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Article

Sensitivity Enhancement for Direct Injection Capillary Electrophoresis to Determine Morphine in Human Serum via In-capillary Derivatization

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Abstract

Rapid and simple micellar electrokinetic chromatography (MEKC) with in-capillary derivatization and fluorescence detection has been developed to determine morphine in human serum. The sample was introduced into a background electrolyte (BGE) containing potassium ferricyanide, whereas morphine was oxidized into highly fluorescent product, pseudomorphine. Different parameters for derivatization and subsequent separation were systematically investigated for the analysis of morphine in serum. Efficient performance of the developed MEKC system was carried out in a single run using BGE made up of 70 mM sodium tetraborate decahydrate (pH 10.5), 0.30 mM potassium ferricyanide, 80 mM sodium dodecyl sulfate, and applied voltage of 9 kV. The combination of MEKC with in-capillary derivatization of morphine was successfully achieved with a high degree of sensitivity. The validation of the method showed good linearity between areas of morphine and the corresponding concentrations over the range of 5-5000 ng/mL. Excellent accuracy and precision were obtained at all concentration levels. The mean recoveries of morphine were ranging from 83.86 to 94.45%. The validated MEKC method successfully permitted determination of morphine in clinical samples after a single oral dose of controlled release morphine sulfate tablets.

Introduction

Morphine is a relatively strong opioid that is routinely used to manage acute and chronic pain (1). Despite its effectiveness in pain relief, it may cause serious side effects which necessitate close monitoring of its levels in biological fluids routinely (2). Several analytical techniques, such as gas chromatography (GC) (3–6) and high-performance liquid chromatography (HPLC) (7–15), have been employed to detect and quantify morphine in biological fluids. However, these methods have drawbacks such as lengthy analysis time and tedious dispensing steps for sample preparation, which makes them inconvenient and increase the chances of error. Moreover, extensive derivatization before analysis is essential in most cases of GC. In an attempt to eliminate these problems, immunological assays such as radioimmunoassay (16) and enzyme immunoassay (17) were the preferred methods for therapeutic drug monitoring, because they were rapid, specific, sensitive and could analyze biological fluids directly without prior sample pretreatment procedures. Unfortunately, immunoassay methods are only limited

to specific drugs and can be impaired by specific and non-specific interferences.

Capillary electrophoresis (CE) has become an increasingly attractive separation technique for the determination of drugs and their metabolites in biological fluids. A variety of CE techniques have been successfully applied to determine a broad range of opioids and their metabolites in various biological matrices (18–26). CE tools are capable of the required sensitivity and accuracy, with characteristics similar to that of HPLC. In addition, CE will offer other advantages, including high efficiency, fast separation, relatively cheap and long-lasting capillary columns, low reagent consumption and very small sample volume requirements. A key feature of CE is the availability of different modes, which expand the scope of its application. The most common modes are capillary zone electrophoresis (CZE) (20, 23) and micellar electrokinetic chromatography (MEKC) (18, 26). It is well known that CE methods, while having some attractive advantages, also have some shortcomings compared to modern chromatographic methods. While the use of small internal diameter capillary is responsible, in part, for high separation efficiency achievable in CE, it also places limitation on the total amount of analyte that can be loaded onto the capillary. Moreover, the most commonly used detector for CE is UV–Vis which suffers from relatively low sensitivity due to the short optical path length provided by the smaller capillary diameter. The small quantities of analyte that are involved in CE demand that only very sensitive detection techniques should be used to fully utilize the power of CE. Recently, mass spectrometric detection in combination with CE (21, 24) has also been increasingly used but, this method are not ideal for routine clinical purposes, because the procedures are time consuming and the instrumentation is not always available in routine clinical laboratories.

Automated methods for analysis are now urgently needed and should possess enough throughput, robustness, selectivity and sensitivity to determine therapeutic drug levels in variety of fields and applications. To this end, various analytical techniques were developed, among them; MEKC stands out due to its flexibility, which expands the application range of CE techniques to separate analytes in bio-fluids by direct injection (18, 26). In MEKC, surfactants above their critical micellar concentrations are added into the background electrolyte (BGE) to form micelles. One of the most widely used surfactants in MEKC is sodium dodecyl sulfate (SDS) micelles (26), added as pseudo-stationary phase. Separation occurs by differential partitioning of the analytes between the aqueous BGE and the pseudo-stationary phase. Thus, MEKC technique can be considered as a hybrid of electrophoresis and chromatography.

Moreover, chemical derivatization can improve upon the sensitivity and selective of CE-based methods. The derivatization can be carried out before introducing the sample into the capillary (pre-capillary), inside the capillary (in-capillary), or between the outlet of the capillary and the detection point (post-capillary) (27). Unfortunately, off-line chemical modifications in CE can be time-consuming and unreliable, resulting in the dilution of the sample and affecting the separation process. On the other hand, in-capillary derivatization techniques, where the capillary is used as a small reaction chamber, have been widely applied due to its remarkable advantages over the conventional pre- and post-capillary derivatization. Among these advantages are: the low consumption of reagents and sample, short reaction time, and the possibility of automation without additional equipment. In-capillary derivatization can be achieved through different strategies which can be classified into three groups: zone-passing (28), at-inlet (29) and through the electrophoretic solutions containing the reagents (30).

