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# Application of Micellar Electrokinetic Capillary Chromatography to Forensic Analysis of Barbiturates in Biological Fluids

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**ABSTRACT:** Micellar electrokinetic capillary chromatography (MECC) is a form of capillary zone electrophoresis. Addition of a surfactant produces micelles in an aqueous/organic buffer. Separation of drugs is obtained via differences in the electrophoretic mobilities of the analytes within the capillary, resulting from their electrophoretic velocity and the electroosmotic flow of the buffer in a given electric field. The migration order is determined by the differential partitioning of the drugs between the micelles and the aqueous/organic phase.

Barbiturates were extracted from various biological fluids at pH 4.5 with TOXI-TUBES B. MECC analyses were performed using a Waters Quanta 4000 Capillary Electrophoretic System with a 745 Data Module with a 75 μ × 60 cm capillary and an aqueous/organic buffer of 85% 10 mM borate, 10 mM phosphate, 100 mM sodium dodecyl sulfate and 15% acetonitrile at a pH of 8.5 with a voltage of 20 kV using ultraviolet absorption detection at 214 nm. Migration times were: phenobarbital, 7.78 min.; butalbital, 8.01 min.; butabarbital, 8.23 min.; mephobarbital (internal standard), 8.88 min.; amobarbital, 9.41 min.; pentobarbital, 10.03 min. and secobarbital, 10.79 min. Correlation coefficients ($r$) between peak areas and concentration ranges of 3 to 60 μg/mL were from 0.964 to 0.999. Coefficients of variation (CV) ranged from 2.6 to 8.6% between days and 2.3 to 9.8% within day. Application of this methodology to four forensic cases of butalbital intoxication detected concentrations of 0.7 to 12.7 μg/mL in blood; 0.8 to 1.9 μg/mL in vitreous humor and 1.5 to 7.6 μg/mL in urine. MECC is applicable to forensic analysis of barbiturates extracted from biological fluids.

**KEYWORDS:** toxicology, micellar electrokinetic capillary electrophoresis (MECC), barbiturates, butalbital, overdose, poisoning

Micellar electrokinetic capillary chromatography (MECC) is a form of capillary zone electrophoresis in which the addition of a surfactant produces micelles in an aqueous/organic buffer. Separation of analytes results from differences in their electrophoretic mobilities within the capillary, due to their electrophoretic velocity and the electroosmotic flow of the buffer in a given electric field. The migration order is determined by differential partitioning of the drugs between the micelles and the aqueous/organic phase. Analytes are injected into the capillary by hydrostatic sampling and their migration and quantitation is determined by their ultraviolet absorption within the capillary.

MECC has been used for analysis of pharmaceuticals [*1*], identification of illicit drug substances [*2,3*] and detection and quantitation of xanthines in biological fluids [*4*], cicletanine enantiomers in plasma [*5*], cimetidine in serum [*6*], thiopental in serum or plasma [*7*], cefpiramide in plasma [*8*], and illicit drugs in urine [*9*]. The purpose of this paper is to present the application of MECC methodology for determination of barbiturate concentrations in biological fluids. Results of the use of MECC in four actual forensic cases are presented.

## Materials and Methods

### *Analytical Reagents*

1. Buffer: 10 mM borate/10 mM phosphate/100 mM SDS at pH 8.5. Mix 3.8 g sodium borate, 10 g hydrate ($Na_2B_4O_7 \cdot 10H_2O$, Baker Chemical Co., Phillipsburg, NJ), 1.4 g sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$, Fisher Scientific Co., Fair Lawn, NJ), 28.8 g sodium dodecyl sulfate (SDS, Lauryl sulfate-electrophoresis reagent, Sigma Chemical Co., St. Louis, MO) and dilute to one liter with deionized water.
2. Acetonitrile (HPLC grade, Fisher Scientific Co., Fair Lawn, NJ).
3. Capillary Electrophoresis "Run" Buffer: Mix 85% buffer with 15% acetonitrile (vol/vol) and adjust pH to 8.5 with 1.0 M phosphoric acid. Run buffer was filtered and degassed (under vacuum) with Whatman GF/F filters (Whatman International, Maidstone, England).
4. TOXI-TUBES B (Toxi-Lab Inc., Irvine, CA).
5. Internal standard: mephobarbital (100 μg/mL). Mephobarbital (2 mg, crystalline, Sigma Chemical Co., St. Louis, MO) was made up to 20 mL with deionized water after addition of two drops of absolute ethanol (Florida Distiller's, Lake Alfred, FL) for stability.
6. Analytical standards: Amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital (Sigma Chemical Co., St. Louis, MO) were made to concentrations of 1 mg/mL in ethanol then diluted as necessary.

