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Determination of caffeine and its metabolites by micellar electrokinetic capillary electrophoresis

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Abstract

The determination of caffeine and its analogues is important for a wide variety of analyses and is performed in an assortment of matrices ranging from food to clinical samples. While reversed-phase HPLC has become the standard analysis protocol in most laboratories, capillary electrophoresis has the advantages of higher separation efficiency and shorter separation time. The micellar capillary electrophoresis (MECC) separation of caffeine and its metabolites, theobromine, paraxanthine, theophylline and 1,3,7-trimethyluric acid was investigated using sodium dodecyl sulphate (SDS) as the micellar phase. The effects of pH, micelle concentration, buffer concentration, ionic strength, buffer salts, applied voltage and injection time were studied to select the optimum conditions for the determination of caffeine and its four analogues in drugs, foods and body fluids. Caffeine and its three analogues were resolved within 120 s with detection limits less than 1 μg/ml. Samples could be analyzed utilizing direct injection with satisfactory resolution and reproducibility.

Keywords: Caffeine; Theobromine; Paraxanthine; Theophylline; 1,3,7-Trimethyluric acid

1. Introduction

Capillary electrophoresis (CE) has been developed as a technique for the analysis of charged molecules, including proteins, peptides and oligonucleotides. Compared to conventional separation methods such as HPLC, CE provides higher efficiency (approaching or exceeding 1 000 000 theoretical plates), shorter analysis time, smaller sample volume requirements and low running cost. The application of this method was extended to neutral molecules by the introduction of micellar capillary electrophoresis (MECC) as first developed by Terabe et al. [1]. A psuedophase is formed by adding a reagent such as sodium dodecyl sulfate (SDS) to the electrophoresis run buffer at concentrations sufficient to form micelles. In MECC, analytes are separated on the basis of both their hydrophobicity and electrophoretic mobility. Solutes can differentially partition between the hydrophobic interior of the micelles and the aqueous phase leading to different migration rates in the MECC system. MECC has been applied to the separation of both neutral and charged organic molecules, including derivatized amino acids [2], water-soluble vitamins [3], pharmaceuticals [4], and nucleic acids [5].

Caffeine and its metabolites are widely found in the human diet and pharmaceutical formulations. The xanthines have a wide range of therapeutic activity. Theophylline is a bronchodilator used in the treatment of chronic asthma, caffeine is a central nervous system stimulant, theobromine and theophylline are

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diuretics, and theophylline is a respiration stimulant and smooth muscle relaxant [6]. The physiological effects produced by many non-alcoholic beverages such as tea, coffee and coca depend mainly on their natural xanthine content. Caffeine is also used as a probe drug for the assessment of metabolic capacity. Wang et al. [7] have reported the possibility of predicting the extent of hepatic disorder by studying changes in the blood concentrations of caffeine and its three primary metabolites after caffeine administration in patients with liver cirrhosis. Production of paraxanthine, theophylline and theobromine was suppressed in patients with liver disease. These measurements may be a useful tool to assess liver function.

Several techniques have been reported for the analysis of caffeine and its major metabolites in biological fluids [8,9], pharmaceutical tablets [10], and foodstuffs [11], based mainly on HPLC. All of these HPLC methods required a clean-up step prior to chromatographic analysis. On-line analysis, therapeutic drug monitoring, and clinical analysis require a method which is fast, with little or no sample preparation. These criteria can be satisfied by the use of MECC. Lee et al. [12] have described the separation of theophylline and its analogues by MECC with UV detection in human plasma. The separation took 12 min and an extraction of the sample with ethyl acetate was required. Thormann et al. [13] reported the determination of substituted purines in serum by MECC with direct sample injection. The separation took 14 min and only theophylline and uric acid were resolved. Chen and Lunte [14] have developed an HPLC method to determine caffeine and its metabolites in body fluids in less than 60 s but theophylline could not be separated from theobromine. To date no method has appeared in the literature which could separate caffeine, theobromine, paraxanthine, 1,3,7-trimethyluric acid and theophylline in less than 2 min and not require a sample clean-up step.

This report describes a method for separating caffeine and its metabolites by MECC. The method has been applied to the analysis of caffeine and it metabolites in beverages, pharmaceutical tablets, and body fluid. The analysis required less than 2 min with no sample preparation necessary other than filtration.