Intact morphine possesses low native fluorescence; however, it can be oxidized into highly fluorescence pseudomorphine by reaction with ferricyanide in an alkaline medium (13, 31–33). Accordingly, derivatization of morphine on-line into a highly fluorescent product before performing analysis in biological fluids is needed. For this type of derivatization, in-capillary mode was a better choice for MEKC and fluorescence detection of morphine in serum. With a more efficient therapeutic application of various drugs and the need to examine their concentrations in body fluids for diagnostic and research purposes, there has evolved the need for reliable and automated analytical procedures. Therefore, it was important to investigate the potential of MEKC for the determination of morphine in human serum and to establish suitable conditions for automated derivatization and separation in a single run without any sample pretreatment step. The applicability of the developed method to practical serum samples was also emphasized in the present work.

Experimental

Instrumentation and general procedure

Separation was performed in a fused-silica capillary covered with a polyamide-coating layer (Polymicro Technologies, Phoenix, AZ, USA). The total length of the capillary was 90 cm, and the length from the inlet end to detection point was 80 cm with 75 μm i.d. and 360 μm o.d. The detector was a FP-920 fluorescence detector (Jasco), equipped with the flow cell unit for HPLC replaced by the capillary cell unit for CE (Jasco, Tokyo, Japan). The electropherograms were recorded by monitoring the fluorescence intensity at 450 nm (excited at 340 nm). Both ends of the capillary were separately dipped in the anodic and cathodic solutions, having the same composition as the BGE, and the surface of these electrode solutions were adjusted to the same level. The system is equipped with A model HCZE-30 PNO 25-LDS stabilized high voltage power supply to apply voltage up to 30 kV (Matsusada Precision Devices, Japan). The high-voltage end of the capillary was enclosed in an acrylic glass enclosure as a safety precaution.

The CE system was operated using normal polarity (the cathode was located on the detector side). Injection was done in a hydrodynamic fashion by dipping the injection end of the capillary in the sample reservoir for 12 s while it rested 15 cm above the cathodic end reservoir. After the sample was introduced, the capillary was placed back into the BGE vial, and a potential of 9 kV was applied between both ends of the capillary, to move morphine for derivatization and to transport the resulting fluorescent product to the detector. Electropherograms were processed and recorded on a chromatopack integrator C-R6A (Shimadzu, Kyoto, Japan). The CE instrument was operated at ambient temperature (22°C ± 1°C). The CE system used a BGE consisting of 70 mM sodium tetraborate decahydrate (pH 10.5), 0.30 mM potassium ferricyanide and 80 mM SDS.

The electrophoretic mobility of morphine and the first serum peak was calculated according to the formula:

$$\mu = [(L_t/t_m) - (L_t/t_{eo})]/(V/L_d),$$

where μ is the electrophoretic mobility of the analyte, t_m the migration time measured directly from the electropherogram, t_{eo} the

migration time of the electro-osmotic flow (EOF) marker, L_t the total length of the capillary, L_d the length of the capillary between injection and detection, and V is the applied voltage.

All new uncoated fused-silica capillaries were previously flushed for 20 min with 1 M NaOH solution, 5 min with 0.1 M NaOH solution, 5 min with Milli-Q deionized water, followed by 15 min with the BGE. When the CE system was not in use, the BGE in the capillary and in both reservoirs was replaced with Milli-Q deionized water.

Materials and reagents

All chemicals were obtained at the highest purity grade available from the manufacturers, and used without additional purification. Sodium hydroxide and disodium tetraborate decahydrate were purchased from Fisher Scientific (Spring field, NJ, USA). SDS and potassium ferricyanide were obtained from Sigma (St Louis, MO, USA). Morphine sulfate, a certified pharmaceutics sample of >99.63% purity and oral controlled-release morphine sulfate tablets (MST Continus, Napp Laboratories, United Kingdom) were gifts from the Department of Anesthesia, Faculty of Medicine, Assiut University (Assiut, Egypt) and used as received.

Borate solution

The borate solutions over the concentration range of 10–100 mM disodium tetraborate decahydrate were prepared. The pH of borate solution (70 mM) was adjusted over the range from 7.5 to 12. All reagent solutions were prepared with distilled deionized water purified with an ultra-pure water Milli-Q system and filtered through a 0.45-μm Millipore membrane filter (Millipore, Bedford, MA, USA). The pH of borate solutions were determined by a Ross combination pH electrode (Orion, Boston, MA, USA).

Calibration standards and quality control samples

A stock solution of morphine sulfate was prepared at a concentration of 10 mg/ml by dissolving an appropriate amount of the drug in ultra-pure deionized water. Standard solutions of morphine were prepared from the stock solution with the same solvent to achieve the concentration range of 0.05–50 μg/mL. The standard solutions were protected from light by wrapping the containers with aluminum foil. Each standard solution was diluted 10-fold into drug-free human serum to obtain the concentration range of 5–5,000 ng/mL. Calibration standards were freshly prepared and stored at −20°C. Prior to assay, frozen serum samples were thawed at ambient temperature and centrifuged at 2,000 g for 5 min to precipitate solids followed by filtration of the supernatant through 0.45 μm Millipore filters. Five replicates of each sample were injected into the CE system.

