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Research Article

Capillary Electrophoresis with Laser Induced Fluorescence for Determination of Morphine, M3G and M6G in their Pharmaceutical Preparations

Sebaiy MM*

Medicinal Chemistry Department, Faculty of Pharmacy, Zagazig University, Zagazig, Sharkia, Egypt

***Corresponding author:** Sebaiy MM, Medicinal Chemistry Department, Faculty of Pharmacy, Zagazig University, Zagazig, Sharki, Egypt

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Abstract

Novel method for labeling and rapid separation of morphine, Morphine-3-Glucuronide (M3G) and Morphine-6-Glucuronide (M6G) in their pharmaceutical forms by a new boronic acid functionalized Squarylium dye (SQ-BA3) through on-column labeling mode using Capillary Electrophoresis with Laser Induced Fluorescence (CE-LIF) is described. Preliminary spectral studies including absorbance and fluorescence ones has been carried out before separation techniques. SQ-BA3 itself is unstable and weakly fluorescent in aqueous buffers due to soluble aggregates formation but an increase in fluorescence intensity has been reported upon the addition of M3G, morphine and M6G, respectively. This method showed a linearity range of 2.50 x 10^{-4}M - 2.50 x 10^{-3}M for morphine, 1.50 x 10^{-4}M - 1.50 x 10^{-3}M for M3G and 5.00 x 10^{-4}M - 2.00 x 10^{-3}M for M6G and a sensitivity of 1.37 x 10^{10}, 4.98 x 10^{10} and 6.87 x 10^{9} for morphine, M3G and M6G, respectively. These results are considered promising for our next work in the clinical field using biological fluids.

Keywords: Morphine; Morphine-3-Glucuronide; Morphine-6-Glucuronide; SQ-BA3; On-Column Labeling; CE-LIF

Introduction

Morphine is an opioid analgesic drug used for the treatment of moderate to severe pain. It is recommended by the World Health Organisation for the relief of moderate cancer-related pain. The primary elimination pathway of morphine involves the addition of glucuronic acid at the 3- or 6-carbon of the molecule resulting in the formation of Morphine-3-Glucuronide (M3G) and Morphine-6-Glucuronide (M6G) [1] as shown in Figure 1. M6G has been proven to exhibit activity at the μ-opioid receptor to a potency that may surpass morphine while M3G has no analgesic activity [2,3]. Some studies indicated that M3G might be responsible for side-effects after morphine-treatment [4] while other ones suggested that it is a functional antagonist of morphine and M6G [5,6].

Measurement of morphine and its major metabolites, M3G and M6G had been routinely carried out by various methods including chromatographic methods mainly HPLC technique which used Ultraviolet-Visible (UV), Diode Array Detection (DAD), fluorescence, electrochemical and Mass Spectrometry (MS) detectors or their combinations [7-16]. On the other hand, CE is probably the most rapidly growing analytical technology that has appeared in the last two decades. Despite this, there was no any reported method regarding separation of morphine and its major metabolites, M3G and M6G but few papers discussed CE of morphine either alone [17] or in combination with other opiates [18-21]. Regarding that, we have used CE-LIF technique to develop a new method which was capable of separation and quantitative determination of morphine and its major metabolites, M3G and M6G through labeling by a new boronic acid functionalized squarylium dye SQ-BA3 (Figure 1). As a class of cyanine dyes, squarylium dyes were first synthesized over 30 years ago and are 1,3-disubstituted products of the condensation of squaric acid with two aromatic and/or heterocyclic compounds [22,23]. These dyes exhibit effective light absorption and are resistant to photo degradation, and have found use in applications such as photoconductors in copying devices, organic solar cells, and optical recording media [24-27].

Materials and Methods

Reagents, buffers, and sample solutions

A sample of SQ-BA3 dye was generously provided by H. Nakazumi and coworkers (Osaka Prefecture University, Osaka, Japan) and was used as-received. A 4.00 x 10^{-4}M stock solution of SQ-BA3 was prepared by weighing out an appropriate amount of solid dye and dissolving in dimethylformamide, DMF (Fisher Scientific, Fair Lawn, NJ, USA) and was stored in the dark at 4°C as when not in use. The stock solution is stable in the dark for several months or longer specially when stored at 4°c.