2. Experimental

2.1. Chemicals

Caffeine, theobromine, theophylline, paraxanthine, 1,3,7-trimethyluric acid, antipyrine, sodium dodecyl sulfate and sodium phosphate monobasic were purchased from Sigma (St. Louis, MO, USA). HPLC-grade solvents and doubly distilled water were used throughout these studies. All other chemicals were reagent grade or better and used as received.

2.2. Apparatus

An ISCO Laboratory Instruments (Lincoln, NE, USA) Model 3850 capillary electrophoresis system with a 60 cm (25 cm to detector, 50 μm I.D.) fused-silica capillary (Polymicro Technology, Phoenix, AZ, USA) was used for separation. The on-column UV detector was operated at 274 nm with a datajet integrator (Model SP4600, Spectra-Physics, San Jose, CA, USA) connected to a WINner/386 workstation was used for data acquisition. Prior to running, the capillary was sequentially rinsed hydrodynamically with 0.5 *M* EDTA (pH 13), water and running buffer at 520 kPa for 30, 20 and 10 min, respectively, and then filled with running buffer. All solutions were filtered through a 0.45-μm filter. Samples were introduced by vacuum injection using the built-in injection system. Injection volumes were determined by the time necessary for sample to reach the detector using only hydrodynamic introduction.

2.3. Sample Preparation

2.3.1. Beverages

Brewed beverages, coffee and tea, were prepared according to manufacturers instructions. Carbonated beverages were degassed by purging with argon. All beverages were filtered through a 0.25-μm filter before determination.

2.3.2. Pharmaceutical tablets

One tablet of Excedrin (Bristol-Myers Squibb, New York, NY, USA), Anacin (Whitehall Laboratory, New York, NY, USA), Nodoz (Bristol-Myers Squibb), or Super Pain Relief (Fastpro International, Kansas City, KS, USA) was dissolved in 200 ml distilled water and filtered through a 0.25-μm filter.

2.3.3. Rat blood

Blood was collected from the jugular vein of a healthy rat. Serum was prepared by centrifugation for 5 min at 3600 *g*. For direct analysis the serum sample was filtered through a 0.25-μm filter and then injected into the CE system. Direct analysis was compared to the extraction described by Lee et al. [12]. For the extraction, 600 μl of serum was added to 900 μl of ethyl acetate in a test tube, vortex-mixed vigorously for 40 s, and centrifuged to achieve phase separation. After centrifugation, 500 μl of the organic phase was transferred to a clean test tube. An additional 500 μl of ethyl acetate was added to the serum sample and the extraction repeated. This procedure was repeated with the entire organic phase collected after the final extraction for a total volume of 1.9 ml of ethyl acetate. The ethyl acetate was evaporated under a stream of argon and the residue reconstituted with 20 μl of distilled water which was injected onto the CE system.

3. Results and discussion

3.1. pH of running buffer

Using a fixed surfactant concentration of 60 m*M* SDS, the pH of the run buffer was varied. The variation of capacity factor with buffer pH is shown in Fig. 1. Below pH 8 caffeine and paraxanthine could not be resolved. A reasonable separation was obtained at pH 8.1 using a borate buffer as shown in Fig. 2A. The order of migration time of the compounds is theobromine, caffeine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid. At pH 9 theobromine and caffeine comigrated while between pH 10 and pH 11 theophylline and 1,3,7-trimethyluric acid were not resolved. A good separation was achieved at pH 11 using a phosphate buffer as shown in Fig. 2A. Above pH 9, the migration order of theobromine and caffeine reverse. Likewise, above pH 11 the migration of 1,3,7-trimethyluric acid increases dramatically relative to both theophylline and paraxanthine and these compounds reverse elution order. Efficiency, resolution, and sensitivity were all better using the pH 11 phosphate buffer relative to the pH 8.1 borate buffer (Table 1) therefore the pH 11 phosphate buffer was used for further optimization.