### *Extraction*

Two milliliters of sample (serum, blood, vitreous humor, gastric contents (diluted 1:10) or urine), 2.5 mL water and 200 μL internal standard were added to the TOXI-TUBE B. Tubes were mixed

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for 10 minutes on a sample rocker (Baxter Scientific, McGraw Park, IL) then centrifuged at 2000 rpm for 5 minutes. The organic layer was transferred to a concentration cup and dried under a stream of nitrogen at room temperature. Residue was reconstituted in 100 μL of run buffer and vortexed for 30 s then transferred to 0.5 mL polypropylene sample vials (Waters, Milford, MA) and placed in the sample carousel of the Quanta 4000.

*Instrumentation*

Samples were analyzed on a Waters Quanta 4000 Capillary Electrophoresis System with a Waters 745 Data Module. Analytical conditions were: hydrostatic sampling time, 10 s; run time, 15 min; voltage, 20 KV; auto purge between samples, 1 min; ultraviolet detection, 214 nm; absorbance range, 0.01 full scale; and time constant, 0.01. A Waters Accusep Capillary (75 μ × 60 cm) was used for these analyses. The 745 Data Module settings were attenuation, 8; chart speed, 0.5; and peak width, 3.

*Linearity and Reproducibility Experiments*

Serum and urine samples were spiked with amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital to concentrations of 3 to 60 μg/mL for determination of linearity. Aliquoted samples of each barbiturate (10 and 20 μg/mL) were analyzed to determine within day ($n$ = 4) and between day ($n$ = 4) coefficients of variation.

*Calculations*

Linear regressions for the standard curves of concentrations and peak area ratios (peak area of analyte/peak area of internal standard) as well as means and standard deviations and errors were calculated using a model 1860 statistical programmable printing calculator (Monroe, The Calculator Co., Orange, NJ). Specimen concentrations were determined by interpolation of their peak area ratios from those of the standard curves. Coefficients of variation were determined by dividing the standard deviations by the means and multiplying by one hundred.

*Autopsy Specimen Collection and Analyses*

Specimens were collected on the autopsies as they were available. Blood (60 mL) was collected by needle aspiration from the subclavian vein and stored in six (10 mL) Vacutainer tubes: two of the tubes were sterile, two tubes contained potassium ethylenediaminetetra-acetate ($K_3$EDTA), and two tubes contained sodium fluoride and potassium oxalate. Vitreous humor was collected by needle puncture of the posterior chambers of both eyes and stored in a sterile Vacutainer tube. Urine was collected by bladder puncture and stored in a plastic specimen container. Gastric contents were collected and stored in a plastic specimen container. Plasma and serum were separated by centrifugation at 2000 rpm for 10 min. The biological fluids were stored at 2 to 4°C until analyses.

The blood and urine specimens were tested for ethanol using the Abbott TDx/Radiative Energy Attenuation (REA) ethanol assay (Abbott Laboratories, North Chicago, IL). The ethanol concentrations were determined by gas-liquid chromatography [*10*]. The biological fluids and gastric contents were analyzed for numerous acidic, basic, and neutral drugs and metabolites including narcotics and other analgesics; barbiturates and other sedative hypnotics; benzodiazepines; cannabinoids; cocaine; phencyclidine; phenothiazines; sympathomimetic amines; and tricyclic antidepressants by a combination of thin-layer chromatography (TLC) (Toxi-Lab System, Toxi-Lab Inc., Irvine, CA), gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), enzyme-multiplied immunoassay technique (EMIT, Syva Co., San Jose, CA), fluorescence polarized immunoassay (FPIA, Abbott Laboratories, North Chicago, IL), and specific colorimetric procedures.