Recovery

Working standard solutions at four different concentration levels (0.05, 0.5, 5 and 10 μg/mL) were diluted 10-fold into drug-free human serum to obtain quality control (QC) serum samples of 5, 50, 500 and 1,000 ng/mL. The recovery of the drug from serum sample was calculated using the following equation:

$$\%\ \text{Recovery} = \left[\frac{(\text{mean measured concentration})}{(\text{nominal concentration})}\right] \times 100.$$

Precision and accuracy

Precision and accuracy were determined by analyzing QC serum samples at 5, 50, 500 and 1,000 ng/mL morphine. Intra-assay precision was calculated as the relative standard deviation (RSD %) of the mean concentration resulting from the same day. Inter-assay precision was assessed by the RSD % of the mean concentration on five consecutive days. The accuracy was determined by the percent of the relative errors (RE %) of the mean measured concentrations according to the following equation:

$$\text{RE}\% = \left[\frac{(\text{mean measured concentration} - \text{nominal concentration})}{\text{nominal concentration}}\right] \times 100.$$

Results

Borate electrolyte

The reaction of morphine with ferricyanide was always carried out in alkaline condition. Accordingly, the pH of the BGE is a dominant factor when determining morphine using CE with in-capillary derivatization technique. In the present study, the potential of disodium tetraborate decahydrate as a running electrolyte to perform in-capillary derivatization was examined. The effect of pH on the fluorescence intensity was studied over the range of 7.5–12 (corresponding borate concentration was 70 mM). Figure 1 shows that the fluorescence intensity of morphine was low at pH values <9.5, whereas the detector response could be improved with increasing pH values up to 10.5, above which, it decreased gradually.

The variations of the electrophoretic mobility of morphine and the first serum peak as a function of pH were further investigated over the range 7.5–12 (corresponding borate concentration was 70 mM) (Figure 2). It was noticed that, the electrophoretic mobility of the serum peak was lower than that of morphine (Figure 2). Hence, the migration times of the serum peak were prolonged and the resolution of morphine from the serum peak was improved at pH values >10. Good baseline resolution and peak sharpness were

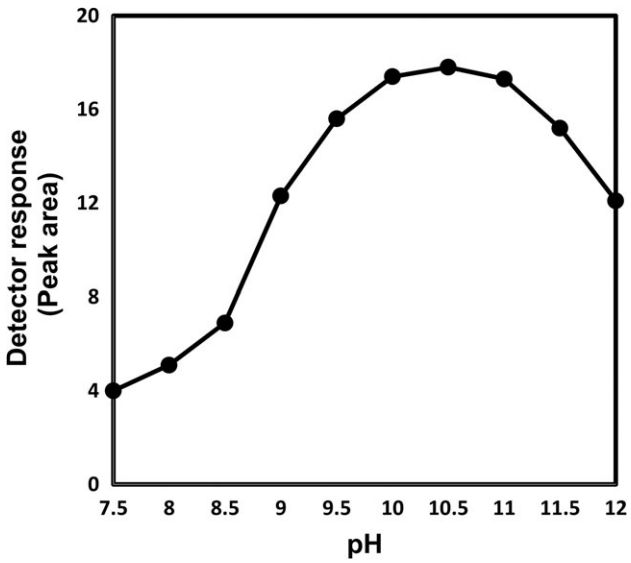

Figure 1. Dependence of the fluorescence intensity of morphine spiked in drug-free human serum on the pH of 70 mM disodium tetraborate decahydrate.

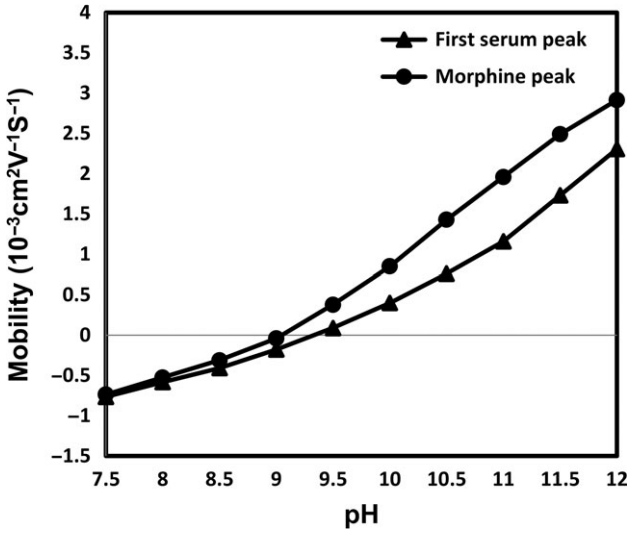

Figure 2. Dependence of the electrophoretic mobility of morphine spiked in drug-free human serum on the pH of 70 mM disodium tetraborate decahydrate.