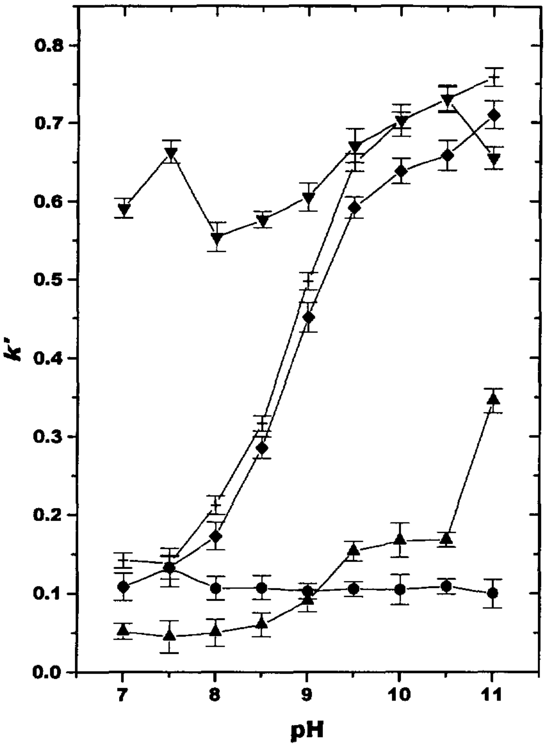

Fig. 1. Effect of run buffer pH on capacity factor. Electrophoretic conditions: buffer, 20 m*M* phosphate buffer containing 40 m*M* SDS; applied voltage, 25 kV; separation capillary, 600×0.05 mm I.D. (300 mm to detector) fused-silica; 3 nl of a 0.5 m*M* sample was injected. Error bars indicate a standard deviation with $n=4$. (●) Caffeine; (▲) theobromine; (▼) 1,3,7-trimethyluric acid; (♦) paraxanthine; (+) theophylline.

3.2. Concentration of SDS

The concentration of SDS was then investigated. At pH 11 the xanthines are all anions except caffeine which is neutral. In the absence of SDS the xanthines can be resolved, however, caffeine migrates with the electroosmotic flow and is not resolved from other neutral compounds in the sample. As can be seen in Fig. 3 the migration time of caffeine remains relatively constant at all SDS concentrations, however, the capacity factor is increasing with increasing SDS. The other xanthines exhibit decreasing migration

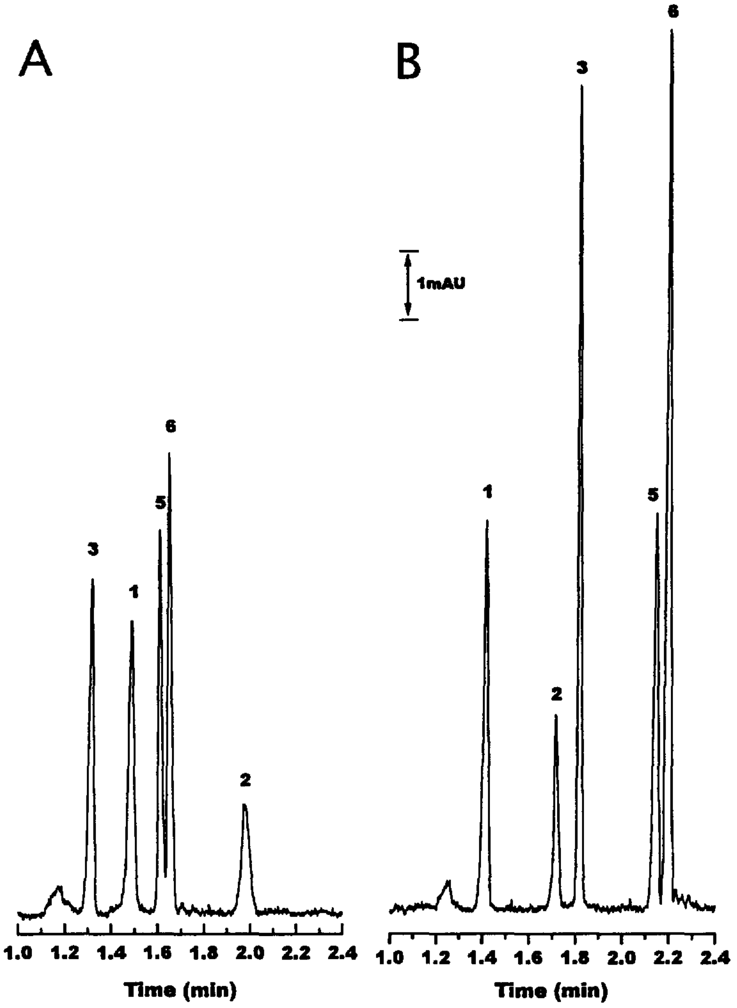

Fig. 2. Electropherograms using a 20 m*M* borate buffer, pH 8.1 (A) compared to a 20 m*M* phosphate buffer, pH 11.0 (B). Other separation conditions as in Fig. 1. Peaks: 1=caffeine; 2=antipyrine (internal standard); 3=theobromine; 5=paraxanthine; 6=theophylline.

