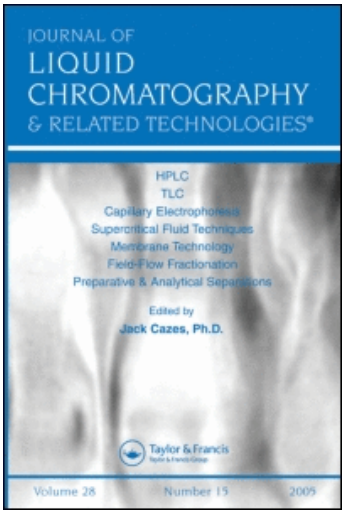

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Applications of Capillary Zone Electrophoresis and Micellar Electrokinetic Chromatography in Cancer Research

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APPLICATIONS OF CAPILLARY ZONE ELECTROPHORESIS AND MICELLAR ELECTROKINETIC CHROMATOGRAPHY IN CANCER RESEARCH[1]

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ABSTRACT

The purpose of this communication is to illustrate the utility and advantages of capillary zone electrophoresis and micellar electrokinetic chromatography in cancer research. In our laboratory, both techniques have been used to explore many aspects of cancer research from the search for biological markers in urine, serum and tissue, to epidemiological studies, to the exploration of anticancer drugs in plant materials and marine organisms. Both CE and MEKC have proven to be useful in solving problems which faced us. We present a series of examples which illustrates the application of these techniques to problems faced by our laboratory.

INTRODUCTION

Analytical chemistry is an integral part of many aspects of cancer research, from testing of a chemical's purity and identity to pharmacokinetic, environmental, epidemiological, chemotherapeutic studies and the search for anticancer drugs and cancer markers. The rapid increase in the number and quantity of

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chemicals found in the environment, some of which pose a carcinogenic risk to animals as well as humans, require the continuous monitoring for their effect. The term chemical carcinogen refers to the response in experimental animals to a carcinogen which may be observed in several forms: a) as a significant increase in the frequency of one or several types of neoplasm, as compared with other than zero frequency in control animals; b) as the occurrence of neoplasma not observed in experimental animals; c) as a decreased latent period as compared with control animals; and d) as a combination of (a) and (c). Evidence that a particular chemical is carcinogenic in humans depends largely on epidemiological data. Analytical chemists (chromatographers, spectroscopists, etc.) have a role to play by developing methods for the extraction, separation, quantification, and structure elucidation of chemicals and their metabolites from environmental, biological, and clinical specimens.

Capillary zone electrophoresis (CZE) is a powerful micro-analytical technique which gives high resolution of large biomolecules as well as small ions. Micellar electrokinetic chromatography (MEKC) can be used to resolve mixtures of neutral as well as charged molecules. In our laboratory, CZE and MEKC have been used for the separation of varied groups of compounds of biomedical and clinical interest. In this review we will show the utility of CZE and MEKC as an important and useful analytical tool in cancer research. CZE and MEKC with UV-laser induced fluorescence (LIF), and photodiode array detection have been used for the separation and detection of proteins, purity determination of synthetic peptides and separation of amino acids in urine, and for the determination of cancer markers, anti-cancer drugs and other compounds and metabolites of interest to cancer researchers.

High performance liquid chromatography (HPLC) and gas chromatography (GC) are the main separation techniques used in solving analytical problems related to chemical carcinogen determination and quantification (1). Today, capillary electrophoresis (CE) is being used to solve many of the analytical problems facing cancer researchers. We present here a few examples which will illustrate the utility and effectiveness of CE and MEKC as an analytical tool in different aspects of cancer research.