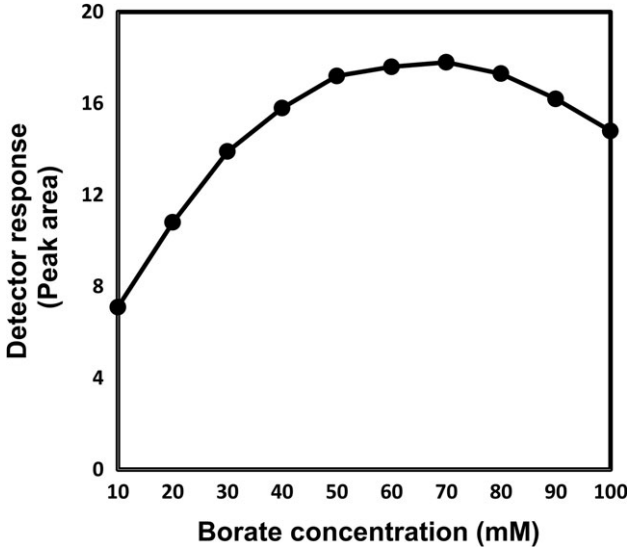

Figure 3. Dependence of the fluorescence intensity of morphine spiked in drug-free human serum on the concentration of disodium tetraborate decahydrate at pH 10.5.

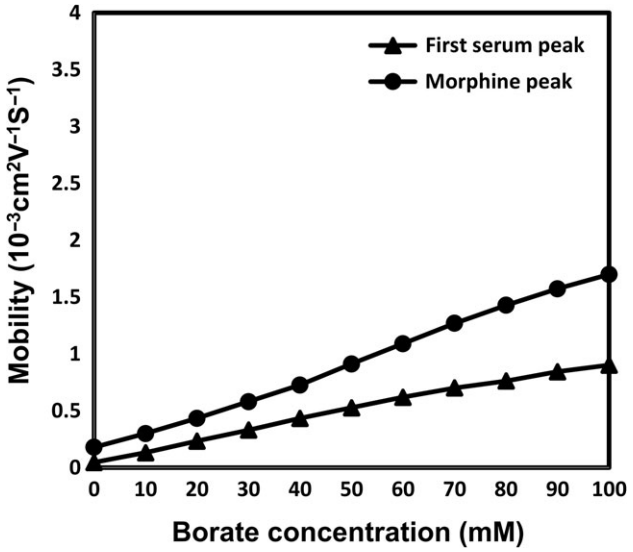

Figure 4. Dependence of the electrophoretic mobility of morphine spiked in drug-free human serum on the concentration of disodium tetraborate decahydrate at pH 10.5.

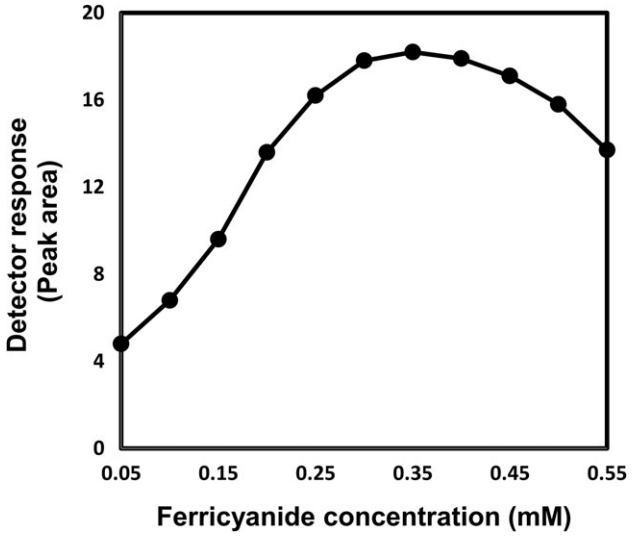

Figure 5. Dependence of the fluorescence intensity of morphine spiked in drug-free human serum on the potassium ferricyanide concentration.

obtained at pH 10.5, while pH values >10.5 resulted in more baseline noise. As a result, pH of 10.5 was required to achieve high fluorescence intensity with acceptable baseline resolution.

The variations of the fluorescence intensity and electrophoretic mobility of morphine, as a function of borate concentration, were studied over the range of 10–100 mM (pH 10.5). As shown in Figure 3, the fluorescence intensity increased with increasing borate concentration from 10 to 70 mM, and then decreased gradually. Increasing borate concentration above 70 mM resulted in higher current flow which in turn led to increasing Joule heating and decreasing fluorescence intensity. While results in Figure 4 showed that, the mobility difference between morphine and the first serum peak was improved gradually with increasing borate concentration up to 100 mM. Based on these results and to balance between the system efficiency and Joule heating, 70 mM was selected as the optimum concentration to give high fluorescence intensity with acceptable baseline resolution.

Potassium ferricyanide concentration

To establish optimal reagent concentration, the dependence of fluorescence intensity on the potassium ferricyanide was examined over concentration range of 0.05–0.55 mM (Figure 5). Our studies revealed that an increase in ferricyanide concentration significantly increased the fluorescent signal of morphine up to 0.35 mM above which the detector response decreased gradually. At the same time, the effect of baseline noise on the system performance increased when the concentration of ferricyanide was >0.30 mM. As a result,

0.30 mM ferricyanide was selected as an optimum concentration to compromise between higher sensitivity and lower baseline noise.

Sodium dodecyl sulfate

The effect of SDS on the electrophoretic mobility of morphine in serum sample was studied over the range of 10–100 mM. As shown in Figure 6, the mobility of serum proteins decreased significantly with the increase of SDS concentration. Hence, the proteins migrated later than morphine and a great improvement in resolution was observed over the concentration range of 40–100 mM. A concentration of 80 mM of SDS presented in the BGE was efficient for base line resolution of morphine when serum samples were directly injected without any sample pretreatment step.