times but constant capacity factors as the SDS concentration increases. Increasing the concentration of SDS results in increasing capacity factor for caffeine as it partitions into the SDS micelles while the anionic xanthines exhibit no evidence of partitioning into the micelles. Because the observed migration time (and therefore analysis time) is a function of capacity as well as the electroosmotic flow, increasing the SDS concentration decreases the migration times of the anionic compounds due to increased electroosmotic flow. A concentration of 40 m*M* SDS provided good resolution of caffeine from the electroosmotic flow and a short analysis time while maintaining good resolution.

3.3. Applied voltage

The effect of applied voltage on the electrophoretic separation was investigated with the optimized buffer conditions of pH 11 phosphate and 40 m*M* SDS. The applied voltage had no effect on the resolution of the xanthines below 27 kV. At higher

Table 1
Comparsion of separations using phosphate and borate buffers

Buffer	Compound	Sensitivity (mAU/m*M*)	Efficiency (plates/m)	Resolution
20m*M* Borate pH=8.1	Theobromine	13.7± 0.7	63 820±698	
	Caffeine	11.5±0.9	50 430±560	2.29±0.03
	Paraxanthine	15.3±1.1	76 830±656	3.48±0.03
	Theophylline	19.0±1.3	81 770±564	1.11±0.06
	Antipyrine	4.45±0.1	26 750±252	4.83±0.04
20m*M* Phosphate pH=11.0	Caffeine	15.0±0.7	61 190±117	
	Antipyrine	7.31±0.4	64 980±135	7.50±0.01
	Theobromine	24.5±1.3	104 200±382	3.25±0.02
	Paraxanthine	15.8±0.1	142 270±357	7.75±0.04
	Theophylline	34.4±0.6	148 960±305	1.33±0.01

n=3.