Extraction of Anti-Cancer Natural Products:

Extraction and isolation of natural products from plant material and marine organism is an important aspect of drug discovery against cancer. The first example is that of the separation by MEKC of the anti-cancer drug taxol from the needles and bark of the Western Yew, taxus brevifolia (taxacaea). Taxol shows unique anti-tumor and anti-leukemic activities (2,3). It has been shown to produce responses in patients with different types of cancer, such as ovarian (4), breast (5), lung (6), head and neck region (7) and malignant melanoma (8). HPLC (9) and TLC (10) have been used, however, MEKC gave a baseline separation of taxol from other coeluting compounds. We were also able to resolve in the same experiment from needle and bark extracts taxol, baccatin III, cephalomannine and other closely related compounds which were not separated by HPLC (11). Figure 1 is a comparison of the separation of a needle extract by both HPLC and MEKC. Other natural products which were resolved by CZE (12) are bryostatin I and bryostatin II which are members of a group of closely related macrocyclic

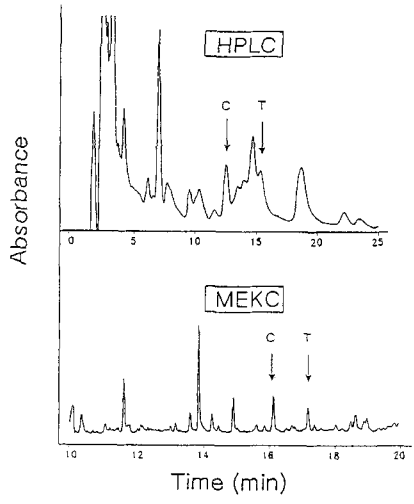


FIGURE 1. HPLC chromatogram and MEKC electropherograms of a crude needle extract from the taxus species. T = Taxol; C = depha-lomannine; B = baccatin III.

lactones which have been isolated from the marine Bryozoa Bugula neritina L. (Bugulidae). Bryostatins are activators of protein kinase C and show interesting antineoplastic and immunomoculating properties. These compounds were previously resolved by TLC (13) and HPLC (14). The advantages of MEKC over HPLC and TLC is the speed of analysis, the high resolution, and the negligible amount of organic solvent waste produced by MEKC.

Epidemeological Applications:

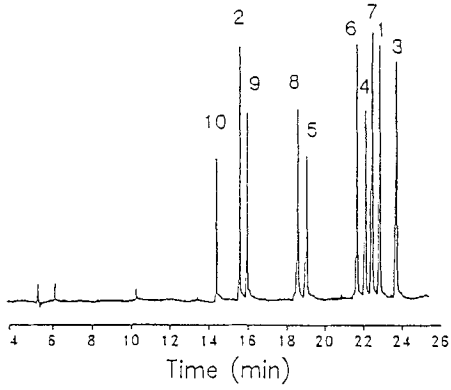
The modern epidemeologic study of hormone-dependent cancers, like breast, prostate and endometrial cancers, require the capability of highly accurate, specific and sensitive assay for steroid, peptide and protein hormones as well as growth factors. MEKC has been used in our laboratory for the separation of a group of endogenous and exogenous urinary estrogens which include estrone, estradiol, estriol, 2-hydroxyestrone, 2-hydroxyestradiol and others (15). Figure 2 shows the separation of 10 estrogens which have closely related structures.

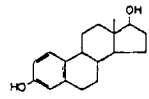
Retinoic Acids:

Retinoic acids (RAs) are metabolites or analogues of vitamin A that show important physiological functions. A variety of RAs have been shown to be capable of promoting growth and differentiation of epithelial cells and inhibiting tumorigenesis. A number of physiologically relevant RAs have been identified including all-trans-RA, 13-cis-RA, 4-oxo-RA and 13-cis-4-oxo-RA. Recently, the isomer 9-cis-RA has been identified as a high affinity ligand for the retinoid X receptor. Unfortunately, some RAs are also able to act as teratogens. Efficient separation among those isomers is needed for the assessment of their biological activities and for the most part high-performance liquid chromatography has been the method of choice. The separation of 5 retenoic acid isomers and internal standard from spiked rat urine by MEKC was achieved (16) using a micellar buffer made of 20 mM tris-borate (pH = 8.5), 25 mM SDS and 20% acetonitrile (figure 3).