Applied voltage

The applied voltage for high in-capillary derivatization was another important factor as it directly affected the electrophoretic mobility of morphine through the BGE, which led to a change in the fluorescence intensity. Accordingly, the dependence of fluorescence intensity and baseline resolution of morphine on the applied voltage was examined over the range from 7 to 14 kV. The electrophoretic mobility was relatively constant, with a rising tendency, when the voltage was increased from 7 to 11 kV. Above 12 kV an even shorter migration time could be achieved, but a more noise was observed resulting in a lower signal-to-noise ratio. An optimum applied voltage to compromise between fluorescence intensity and baseline resolution was found to be 9 kV.

Sample size

In order to improve sensitivity, the amount of injected sample was increased by increasing the injection time (4–20 s). It was observed that the length of the sample plug, reflected by the sample injection time, was one of the critical factors affecting the sensitivity of the MEKC method. The results clearly indicated that an increase in the injection time led to an increase in the detector response, but when this parameter was higher than 12 s, peak signal was not increased linearly and became broader. For this reason, the value of 12 s was recommended as an optimum time for sample injection.

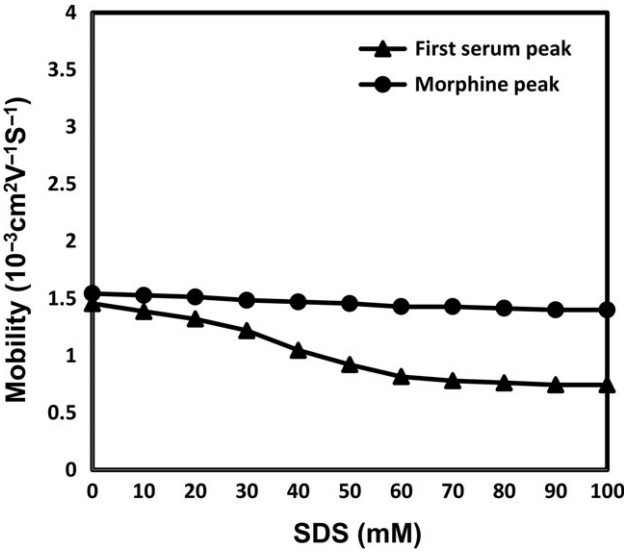

Figure 6. Dependence of the electrophoretic mobility of morphine spiked in drug-free human serum on SDS concentration.

Alkaline rinsing procedure

The reliability of MEKC method was an important concern to be assessed in the present study. Regular reactivation of the internal surface of capillary wall by rinsing with alkaline solution to improve both the efficiency and reproducibility was required for the validation of the method. To test the long-term reproducibility with the running BGE, the spiked serum sample containing 100 ng/mL morphine was injected every 30 min. After measurement for 5 h, migration time increased by ~16%. The peak was broad and the signal-to-noise ratio was reduced to ~18% with respect to the initial value. The resulting problems could be overcame by using between-run wash-cycle using Milli-Q deionized water for 0.5 min, 0.1 M NaOH for 1 min, followed by Milli-Q deionized water for 0.5 min, afterwards, the capillary was filled with the running BGE.

Method validation

Linearity

To evaluate the linearity of the developed method, seven different concentrations of analyte spiked in drug-free human serum were analyzed. Calibration curve was constructed by plotting the measured peak areas against the concentrations of morphine in the range of 5–5,000 ng/mL. The calibration curve was linear, with a correlation coefficient of 0.9995. Sample concentration was determined by the least-squares linear regression equation, using the formula $Y = a + bC$, where Y = peak area, C = concentration of morphine in ng/mL, b = the slope of the curve and a = the intercept with Y axis. The limit of detection (LOD), defined as the smallest concentration of the analyte that can be reliably detected above the base line signal, is evaluated as three-times the signal-to-noise ratio. The LOD was determined ($n = 6$) by injection of spiked drug-free human serum samples with morphine in decreasing concentrations. The limit of quantification (LOQ) is defined as 10 times the signal/noise ratio. The LOQ was determined ($n = 6$) by injection of spiked drug-free human serum samples with morphine in decreasing concentrations. The LOQ was calculated as the concentration that resulted in precision below 20%. All obtained analytical data including linearity, correlation coefficient and others are presented in Tables I. The values of LOD and LOQ confirmed that MEKC approach offered real option to determine morphine in human serum.

Recovery, precision and accuracy

For evaluation of precision and accuracy of the developed MEKC method, the recovery, RSD and RE of morphine spiked in drug-free human serum were investigated at three different concentration

Table I. Characteristic Parameters for the Regression Equation of the MEKC Method

Parameters	MEKC
Calibration range (ng/mL)	5–5,000
Detection limit (ng/mL)	1.14
Quantitation limit (ng/mL)	3.78
Slope (b)	0.1335
Intercept (a)	−0.2063
Correlation coefficient (r^2)	0.9995

$Y = a + bC$, where C is the concentration of morphine in ng/mL and Y is the peak area.