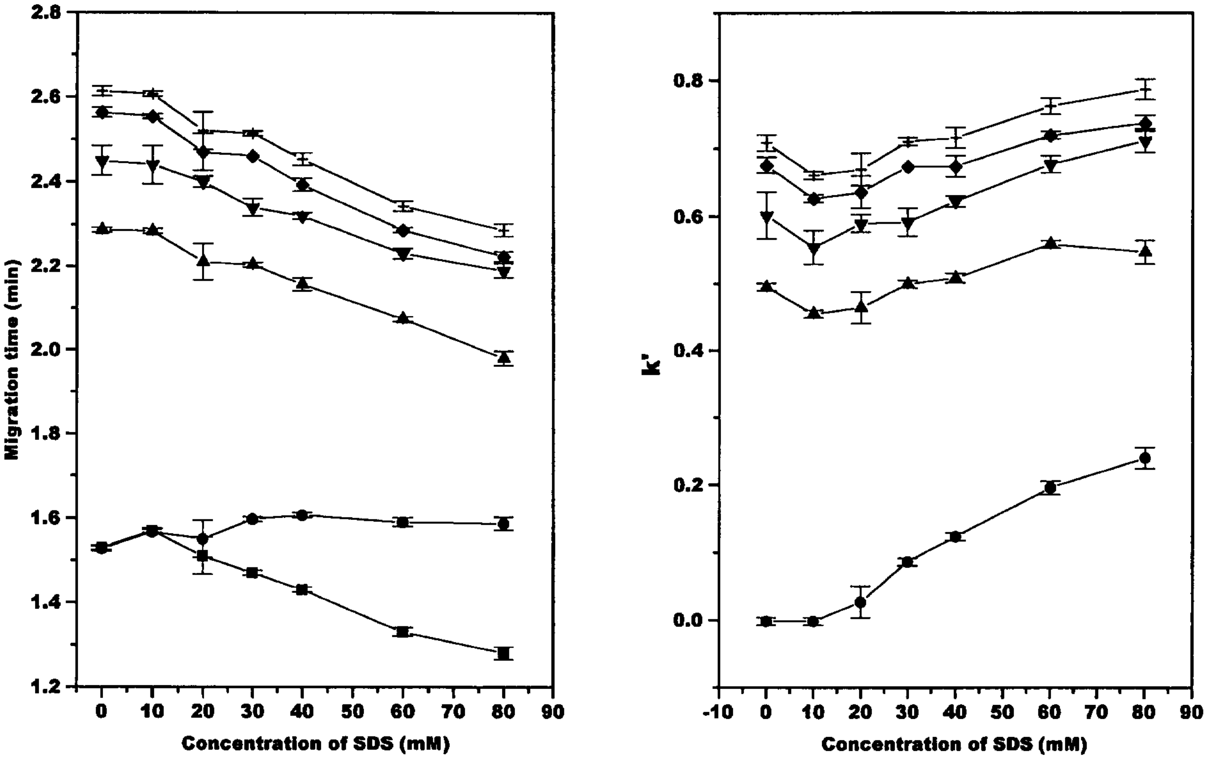

Fig. 3. Effect of SDS concentration on migration time and capacity factor. Separation conditions as Fig. 1 except that a 20 m*M* phosphate buffer, pH 11.0, and a 4-nl injection were used. Error bars indicate a standard deviation with *n*=4. Symbols are as in Fig. 1 with (■) electroosmotic flow.

voltages the migration time of caffeine becomes too short to allow partitioning into the micelles and coelution with neutrals occurs. The resolution of the other xanthines also deteriorates at applied voltages above 27 kV because of lower efficiencies. This is likely due to Joule heating problems at these high applied voltages. An applied voltage of 27 kV provided good resolution of the xanthines in less than 3 min.

3.4. Concentration of phosphate buffer

Increasing the concentration of the phosphate buffer increased the electroosmotic flow resulting in decreased migration times. This effect is due to differences in ionic strength at different phosphate concentrations. The separation was not affected by the buffer concentration (Fig. 4) except that at concentrations above 50 m*M* phosphate caffeine comigrated with the neutral compounds. A buffer concentration of 20 m*M* was found to provide good buffering, reasonable electroosmotic flow, and sufficient separation of caffeine from neutral compounds.

3.5. Injection volume

The effect of injection volume on the separation efficiency was investigated in order to determine the maximum sample that could be injected. The efficiency was independent of sample volume injected below 3 nl. At volumes larger than 7 nl, the efficiency decreased dramatically as the injection volume increased (Table 2). Response based on peak height became non-linear with injection volumes greater than 5 nl due to band broadening from lower separation efficiency. As expected the efficiencies for the charged compounds were much higher than for the neutral caffeine. The high efficiencies for the anionic xanthines indicate that these compounds do not significantly interact with the hydrophobic micelles and therefore exhibit efficiencies typical for capillary electrophoresis. At larger injection volumes efficiency decreases due to the large injection volume relative to the peak volume as no stacking occurs in this system because the sample is in a highly ionic solution to realistically simulate actual samples. Caffeine, being neutral, interacts significantly with the micelles and therefore exhibits