Amino Acids, Peptides and Proteins:

Amino acids, peptides and proteins play an important role in cancer research. Hydorxyproline (Hyp) is a secondary amino acid

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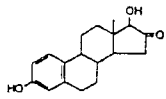
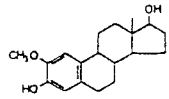

[2] 16-Keto-17β-estradiol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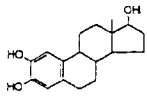
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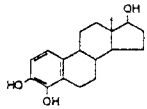
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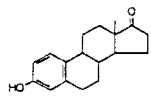
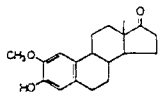

[6] Estrone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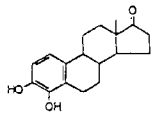
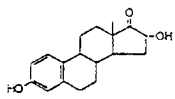

[8] 4-Hydroxyestrone


[9] 16α-Hydroxyestrone

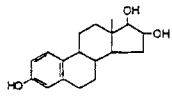

[10] Estriol

FIGURE 2. MEKC of ten estrogens. Buffers: 10 mM sodium phosphate (pH 7.0) containing 50 mM SDS and 20% methanol; capillary: 50 μm x 47 cm; voltage: 20 kV; pressure injection: 2S; detection: absorption at 200 nm.

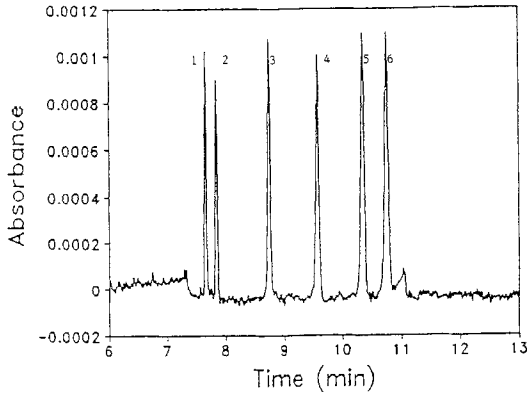


FIGURE 3. Separation of retinoic acid mixture from spiked rat plasma extract. Capillary: 75 μm x 47 cm; buffer: 20 mM Tris-borate (pH 8.5), 25 mM SDS, 20% acetonitrile; applied voltage: 13 kV; injection: 4S, pressure; peaks: 1 = 13-cis-4-oxo-RA, 2 = 4-oxo-RA, 3 = 13-cis-acitretin, 4 = 13-cis-RA; 5 = 9-cis-RA, 6 = all-trans-RA.

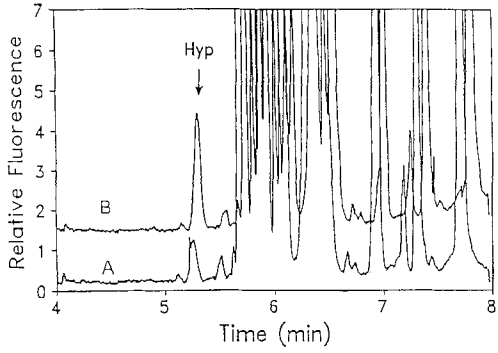


FIGURE 4. Electropherograms of a FMOC-derivatized serum (A), and a Hyp-spiked (~$6 \cdot 10^{-8}$ M) serum sample (B). The serum was diluted ca. 500-fold. Running conditions were: Capillary:60 cm x 50 μm I.D.; buffer: 20 mM sodium borate (pH 9.2) containing 25 mM SDS; applied voltage: 2 kV; injection: 15 s, gravity.

that reflects the metabolism of collagen. The determination of Hyp is important because it has been suggested as a marker for certain cancers and bone diseases. The detection of Hyp in biological samples is difficult due to its relatively low concentration compared to other amino acids and its lack of any strong spectroscopic property. Sensitive detection is possible by modifying the molecule using chemical derivatization. Micellar electrokinetic chromatography with UV laser-induced fluorescence (LIF) was used for the rapid and sensitive detection of Hyp in biological samples after pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC) (figure 4). Also, the application of the combined orthophthalic aldehyde (OPA)/FMOC derivatization in MEKC for the selective detection of secondary amino acids was achieved. The described MEKC-LIF method (17) allows rapid and sensitive detection of Hyp in serum and hydrolyzed urine that were derivatized with FMOC. The combined OPA/FMOC derivatization simplified the method (17) for the selective detection of proline (Pro) in serum and the simultaneous detection of Hyp, Pro, and sarcosine in hydrolyzed urine (figure 5). Also, the separation of a chiral mixture of amino acids was accomplished (18). Issaq and Chan (19) reviewed the separation and detection of amino acids and their enantiomers by capillary electrophoresis.