Table II. Precision and Accuracy Validation of the MEKC Method

Nominal concentration (ng/mL)	Recovery (%)[a] ± SD	RSD (%)	Mean RE (%)
Intra-assay			
5	83.86 ± 7.83	9.34	−16.14
50	87.14 ± 6.95	7.98	−12.86
500	93.32 ± 4.14	4.44	−6.68
1,000	94.45 ± 3.42	3.62	−5.55
Inter-assay			
5	82.98 ± 8.35	10.06	−17.02
50	86.42 ± 7.64	8.84	−13.58
500	92.70 ± 4.65	5.02	−7.30
1,000	94.01 ± 3.87	4.12	−5.99

[a]Average of five determinations.

levels. The intra-day precision and accuracy were good, with % RSD ranging from 3.62 to 9.34% and with mean RE % ranging from −5.55 to −16.14% (Table II). At the same concentration levels, the inter-day %RSD was in the range of 4.12–10.06% and the mean %RE ranged from −5.99 to −17.02%. Repeatability and reproducibility of morphine in serum samples with high and low concentration levels indicated the reliability of measurement using the proposed MEKC method. The results of the recovery experiments were adequate; the mean recovery values of morphine were found to be consistent and ranged from 82.98 to 94.45%.

Selectivity

The possible occurrence of endogenous compounds in serum samples that would co-migrate with morphine was the first aspect of the MEKC method to be investigated. Therefore, several drug-free human serum samples of different health subjects and samples of drug-free human serum spiked with morphine (50 ng/mL) have been tested for the absence of interferences. Good selectivity for the method was obtained as evidenced by the absent of any peaks at or very near the migration time of morphine (Figure 7). The endogenous species detected in serum have migration times longer than that of morphine.

Stability

Morphine stability studies were performed in human serum to ensure the reliability of results with respect to handling and storage of samples. These studies involve evaluating the freeze and thaw stability, short-term stability, and long-term stability. Stability tests were assessed with two concentrations of QC serum samples, i.e., 50 and 500 ng/mL. In freezing and thawing stability test, samples were stored at −20°C for 24 h and thawed at room temperature for approximately 1 h. Triplicate analyses of the QC serum samples at each concentration were done. For the next study day, samples were immediately re-frozen at −20°C. This cycle was repeated for three consecutive days. The short-term stability was evaluated after storage of QC serum samples at 5°C and at room temperature. Furthermore, measuring morphine concentration after storage at −20°C for 4 weeks was carried out to assess long-term stability. The concentration of morphine after each storage period was related with the initial concentration as determined for the newly prepared sample. Experiments showed that there was no significant difference in the mean peak areas after one, two and three cycles of freezing and thawing and fresh QC serum samples. Also, morphine showed

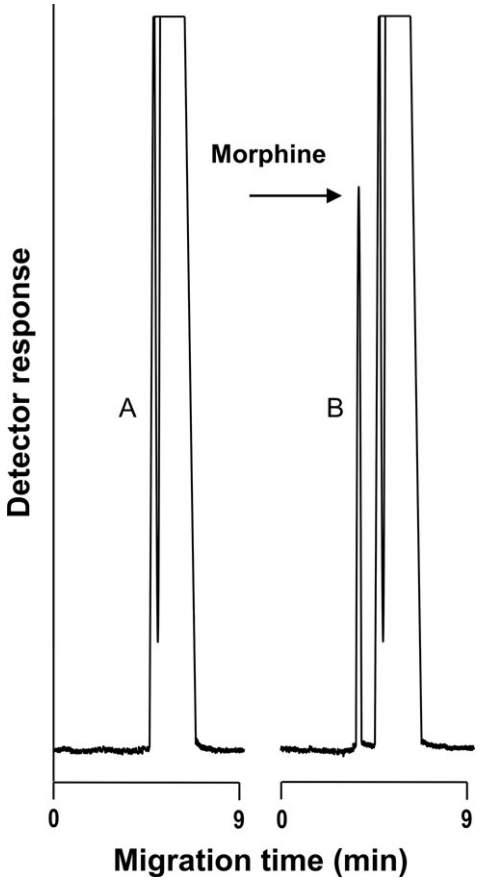

Figure 7. Representative electropherograms for the determination of morphine, A: drug-free human serum; B: drug-free human serum spiked with morphine (50 ng/mL).

no changes in the electropherograms when stored at 5°C for three days and at −20°C for 4 weeks. QC serum samples were found to be stable at room temperature when standing for at least 8 h.

Application

The validated MEKC method was applied to determine the concentration of morphine after a single oral dose of oral controlled-release morphine sulfate tablets (MST Continus, Napp Laboratories, UK, 30 mg/tablet). Figure 8 shows electropherogram of a drug-free human serum sample taken from the volunteer before administrating the drug, as well as the electropherogram of the clinical serum sample collected after 2 h from orally administering 30 mg morphine.