A working solution of the dye was prepared by further dilution of the stock solution in DMF to a concentration of 1.00 x 10^{-4}M just before use. Aliquots of this working solution were added to the appropriate buffer to serve as the Background Electrolyte (BGE) solution for CE studies. However, the final dye concentration was 1.00 x 10^{-6}M for spectral studies and up to 5.00 x 10^{-6}M for CE optimization studies.

Morphine, Morphine-3-Glucuronide (M3G) and Morphine-6-Glucuronide (M6G) ampoules (1mg/ml) were used as received from Cerilliant (Round Rock, TX, USA). Morphine was formulated in 1ml methanol, M3G in 1ml methanol w/0.05% NaOH while M6G was

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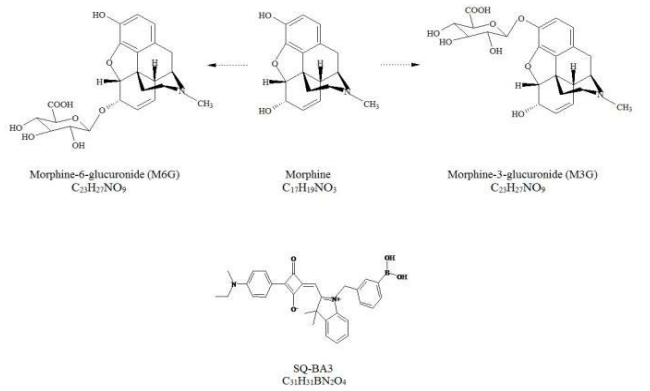

Figure 1: Molecular structures of SQ-BA3 (M.Wt. 506.40g/mol), morphine (M.Wt. 285.34g/mol) and its major metabolites M6G and M3G (M.Wt. 461.46g/mol).

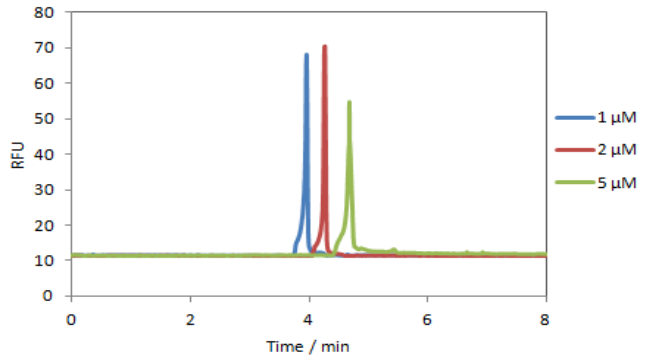

Figure 2: Electropherograms of 5.00 x 10^{-4}M morphine labeled on-column with different concentrations of SQ-BA3 in 25mM ammonium phosphate buffer with 10mM phytic acid (pH 10.50). Dye concentrations as follow: 1.00 x 10^{-6}M (blue line), 2.00 x 10^{-6}M (red line) and 5.00 x 10^{-6}M (green line). Other optimized parameters included a 50µm ID x 70cm total length x 60cm effective length capillary, a separation voltage of 30kV, a pressure injection of 5 psi*10 sec and sample temperatures were held at 25°C.

dissolved in 1ml water: methanol (80:20 v/v). Morphine and M6G were kept in the freezer while M3G was kept in the refrigerator while not in use. A dilute working solution was prepared from the ampoules by dilution with water to the desired concentration just before use for spectral and CE-LIF studies.

Buffers for Fluorescence studies were prepared to span the pH range from acidic to basic one from citric acid (Sigma Aldrich, St. Louis, MO, USA), tris (hydroxymethyl) aminomethane (AMRESCO, Solon, OH, USA), or ammonium dihydrogen phosphate (JMC, Royston, Hertfordshire, UK). Buffers were prepared by dissolving the correct amount of reagent in MilliQ distilled, deionized water (Millipore, Bedford, MA, USA) and adjusting the pH by the addition of either 1M NH_4OH or 1M HCl (Fisher, Pittsburgh, PA, USA). For CE studies, phytic acid sodium salt (Sigma, St. Louis, MO, USA) was used as a buffer additive at 10mM concentration. All buffers were stored in plastic bottles at room temperature, and were filtered through 0.20µm nylon syringe filters (VWR International, Houston, TX, USA) prior to use.