## Results

A typical electropherogram for a mixture containing 5 μg/mL of each barbiturate (and mephobarbital, internal standard, 10 μg/mL) extracted from serum is illustrated in Fig. 1. Migration times

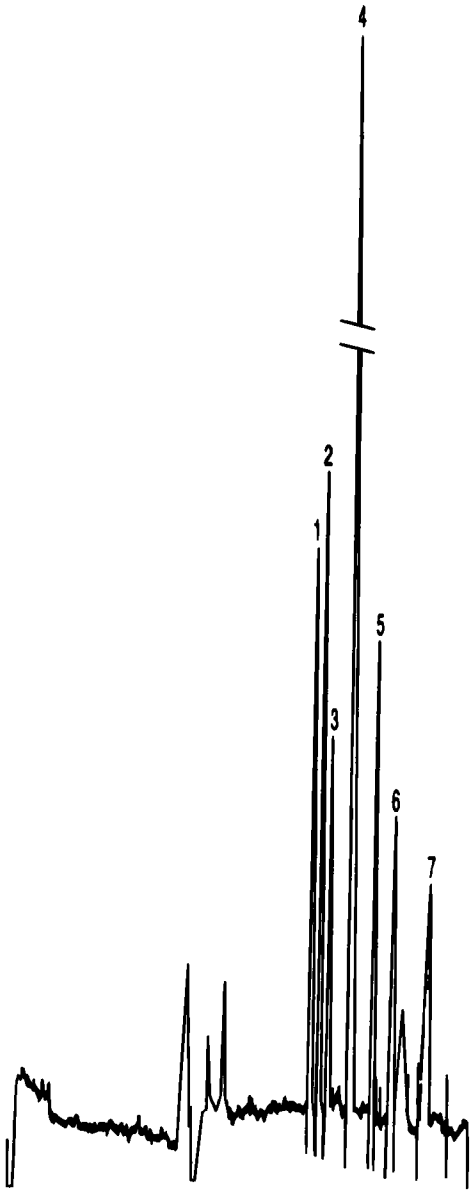


FIG. 1—*Electropherogram of barbiturates (5 μg/mL) extracted from serum. Peak identifications are: (1) phenobarbital (7.78 min), (2) butalbital (8.01 min), (3) butabarbital (8.23 min), (4) mephobarbital (8.88 min, internal standard, 10 μg/mL), (5) amobarbital (9.41 min), (6) pentobarbital (10.03 min) and (7) secobarbital (10.79 min).*

were: phenobarbital, 7.78 min; butalbital, 8.01 min; butabarbital, 8.23 min; mephobarbital (internal standard), 8.88 min; amobarbital, 9.41 min; pentobarbital, 10.03 min; and secobarbital, 10.79 min. Correlation coefficients ($r$) between peak area ratios and concentrations for each barbiturate in serum and urine are given in Table 1. The correlation coefficients ranged from 0.964 to 0.999. Mean detected concentrations and standard errors as well as coefficients of variation, both within day and between days, for each barbiturate in serum are given in Table 2. Coefficients of variation ranged from 7.6 to 8.6% between days and 2.3 to 9.8% within day. The minimum detectable concentration was 0.1 μg/mL. The mean relative migration times of each barbiturate and their standard errors and coefficients of variation are given in Table 3. This methodology was applied to four forensic cases of butalbital intoxication. Butalbital concentrations in various biological fluids as well as other drugs detected and the causes of death in these cases are presented in Table 4.

TABLE 1—*Correlation coefficients.*

| Analyte | Specimen | Concentration Range[a] | Correlation Coefficient[b] |
|---|---|---|---|
| Amobarbital | Serum | 3 to 30 | 0.9990 |
| | Urine | 3 to 30 | 0.9995 |
| Butabarbital | Serum | 3 to 30 | 0.9640 |
| | Urine | 3 to 30 | 0.9999 |
| Butalbital | Serum | 3 to 30 | 0.9962 |
| | Urine | 3 to 30 | 0.9560 |
| Pentobarbital | Serum | 3 to 30 | 0.9999 |
| | Urine | 3 to 30 | 0.9645 |
| Phenobarbital | Serum | 6 to 60 | 0.9978 |
| | Urine | 6 to 60 | 0.9996 |
| Secobarbital | Serum | 3 to 30 | 0.9849 |
| | Urine | 3 to 30 | 0.9988 |

[a]Concentrations were in μg/mL.
[b]Correlation coefficient ($r$).