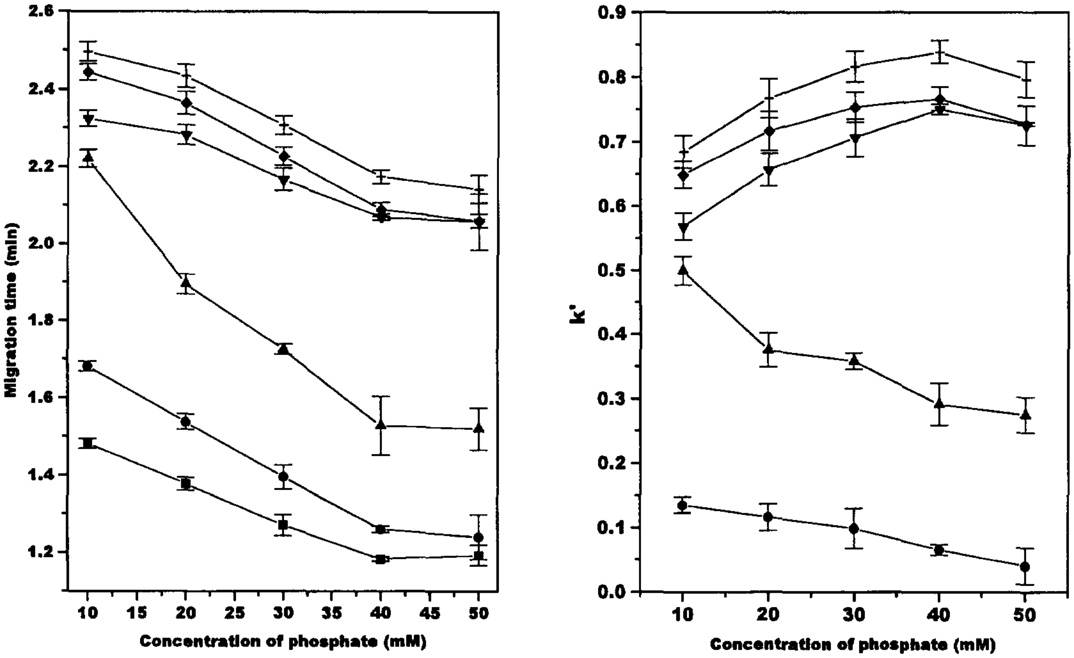

Fig. 4. Effect of phosphate buffer concentration on migration time and capacity factor. Separation conditions as Fig. 3. Error bars indicate a standard deviation with $n=4$. Symbols are as in Fig. 1.

Table 2
Relationship between injection volume and column efficiency

Injection volume	Efficiencey (plates/m)			
	Caffeine	Theobromine	Paraxanthine	Theophylline
1 nl	51 460±107	388 750±1477	469 570±1668	495 900±1668
3 nl	50 980±107	392 060± 148	479 240± 656	503 840± 672
5 nl	51 460±107	98 620± 149	1 202 660± 165	1 272 210± 169
7 nl	22 870±48	99 390± 149	54 170± 73	56 700± 75
9 nl	12 740±27	99 730± 249	54 720± 123	57 490± 126
11 nl	7 980±59	44 810± 189	55 320± 210	58 220± 266
15 nl	5 630±12	45 660± 67	31 610± 84	59 180± 128

n=3.

partitioning behavior and the resulting lower separation efficiency.

3.6. Optimized system

The optimized separation conditions consisted of a run buffer of 20 m*M* sodium phosphate buffer, pH 11.0, containing 40 m*M* SDS, a 60 cm×50 μm I.D. capillary with 25 cm to the detection window, an applied voltage of 29 kV and an injection volume of 2 nl. A typical electropherogram under these conditions is shown in Fig. 5. The entire analysis required less than 2 min with detection limits of less than 1 μg/ml for all compounds except caffeine. The response was linear up to 200 μg/ml based on peak area with a relative standard deviation of less than 5% for all of the compounds (Table 3). The linear range extended to only 50 μg/ml when peak height was used for all compounds except caffeine which still exhibited a linear response to 200 μg/ml. The reproducibility of the migration time was also very good at less than 2% R.S.D.

3.7. Applications

3.7.1. Beverages

No sample preparation other than filtration to prevent clogging of the capillary was necessary for analysis of non-carbonated beverages. For carbonated beverages degassing by purging with argon was required prior to filtration. The only xanthine detected in beverage samples was caffeine. Representative electropherograms are shown in Fig. 6A-D. Using antipyrine as an internal standard, the CE