Discussion

The feasibility of the in-capillary derivatization was explored for the improvement of selectivity, as well as enhancement of sensitivity for morphine analysis. Hence, potassium ferricyanide was selected as an oxidizing agent for chemical derivatization of morphine into highly fluorescent product, pseudomorphine. A running electrolyte consisting of disodium tetraborate decahydrate was chosen as being the most suitable BGE based on morphine derivatization and separation. In the CE system with a fused-silica capillary, the direction of the EOF was from the anode to the cathode and the sample of morphine was introduced at the anodic site. The alkaline BGE allowed a strong EOF such that all the compounds, even those with negative

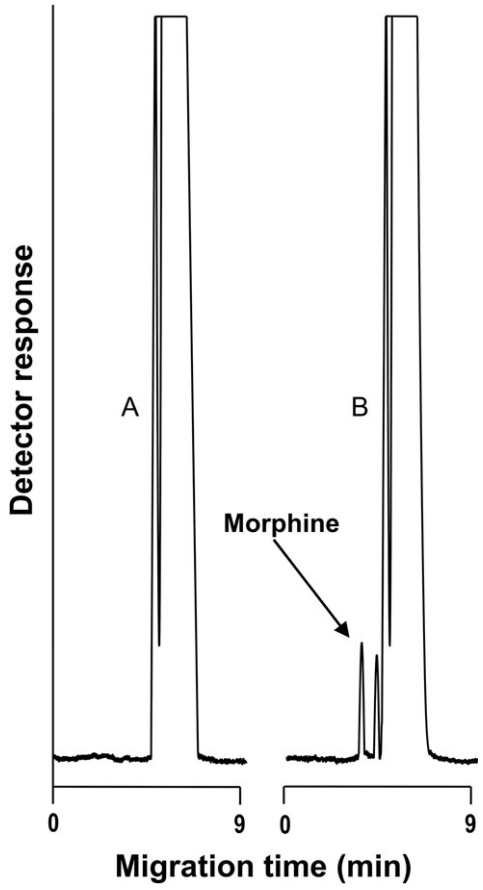

Figure 8. Representative electropherograms for the determination of morphine, A: drug-free human serum; B: serum sample from a subject 2 h after the administration of a single 30 mg dose morphine tablet, in which the concentration was found to be 10 ng/mL.

charge, will move toward the cathode. However, the ionic mobility of the highly negatively charged ferricyanide ions was so large that they would not be carried from the anode to the cathode (34). Consequently, it is important to apply the in-capillary derivatization where the sample zone will migrate toward the cathode in the opposite direction to that of the ferricyanide, and then reacts with the reagent to produce a highly-fluorescent product.

Analysis of morphine in serum by direct injection using the CZE could cause difficulties due to the clogging of capillary columns. This is mainly due to adsorption of proteins on the walls of fused silica capillaries is a well-known and described phenomenon necessitating the pretreatment of serum samples before CZE analysis in order to remove these matrix components. Hence the need to identify the main factors affecting the separation of morphine from endogenous serum constituents without any sample pretreatment procedure. Because of the well-known characteristics of SDS as a protein denaturant, and its effectiveness in preventing adsorption of proteins on the internal capillary wall, it was essential to investigate its use for morphine analysis in serum. Thus, SDS was added to the running BGE to enhance the efficiency of the CE system for drug analysis in serum.

As the formation of pseudomorphine depends on how the derivatization is performed, we have investigated the influence of various parameters to improve the sensitivity and efficiency of MEKC system to determine morphine in human serum. pH and concentration of sodium tetraborate decahydrate, potassium ferricyanide concentration, SDS concentration, applied voltage and time of sample injection were the key parameters.

In MEKC, pH can affect fluorescence intensity of morphine, adsorption of serum proteins onto capillary wall sites and resolution of the analyte from endogenous matrix constituents. The higher pH electrolyte solution eliminated the interactions between the negatively charged proteins and the capillary surface and allowed highly efficient and reproducible capillary electrophoretic separation of morphine from serum matrix. Accordingly, the pH of the BGE was a dominant factor that should be adjusted to optimize baseline resolution and in-capillary derivatization. The influence of pH on the formation of pseudomorphine was studied over the range 7.5–12. The relative fluorescence response was much smaller as the pH values were <9.5, whereas maximum extent of pseudomorphine formation was observed at pH 10.5. At pH values >10.5, the fluorescence intensity decreased gradually due to the short duration stability of the fluorescent product along with increase in baseline noise. On the other hand, when the pH values were lower than 9.5, longer migration times of morphine were obtained due to the reduction in EOF. This could be explained by the fact that, as the pH decreased, the number of surface charges due to silanol ionization on the capillary walls decreased which led to reduction in EOF. At the same time, morphine peak migrated as broad peak and there was an incomplete resolution between morphine and the first eluted serum peak. When pH values were >10, an improvement of the mobility difference between morphine and the first serum peak could be achieved. A convenient compromise between the advantageous effect of high pH on the in-capillary derivatization and separation on one hand and the electrical repulsion of negatively charged proteins with capillary inner surface and the noise of the baseline on the other hand was found to be at pH 10.5.