TABLE 3—*Relative migration times (RMT).*[a]

| Analyte | Mean RMT | $n$ | SE[b] | CV[c] |
|---|---|---|---|---|
| Amobarbital | 1.0584 | 18 | 0.0027 | 1.1 |
| Butabarbital | 0.9197 | 18 | 0.0015 | 0.7 |
| Butalbital | 0.8981 | 18 | 0.0022 | 1.0 |
| Pentobarbital | 1.1317 | 18 | 0.0071 | 2.7 |
| Phenobarbital | 0.8134 | 18 | 0.0119 | 6.2 |
| Secobarbital | 1.2522 | 18 | 0.0056 | 1.9 |

[a]Relative migration time: migration time of analyte/migration time of the internal standard (mephobarbital).
[b]Standard error.
[c]Coefficient of variation (%).

## Discussion and Conclusions

Barbiturates extracted from biological fluids can be identified by a variety of analytical methods including classical ultraviolet spectrophotometry; gas chromatography of derivatives and non-derivatives with flame-ionization, nitrogen-phosphorus or electron-capture detection; high performance liquid chromatography; and various immunoassays [*11*]. The development of the MECC methodology reported herein offers a different qualitative and quantitative analytical technique.

The MECC separation was obtained with a short, small bore capillary column with minimal organic/aqueous buffer solution (<2 mL/analysis) and no derivatization. Baseline separation was obtained for the standard therapeutic barbiturates (Fig. 1) in under eleven minutes. The excellent separation capabilities of MECC are demonstrated by the separation of butalbital and butabarbital. These two barbiturates are often difficult to separate with common chromatographic methods and need special chromatographic procedures for differentiation [*12*]. The hydrostatic sampling (versus electromigratory) and MECC separation produced accurate and precise quantitation (Table 2) as well as excellent reproducibility in migration times of the barbiturates (Table 3). The ultraviolet detection at 214 nm (versus 254 nm) offered an adequate minimum detectable concentration and a linear determination of therapeutic to lethal barbiturate concentrations (Table 1).

The operation of MECC methodology is more similar to high performance liquid chromatography (HPLC) than it is to capillary gas chromatography (CGC). Capillary electrophoresis buffers must be made precisely to achieve good reproducible electropherograms, and filtered and degassed in order to avoid gas bubble formation within the capillary as it heats up under large potentials (especially if they contain significant portions of organic solvents). These

TABLE 2—*Within day and between day statistics.*

| Analyte | Concentration[a] | Within Day | | Between Day | |
|---|---|---|---|---|---|
| | | Mean ± SE[b] | CV[c] | Mean ± SE[b] | CV[c] |
| Amobarbital | 10 | 7.7 ± 0.19 | 5.1 | 8.6 ± 0.37 | 8.6 |
| | 20 | 21.9 ± 0.60 | 5.5 | 20.1 ± 0.54 | 5.4 |
| Butabarbital | 10 | 11.3 ± 0.33 | 6.0 | 12.3 ± 0.17 | 2.9 |
| | 20 | 19.9 ± 0.29 | 3.0 | 20.1 ± 0.36 | 3.4 |
| Butalbital | 10 | 11.8 ± 0.26 | 5.4 | 11.6 ± 0.45 | 7.8 |
| | 20 | 26.8 ± 0.64 | 4.8 | 25.5 ± 0.89 | 6.9 |
| Pentobarbital | 10 | 10.0 ± 0.17 | 3.4 | 11.2 ± 0.28 | 5.1 |
| | 20 | 19.5 ± 0.22 | 2.3 | 20.5 ± 0.26 | 2.6 |
| Phenobarbital | 10 | 8.1 ± 0.39 | 9.8 | 8.5 ± 0.21 | 4.9 |
| | 20 | 17.5 ± 0.47 | 5.5 | 19.1 ± 0.42 | 4.4 |
| Secobarbital | 10 | 9.3 ± 0.39 | 8.5 | 10.8 ± 0.24 | 4.6 |
| | 20 | 19.4 ± 0.30 | 3.1 | 21.0 ± 0.61 | 5.8 |

[a]Concentrations were in μg/mL of serum.
[b]Mean ± standard error.
[c]Coefficient of variation (%).

