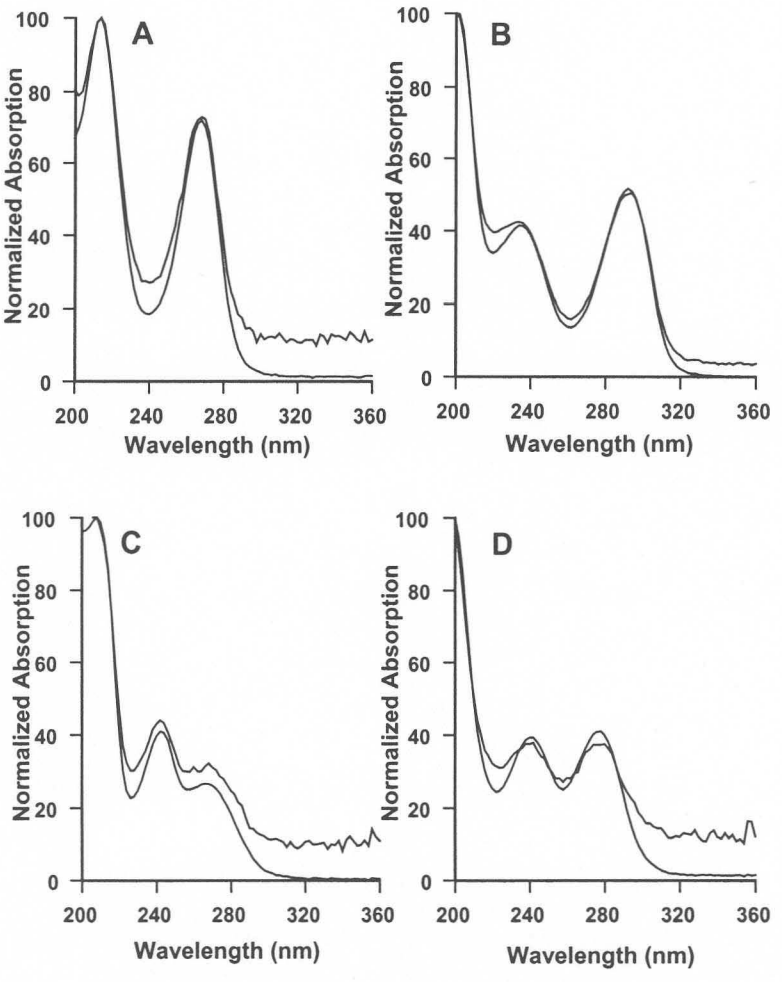


Figure 4.10 Comparison of UV spectra of analytes (**A**: 9-methylxanthine, **B**: uric acid, **C**: oxypurinol, and **D**: xanthine) from rat serum or urine with that of pure standards.

and with micellar liquid chromatography (Haginaka, 1987), (Palmisano, 1989). It was suggested that the analytes interacted with proteins and resulted in two closely related migration times. It may be that low amounts of analytes can interact with residual proteins still left in the sample, causing the double peaks.

Inconsistent migration times can also be due to the high ion concentration in the running buffer required for the stacking process. The positive sodium ions can cause variable screening of the negative silica hydroxyl charges on the column resulting in an erratic EOF. High Joule heating also occurred due to high voltage and current, causing variation in the buffer viscosity. Unfortunately it was impossible to change the buffer, for the high ion concentration required for sample stacking (and better LOD's) decreased the stability of the method due to the reasons mentioned above. In the trade-off it was decided that the LOD's were more important than reproducible migration times since peak identity could be confirmed with the UV spectra.

4 Conclusion

Two different CE modes were compared to assess which one will serve the purpose of analyzing purine metabolites in urine and serum the best. A direct serum injection MEKC method was compared to a CZE method utilizing the principles of sample stacking. Both methods were capable of separating the analytes of interest, but only the CZE method could reach the desired limits of detection. In the end only hypoxanthine, xanthine, uric acid, oxypurinol and 9-methylxanthine were analyzed. Only pH as electrophoretic variable was tested to determine the optimum pH conditions. The CZE method was suitable to detect and monitor drug induced changes in the uric acid and xanthine levels in both rat serum and urine. The total analysis time

varied according to the type of samples analyzed, with urine samples taking the longest. The separation time for the urine samples was 12 minutes with 7 minutes column conditioning. The analytical method and limits of detection may be enhanced when sample pre-concentration or different injection procedures are implemented.

In the end the CZE method was reliable enough to do automated sample analysis of both serum and urine samples from rats treated with drugs altering the purine metabolism. No standard runs were required in between the automated runs since all analytes were identified based on their UV spectrums. A total of more than 50 runs could be done in a 24 hour period.