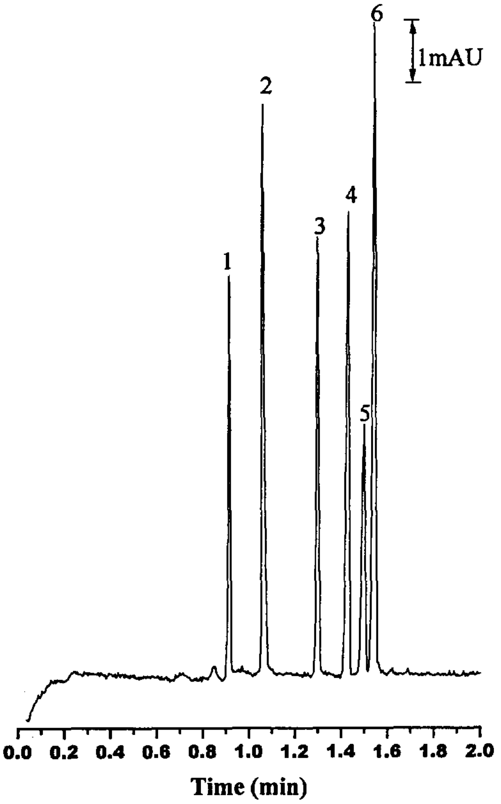

Fig. 5. Typical electropherogram of caffeine and its analogues by MECC. Electrophoretic conditions: buffer, 20 m*M* phosphate buffer, pH 11.0, containing 40 m*M* SDS; applied voltage, 29 kV; separation capillary, 600×0.05 mm I.D. (250 mm to detector) fused-silica; 2 nl of a 2 m*M* sample was injected. Peaks: 1= caffeine; 2=antipyrine (internal standard); 3=theobromine; 4= 1,3,7-trimethyluric acid; 5=paraxanthine; 6=theophylline.

Table 3
Sensitivity and reproducibility

Compound	Detection limit[a] (μg/ml)	Linear range[b] (μg/ml)	*r*	R.S.D.[c]	
				Peak height	Migration time
Caffeine	1.50±0.17	1.5–194	0.999	1.15	0.85
Theobromine	0.70±0.06	0.7–180	0.998	3.58	0.56
TMUA	0.70±0.06	0.7–210	0.998	2.58	0.24
Paraxanthine	0.70±0.08	0.7–180	0.999	2.38	1.01
Theophylline	0.70±0.06	0.7–180	0.999	4.58	1.23

[a] $S/N=3$.

[b] Five injections at eight concentrations.

[c] Calculated from 20 injections of a 100 μg/ml standard.

determination of caffeine had excellent reproducibility (Table 4).

3.7.2. Pharmaceuticals

As with the beverages no sample pretreatment was needed other than filtration. Tablets were dissolved in distilled water and directly injected in to the CE system following filtration. The only xanthine detected was caffeine although acetaminophen was also detected in some formulations (Fig. 6E–H). Acetaminophen does not comigrate with any of the xanthines. This method is therefore also applicable to the determination of acetaminophen in pharmaceuticals. Typical analytical results are given in Table 4 and are in good agreement with the manufacturer's reported concentrations.

3.7.3. Rat serum

Filtered rat serum could be directly injected into the CE system. No interferences for caffeine or its metabolites were observed. A small peak due to an endogenous compound was observed with an elution time of 1.7 min which is near that of 1,3,7-trimethyluric acid but does not co-elute (Fig. 7A). Most endogenous compounds in the blood eluted well after the last of the caffeine metabolites. A serum blank

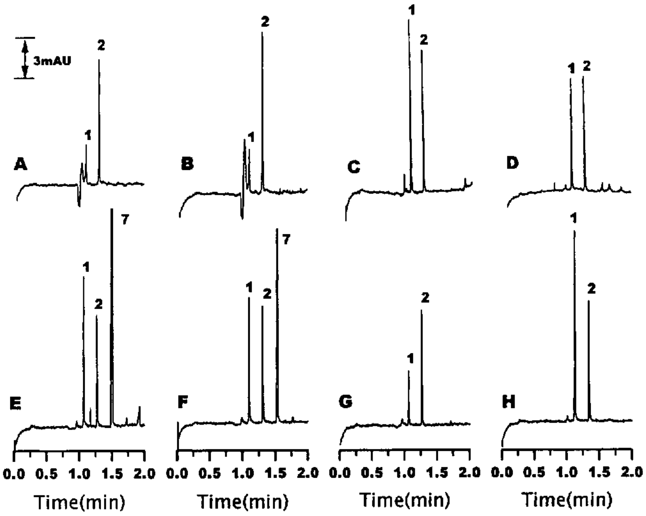

Fig. 6. Typical electropherograms of beverages and pharmaceutical tablets. (A) Pepsi-cola; (B) Coca-cola; (C) coffee; (D) Nestea; (E) Super pain relief; (F) Excedrin; (G) Anacin; (H) Nodoz. Electrophoretic conditions as in Fig. 5 except the applied voltage was 27 kV. Peak identities as in Fig. 5 with peak 7 acetaminophen.