Instrumentation

CE studies were conducted using a Beckman Coulter P/ACE

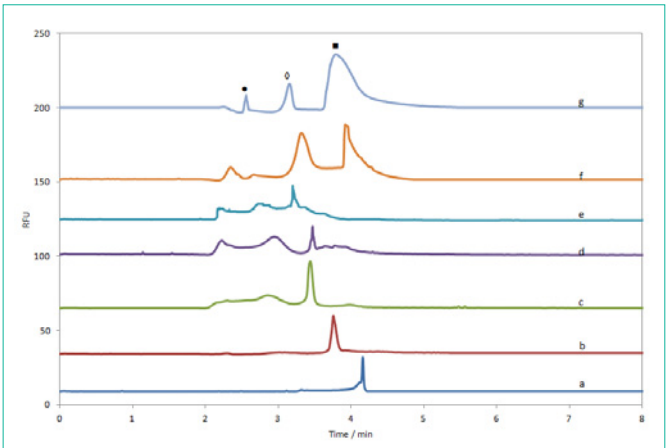

Figure 3: Electropherograms of on-column labeling of a mixture of morphine standards including morphine ◊, M3G ■ and M6G ● (5.00 x 10^{-4}M each) by CE-LIF with 1.00 x 10^{-6}M SQ-BA3 at different buffer pH conditions. (a) 25mM ammonium phosphate buffer pH 9.50, (b-g) 25mM ammonium phosphate buffer with 10mM phytic acid at (b) pH 8.00, (c) pH 8.50, (d) pH 9.00, (e) pH 9.50, (f) pH 10.00 and (g) pH 10.50. The other optimized parameters are mentioned in Figure 2.

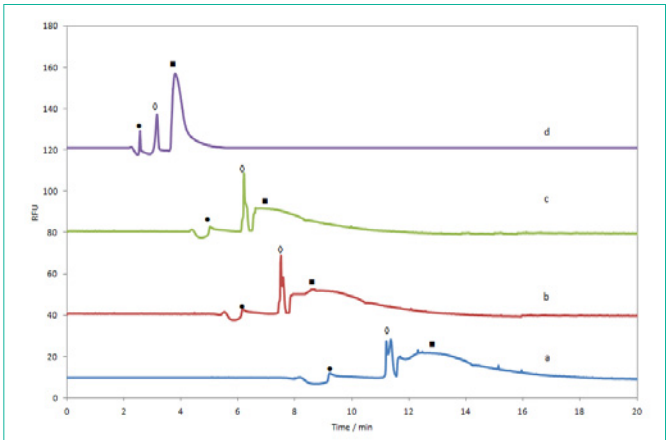

Figure 4: Electropherograms resulting from on-column labeling of morphine standards mixture (morphine ◊, M3G ■ and M6G ● each of 5.00x10^{-4}M) with 1.00x10^{-6}M SQ-BA3 in 25mM ammonium phosphate buffer with 10mM phytic acid (pH 10.50) by applying different voltages: (a) 10kV, (b) 15kV, (c) 20kV and (d) 30kV. Other optimized parameters included a 50µm ID x70cm total length x 60cm effective length capillary, a pressure injection of 5 psi*10 sec and sample temperatures were held at 25°C.

MDQ capillary electrophoresis instrument coupled with an external LIF detector module, a 655nm emission band-pass filter (Beckman Coulter, Brea, CA, USA) and external 635nm diode laser module of 2.5mW (Oz Optics, Ottawa, ON, Canada). Uncoated fused-silica capillaries of 50µm x 70cm x 60cm (ID x total length x effective length) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Variations in capillary dimensions were governed by optimization considerations and system compatibilities. The new capillary was conditioned by a pressure flushing for 20 min using purified water, 20 min using 1M sodium hydroxide, then 20 min using purified water, and finally 30 min using the running buffer. Every Day prior to sample analysis, the capillary was conditioned again but by a pressure flushing for 10min using purified water, 10min using 1M sodium hydroxide, then 10min using purified water, and finally 20min using

Table 1: Absorbance and fluorescence properties for 1.00 x 10^{-6}M SQ-BA3 dye with and without added 5.00 x 10^{-4}M morphine, M3G and M6G in various solvent systems.