The separation of peptides (from dipeptides to 66 residue amino acids) have been accomplished using CZE (20-22). Purity determinations which could not be achieved by HPLC were easily done by CZE. In each case studied CZE was superior to HPLC in resolving the impurities in synthetic peptides (22). Figure 6 is a comparison of the separation by both HPLC and CZE of a 66 residue peptide. The results clearly show the superiority of the CZE separation. Proteins were also resolved by CZE and their native fluorescence was detected by LIF when they contained an aromatic amino acid (21).

Furthermore, CZE was used to study the binding of sequence-specific DNA to recombinant ETS1 oncoproteins (23). A time-dependent shift in the mobility of the P42-DNA complex was observed and the change in mobility was correlated with a net change in charge of the protein-DNA complex.

Catecholamines:

Capillary zone electrophoresis with untreated fused-silica capillaries and acetate buffer was evaluated for the separation and

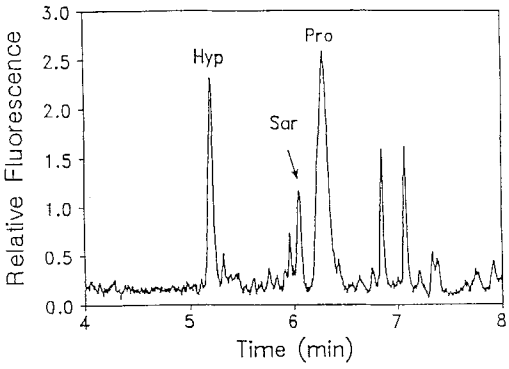


FIGURE 5. Electropherogram of a OPA/FMOC-derivatized urine. The urine sample was diluted ca. 5000-fold. Running conditions were the same as in Figure 4.

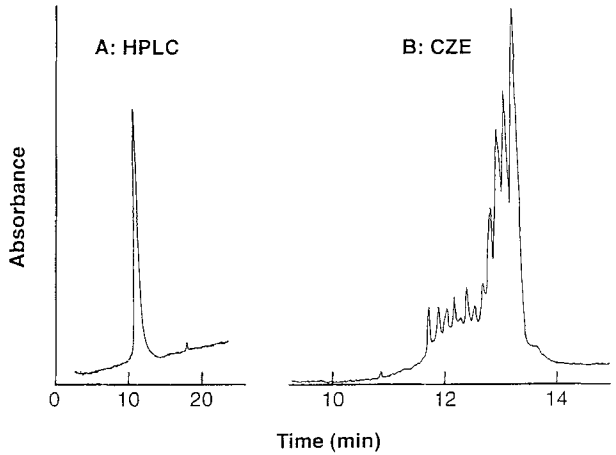


FIGURE 6. Analysis of the purity of a 66-residue peptide, LYQSNPPPNPEGTRQARRNRRRRWRERQRQIHSIZERILSTYLGRSAE-PVPLQLPPLERLTLDCNE-OH by (a) HPLC and (b) CZE. For HPLC the mobile phase program was from 40% (0.1 TFA in acetonitrile/water 4:1 and 60% 0.1 TFA in water to 100% 0.1 TFA in acetonitrile/water 4:1) in 40 min. For CZE the buffer was 50 mM phosphate at pH 3.0, the applied voltage 15 kV, and the current 55 μA. CE was performed with a Beckman Model P/ACE System 2000 fitted with a 57 cm x 75 μm i.d. polyacrylamide-coated fused silica column. Injection was by application of 0.5 psi pressure for 5 s. Detection was monitored at 214 nm.

analysis of catecholamine metabolites. Homovanillic acid and vanillylmandelic acid, which are excreted in abnormally elevated levels in the urine of patients with neuroblastoma, were separated from other possible catecholamine metabolites, using a 200 mM acetate buffer at pH = 4.10 (24). The high buffer concentration was necessary to minimize the peak tailing resulting from analyte-capillary wall interactions.