Table 4
Analysis of real samples

Sample	Caffeine	
	Found	Reference[a]
Coca-Cola	74.4±2.5 μg/ml	N.A.
Mr. Pibb	89.9±4.2 μg/ml	N.A.
Mountain Dew	115.2±5.9 μg/ml	N.A.
Pepsi-Cola	89.6±2.5 μg/ml	N.A.
Coffee (Premium)	309.0±9.5 μg/ml	N.A.
Chinese Green Tea	10.5±0.2 mg/g	N.A.
Nestea (Bag)	49.7±1.4 mg/bag	N.A.
Nestea (Iced)	7.00.4 mg/g	N.A.
Excedrin	67.0±2.7 mg/tablet	65 mg/tablet
Anacin	30.6±1.0 mg/tablet	32 mg/tablet
Nodoz	103.4±2.7 mg/tablet	100 mg/tablet
Super Pain Relief	35.1±1.2 mg/tablet	32.4 mg/tablet

[a] Given by manufacturer; N.A.=not available.

spiked with the analytes is shown in Fig. 7B. The large ill-defined peaks eluting later than 2 min are likely serum proteins. These peaks varied greatly from serum sample to serum sample but did not effect the separation. Fig. 7C shows an electropherogram of direct analysis of rat serum collected 2.5 h after the animal had been dosed with 30 mg/kg of caffeine intravenously. Caffeine can easily be determined in this sample but none of the metabolites are detectable. An extraction procedure which gave a 30-fold concentration enhancement was used for comparison. The electropherogram of a serum extract taken prior to dosing with caffeine is shown in Fig. 7D. No potential interferences were observed in the blank. An electropherogram of a spiked blank serum extract is shown in Fig. 7D. Fig.

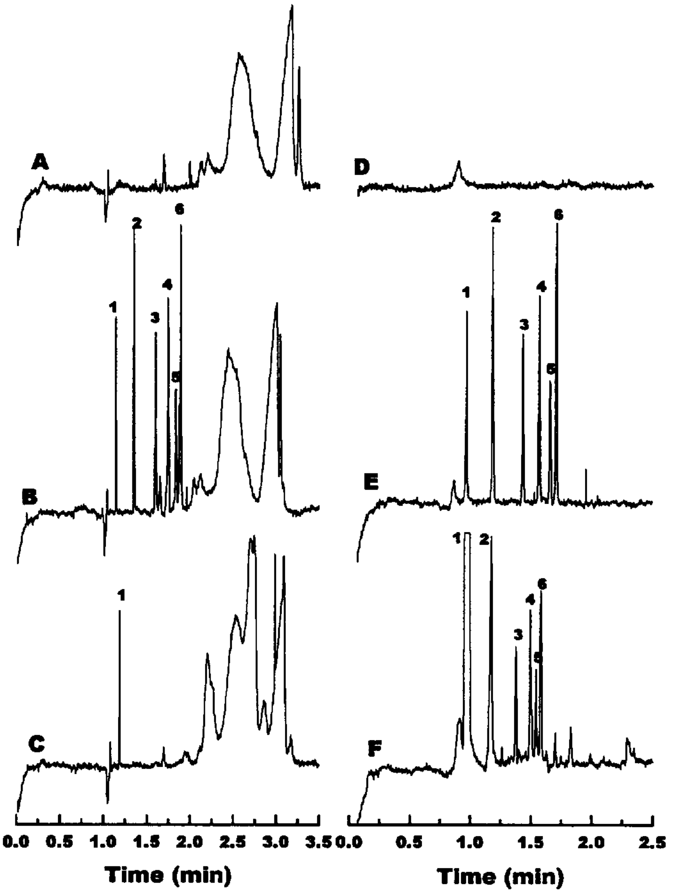

Fig. 7. Electropherograms of rat serum caffeine. (A) Serum blank; (B) spiked serum blank; (C) serum sample; (D) serum extract prior to dosing; (E) spiked serum extract; (F) serum extract after dosing. Electrophoretic conditions as in Fig. 6 except the applied voltage was 26 kV. Peak identities as in Fig. 6.

7E shows a typical electropherogram obtained following extraction of the serum sample collected 2.5 h after dosing 30 mg/kg caffeine intravenously. In this case, not only is caffeine detected but the metabolites are as well. Therefore, while direct injection of a biological sample such as serum into the CE system is possible, overall detection limits are higher. On the other hand, while an extraction step provides the possibility of preconcentration of the analytes it is more time and labor intensive than direct injection.

Acknowledgments

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