TABLE 4—*Results from forensic cases.*

| Butalbital Concentrations[a] | | | | | |
|---|---|---|---|---|---|
| Serum | Vitreous | Gastric | Urine | Other Drugs Detected | Cause of Death |
| 0.7 | 0.8 | ND | 1.5 | Acetaminophen, alcohol, amitriptyline, caffeine, dextromethorphan, nicotine and metabolite, nortriptyline, norpropoxyphene, propoxyphene and salicylates | Exsanguination; multiple trauma; vehicular accident. |
| 2.6 | 1.0 | ND | NA | Caffeine | Exsanguination; multiple trauma; vehicular accident. |
| 12.7 | NA | 1.7 | 7.6 | Acetaminophen, benzodiazepines, nicotine and metabolite | Anoxia due to drug overdose. |
| 1.7 | 1.9 | NA | NA | Benzodiazepines, benzoylecgonine, ethanol, lidocaine, methadone and morphine | Multiple drug poisoning. |

[a] Concentrations in serum, vitreous and urine were μg/mL while gastric was mg/mL.
NA: Specimen not available.
ND: None detected.

bubbles can then produce noisy baselines and false peaks that interfere in the analyses especially with low wavelength ultraviolet detection and can even short out the field and abort the run. The sodium dodecyl sulfate (SDS, lauryl sulfate) is used to form the micelles in this MECC method and it causes significant foaming when the run buffer is filtered and degassed. The foam must be avoided or allowed to dissipate before pipetting buffer into reservoirs for analyses as it can cause the same problems as organic solvent bubbles.

Buffer depletion can occur with capillary electrophoresis, which does not occur with HPLC since the mobile phase is homogeneous and is under continuous flow through the column. Electrophoretic flow of ions occurs from one electrode reservoir to the other through the capillary. Repeated analyses with the same reservoirs can therefore result in buffer or ion depletion, which can effect both injection accuracy and analyte separation. Limiting the number of analyses per buffer aliquot to five or less circumvents this problem. Automation of the analyses makes this relatively simple. The MECC methodology described is automated and as easily operated as automated HPLC or CGC systems encountered by the authors.

Electropherograms are more similar to CGC chromatograms than HPLC chromatograms in peak shape and size characteristics. Separation efficiencies are equal or greater than CGC. The use of short (60 cm) noncoated capillaries, which are relatively inexpensive, is a distinct advantage versus typical capillaries used in CGC or HPLC columns. Bulk capillary tubing can be easily cut to desired length and the optical detection section made by burning the polyamide coating off the fused silica capillary. Capillaries of various bore sizes (10 to 100 μ) can be used which allows for detection of a wide range of analyte concentrations. "Overloading" of the capillary will produce prepeak shouldering in this technique just as is CGC. Since the optical detection "cell" is actually within the capillary itself the bore size has a directly proportional effect on the limit of detection and the separability of the analytes. The internal diameters most useful are 50 to 75 μ, as narrower bores clog easily and larger bores result in increased heat production. Limit of detection with reasonable size biological specimens (up to 2 mL) is no problem when detecting drugs, (such as barbiturates) which are significant in the 0.1 μg/mL to 100 μg/mL concentration range. Detection of lower nanogram per milliliter drug concentrations would be difficult unless larger specimens (5 mL or greater) could be extracted and concentrated. Specialized capillaries are now available with pre-concentration zones at the sampling end; however, we have not used this technique and do not know how much more sensitivity might be achieved.

The MECC methodology described was applied to four actual forensic cases of butalbital intoxication including a lethal overdose of Fiorinal. Numerous other drugs were detected (Table 4) in various specimens from these cases by our comprehensive toxicological investigation. None of the other drugs were found to coelute or interfere with the basic drug extraction and MECC analysis of the barbiturates. MECC analysis offers a new analytical technique for barbiturates that is applicable to forensic analysis of biological fluids.

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