Nitrate and Nitrite Separation:

Capillary zone electrophoresis offers fast and efficient separation of inorganic ions. Nitrite, a meat preservative, reacts in vivo with amines and amides at an acidic pH to form carcinogenic nitrosamines. A limit of 10 mg nitrate per liter is imposed on drinking water to prevent methomoglobin in infants. Furthermore, the recent interest in the physiological importance of nitric oxide have heightened the need for sensitive methods for the determination of nitrite and nitrate in body fluids. Also, cancer patients receiving interluken II were found to have elevated levels of serum nitrate.

The problem with analyzing for nitrate and nitrite in biological samples by high performance ion chromatography is the presence of high concentration of chloride ions which interfere with the determination of nitrite due to lack of resolution and column saturation. The addition of silver reagents or silver-loaded cation exchange resins will react with the chloride and leads to its elimination as an interfering compound. However, it was reported that the silver chloride precipitation process causes a substantial reduction in the performance of ion exchange columns used for anions with insoluble silver salts (25,26). A capillary zone electrophoresis method for the separation and analysis of nitrate and nitrite in water and urine was developed (27). No interference in the electropherogram from other anions is observed by using a polyacrylamide-coated column with a modified phosphate buffer at pH 3 for the separation, and UV-absorption at λ = 214 nm for the detection (figure 7). The method does not require sample pretreatment or the use of organic solvents. The limit of detection for each analyte (S/N = 3), using a 75 μmi.d. capillary, is 0.5 μg/mL.

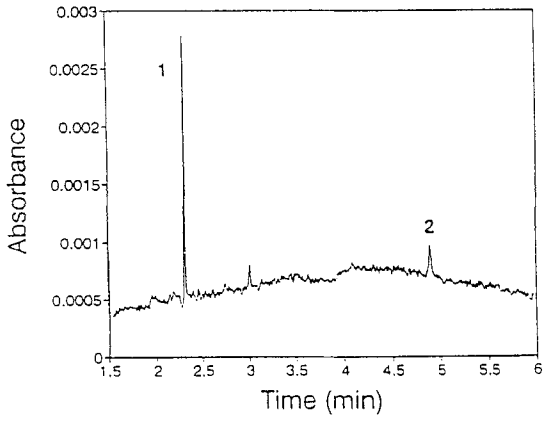


FIGURE 7. Electropherogram of a rat urine sample spiked with 50 μg/ml nitrite then diluted 40:1. Buffer: 25 mM phosphate containing 0.5% DMMAPS and 1.0% Brij-35; applied voltage: -15 kV; column: 10% T, polyacrylamide-coated fused silica [T = (g acrylamide + g N,N'-methylenebisacrylamide)/100 ml solution]; column dimensions: l_{total} = 57 cm, $L_{detection}$ = 50 cm, I.D. = 75 μm; instrument: beckman Model P/ACE System 2000. Detection: 214 nm. Solutes: 1 = nitrate; 2 = nitrite.

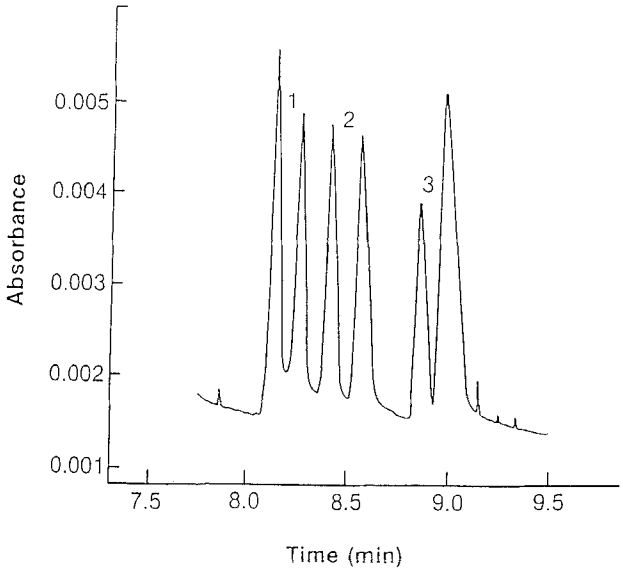
Nitrosoamino Acids:

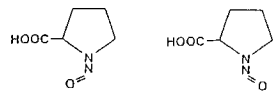
CE was also used for the separation of nitrosoamino acids and their syn and anti-conformers using a polyacrylamide-coated capillary and sub-ambient temperatures (28). The conformers of N-nitrosopro-line, N-nitroso-4-hydroxyproline and N-nitrosothiazolidine-4-carboxylic acid were resolved using CE at 5°C, figure 8.