The concentration of disodium tetraborate decahydrate could affect various aspects of the developed MEKC method. The effect of borate concentration on the fluorescence intensity (Figure 3) and electrophoretic mobility (Figure 4) of morphine was studied over the range of 10–100 mM (pH 10.5). On the basis of fluorescence intensities, the formation of the pseudomorphine appeared to increase gradually with the increase of borate concentration, where the best fluorescent intensity was reached at 70 mM (Figure 3). Further increase of borate concentration resulted in gradual decrease of fluorescence intensity and led to higher current flow. Elevated current flow resulted in increased Joule heating, which could increase baseline noise and reduce the efficiency of the system. The effect of borate concentration on the electrophoretic mobility of morphine and the first serum peak was lower than that of pH. The mobility difference between morphine and the serum peak could be improved by increasing the concentration of borate from 40 to 100 mM (Figure 4). However, the optimum borate concentration for the in-capillary derivatization and separation of morphine from human serum was found to be 70 mM (pH 10.5), which combined an effective derivatization and a sufficient resolution on one hand and reduced levels of baseline noise and Joule heating on the other hand.

It is normally known that, the concentration of derivatization reagents strongly influences the kinetics of the reaction. The feasibility of the in-capillary derivatization was explored in the present study for sensitivity improvement of morphine. Our studies revealed that the fluorescence signal of morphine increased as the concentration of ferricyanide was increased from 0.05 to 0.35 mM and then decreased with concentrations >0.35 mM. Increasing the concentration of ferricyanide

(>0.35 mM) resulted in quenching, and thus, the fluorescence intensity was reduced. When considering the fact that a higher concentration of ferricyanide is useful to maintain a high sensitivity, it is also important to maintain less base line noise. Accordingly, 0.30 mM was selected as an optimum ferricyanide concentration for further studies. The derivatization of morphine was successfully accomplished with about 10-fold sensitivity improvement compared to its native fluorescence at the same concentration level.

The SDS concentration was found to be crucial for the separation of morphine in human serum. A significant improvement in resolution of morphine from endogenous serum constituents was observed when adding SDS to the BGE, of 70 mM disodium tetraborate (pH 10.5), over the concentration range of 40–100 mM SDS. The first serum matrix component migrated slower and later than morphine and a good resolution was achieved. It was found that 80 mM SDS was an optimum concentration to compromise between analysis run time and efficiency of the MEKC system.

The injected sample volume, reflected by the injection time, is one of the most important factors affecting the sensitivity of the in-capillary derivatization mode. Thus, a constant height of 15 cm above the detection end reservoir was maintained, while the sample injection time was varied from 4 to 20 s. The results clearly indicated that the intensity of the fluorescence signal increased with increased injection time. However, peak broadening and poor resolution of morphine from endogenous serum matrix were the main problems upon injection time of more than 12 s. In addition, a long injection time resulted in deviation from linearity, which was due to the fact that, the sample components were too long to be unfavorable to react with ferricyanide reagent, resulting in poor in-capillary derivatization. Thus, 12 s was recommended as an optimal time for the sample injection.

The effect of applied voltage on migration time of morphine was also investigated. It was observed that higher voltage led to lower migration time of morphine with decreasing fluorescence intensity. Higher voltage would also cause large Joule heating, which resulted in an increase in base line noise with LOD being higher than that at the lower voltage. The optimum voltage was determined by performing runs at applied voltages until decline in the system performance was observed. The optimum voltage for the analysis of morphine was found to be 9 kV, which combines sufficient sensitivity with a less baseline noise.

It was observed that capillary inner surface experienced very little changes by the alkaline-wash cycle between runs, which improved the robustness of the method. This is mainly because pH changes during the alkaline-wash cycle activated the capillary inner wall characteristics, which in turn would affect any possible interaction between the capillary surface and the analyte. This is especially important when a negatively charged ferricyanide reagent is used, where there is no interaction of the reagent ions and negatively charged silanol groups on capillary inner wall that should not affect the reproducibility of morphine derivatization. Also, the negatively charged wall repels proteins, which are also negatively charged as their isoelectric point is less than the pH of the operating BGE (pH 10.5). The precision of migration times was also investigated. RSD values were found to be <2% ($n = 5$) for all measurements.

The proposed MEKC method was successfully validated for assay parameters of morphine in serum samples. The selectivity of the method was evaluated by comparing the migration time of morphine reference standard with the results obtained from human serum analysis. In addition, the peak identity of analyte was confirmed by spiking different standard reference solutions of morphine in the drug-free human serum as described in the experimental section.

A high degree of confidence was expressed in the validity of the proposed MEKC method in the appropriateness of this technique to determine the concentration of morphine in human serum after a single oral dose of oral controlled-release morphine sulfate tablets, as there were no potential concomitant interferences arising from the matrix. Preliminary experiments revealed that the concentration of morphine determined by this method was consistent with the values obtained from an alternative method [14], making the present MEKC method promising for pharmacokinetic studies.

Conclusion

Conventional chromatographic separation techniques (e.g., HPLC and GC) often require time-consuming sample preparation protocols for morphine analysis in protein-based bio-fluids. The capability of using MEKC to directly inject proteins including samples such as serum for analysis of morphine has been demonstrated. The combination of MEKC with derivatization of morphine was successfully achieved with a high sensitivity enhancement. The automated derivatization and separation was completed in a single run within 9 min without any sample pretreatment step, eliminating the need for manual labor and giving time for other tasks. A linear calibration curve using spiked drug free human serum with morphine could be achieved over a concentration range of 5–5,000 ng/mL without the addition of an internal standard. Data quality was assured such that, the developed MEKC appeared to provide meaningful technique for clinical and pharmacological research interest and could be considered appropriate for the therapeutic and diagnostic monitoring of morphine.

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