Caffeine and Its Metabolites:

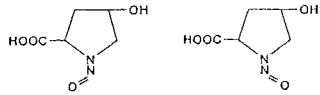
Caffeine (1,3,7-trimethylxanthine) is widely used in the human diet. It can be studied as a probe drug for the assessment of variability in biotransformation capacity. In the 1980's, examining caffeine metabolism to determine genetic acetylator phenotype and genotype of human population groups was common practice (29). Caffeine is particularly well suited as a test drug for many reasons such as availability, ease of administration and safety.

To date, high performance liquid chromatography is the method of choice for the determination of caffeine and its metabolites.

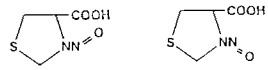




N-nitrosoproline



N-nitroso-4-hydroxyproline



N-nitrosothiazolidine-4-carboxylic acid

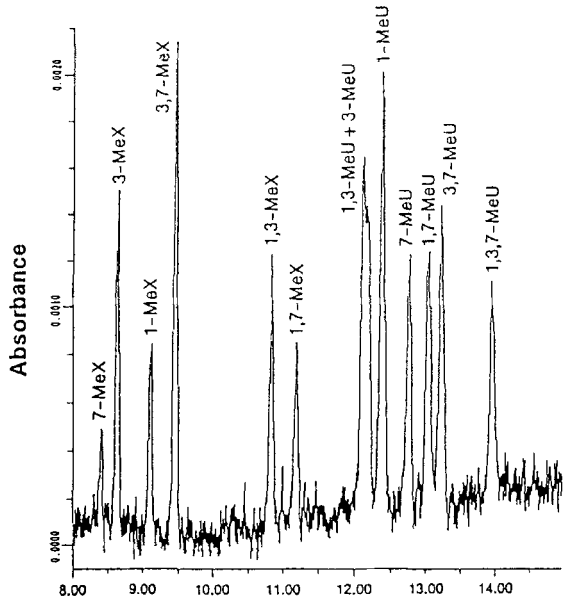
FIGURE 8. Electropherogram showing the separation of syn and anti conformers of selected nitrosoamino acids at 5°C. Solutes: 1 = N-nitrosothiazolidine-4-carboxylic acid, 2 = N=nitrosoproline, and 3 = N-nitroso-4-hydroxyproline; instrument: Beckman Model P/ACE System 5510; detection: 235 nm; column 10% T polyacrylamide-coated fused-silica; column dimensions: L_{total} = 57 cm, $L_{detector}$ = 50 cm, i.d. = 75 μm; buffer: 10 mM phosphate containing 2 mM DMMAPS, and 0.1% Tween 20; pH = 7.2; applied voltage: -25 kV; solute concentration: 2-5 μg/mL.

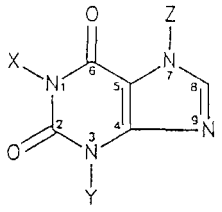
Grant et al. (30) developed a procedure for their extraction and subsequent HPLC separation and quantification. In our laboratory a method was developed for the separation of xanthines and uric acid derivatives which are normally present in human plasma and urine as metabolites of caffeine. The methyl-substituted uric acids were separated using in the CZE mode a 0.05 M sodium phosphate buffer (pH 7.0) at an applied voltage of 10 kV. In contrast, the separation of the methyl-substituted xanthines was only possible in the MEKC mode, where SDS (0.15 M) was added to the buffer system. When both types of compounds were present in the same sample, optimum resolution (12 peaks for 13 standard solutes) was realized in the MEKC mode with 0.15 M SDS added to the 0.05 M, pH 7 phosphate buffer at an applied voltage of 15 kV, figure 9 (31).

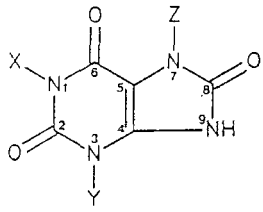
Nicotine and Its Metabolites:

The determination of nicotine and its principal metabolites in biological fluids provides information about exposure to cancer-causing cigarette smoke. Several methods have been published for the analysis of these compounds, of which HPLC is the most widely used (32). A CZE method was developed in our laboratory for the separation of nicotine, and three of its principle metabolites from a human urine matrix. Figure 10A shows the separation of a standard mixture using a polyacrylamide-coated column. Figure 10B shows the electropherogram resulting from a smoker's urine specimen, after sample clean-up according to the procedure of Zuccaro et al. (33) and reconstitution in the running buffer. The method offers: 1) orthogonality to HPLC in the separation mechanism; 2) selectivity, because only positively charged species migrate towards the negative electrode in the zero-electroosmotic flow environment provided by the polyacryl amide coated column; and 3) high resolution. The method is currently under evaluation for the analysis of biological samples.

The above examples illustrate the utility and advantages of both CZE and MEKC over other separation techniques in cancer research.







Solute	X	Y	Z
Xanthine (X)	H	H	H
1-MeX	Me	H	H
3-MeX	H	Me	H
7-MeX	H	H	Me
1,3-Mex	Me	Me	H
1,7-MeX	Me	H	Me
3,7-MeX	H	Me	Me
1,3,7-MeX	Me	Me	Me

Solute	X	Y	Z
Uric Acid (U)	H	H	H
1-MeU	Me	H	H
3-MeU	H	Me	H
7-MeU	H	H	Me
1,3-MeU	Me	Me	H
1,7-MeU	Me	H	Me
3,7-MeU	H	Me	Me

FIGURE 9. Electropherogram of a mixture of seven methyl-substituted xanthines and six methyl-substituted uric acids. Experimental conditions: 50 cm x 75 μm i.d.; buffer: 0.05 M Na_2HPO_4 + 0.15 M SDS; pH 7.0; pressure injection mode: 2 sec at 0.5 psi; temperature: 25°C; detector: UV at 280 nm; voltage: 15 kV. Solute symbols as given in Table I.

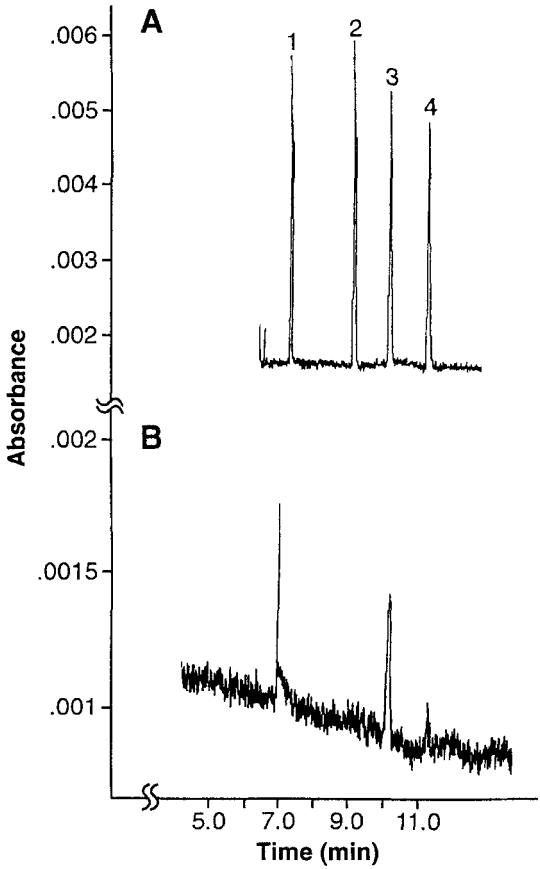


FIGURE 10. Separation of nicotine and three of its main metabolites. A: Standard mix and B: Smoker's urine sample. Buffer: 50 mM sodium acetate; pH: 4.7; voltage: 20 kV; pressure injection: 3S at 0.5 psi; detection: absorption of 260 nm; instrument: Beckman Model P/ACE System 5510; column: 10% T polyacrylamide-coated fused-silica; column dimensions: L_{total} = 47 cm, $L_{detector}$ = 40 cm; i.d. = 75 μm; solute concentration: 2-5 μg/mL in water; solutes: 1-nicotine, 2-demethylcotinine, 3-cotinine and 4-trans-3-hydroxycotinine.

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