Solvent or Buffer	Absorbance λ_{max} (nm)				Emission λ_{max} (nm)			
	SQ-BA3 alone	With morphine	With M3G	With M6G	SQ-BA3 alone	With morphine	With M3G	With M6G
DMF	631	N/A	N/A	N/A	661	N/A	N/A	N/A
Ammonium Citrate (pH= 3.50)	620	621	620	622	659	659	660	659
Tris-HCl (pH=7.50)	619	621	622	623	660	660	661	660
Ammonium Phosphate (pH= 9.50)	618	619	621	621	657	658	659	659
Ammonium Phosphate (pH= 11.30)	617	619	619	618	659	660	659	659

Table 2: Analytical figures of merit derived from on-column labeling of morphine, M3G and M6G with 1.00 x 10^{-6}M SQ-BA3 using CE-LIF method. Experimental conditions are listed in Figure 3g.

	Morphine	M3G	M6G
Linear Regression	y = 1 E+10 x $^{-1}$ E+06	y = 5 E+10 x $^{-7}$ E+06	y = 7 E+09 x $^{-1}$ E+06
Correlation Coefficient R^2	0.9974	0.9965	0.9973
LOD (M)	$1.22\acute{}10^{-4}$	$8.72\acute{}10^{-5}$	$1.15\acute{}10^{-4}$
LOQ (M)	$4.08\acute{}10^{-4}$	$2.91\acute{}10^{-4}$	$3.82\acute{}10^{-4}$
Sensitivity (M^{-1})	$1.37\acute{}10^{+10}$	$4.98\acute{}10^{+10}$	$6.87\acute{}10^{+9}$

the running buffer. Between each run, the capillary was pressure rinsed with purified water for 1min then running buffer for 2min. Injections of samples were by pressure at 5psi for 10sec. The applied separation voltage was 30kV, and the temperature of the capillary, buffer and sample was held constant at 25°C. Other separation conditions are specified in the relevant figures and/or text to follow.

Fluorometric studies were conducted using a Perkin-Elmer LS50B Luminescence Spectrometer (Shelton, CT, USA). Excitation and emission slit widths were 10nm, the scan rate was 250nm/min and the scan range was from 640nm to 800nm. Fluorescence spectra of the free dye and dye-complex solutions were measured using a 1cm semi-micro (total volume 500µL) quartz cuvettes (Perkin-Elmer, Shelton, CT, USA).

Absorbance studies were conducted over a scan range of 500-800 nm with an integration time of 0.5sec and an interval of 1nm using a Hewlett Packard HP8453 UV/Vis spectrometer (Waldbronn, Germany) and a 1cm semi-micro (total volume 500µL) quartz cuvettes (Perkin-Elmer, Shelton, CT, USA).

Results and Discussion

Preliminary absorbance and fluorescence studies

Spectral properties of the novel squarylium dye (1.00 x 10^{-6}M SQ-BA3) were determined under various solution conditions in order to assess its suitability as a fluorescent probe for morphine & its metabolites (5.00 x 10^{-4}M each). The absorbance curve for SQ-BA3 in aqueous solutions was very broad and weak in comparison with the dye in DMF which suggests that the dye forms water-soluble aggregates in agreement with results reported by other researchers [28,29]. However, absorbance was slightly enhanced after addition of M3G, morphine and M6G respectively (Data not shown). A summary of the wavelengths of maximum absorbance and emission of the dye, with and without added morphine, M3G and M6G is presented in Table 1. Significant differences between absorbance and emission wavelengths for the dye and its comlex, for example 39nm in the case of SQ-BA3 complex with morphine in amm. phosphate buffer, indicate good analytical discrimination between excitation and emission signals, thus allowing for more sensitive detection. Although the low fluorescence of SQ-BA3 in aqueous solutions would seem to preclude its use as a fluorescent label since it would provide a low background signal, the dye was still be able to strongly bind with morphine, M3G and M6G under these conditions with a subsequent increase in fluorescence. Buffer range selection was studied and the greatest fluorescence enhancements were observed for SQ-BA3 with added morphine, M3G or M6G under basic pH conditions at pH 9.50. Since neither morphine nor its glucuronide metabolites possess cis-diol functionalities, they can't perform covalent interactions with the SQ-BA3 dye but evidence of dye-analyte interaction is clearly provided by the significant change in the emission of the dye-analyte complex relative to the emission of the free dye as monitored by fluorimetry.

CE-LIF optimization of morphine & its metabolites mixture

CE-LIF can be used analogously to fluorimetry to monitor changes in fluorescence emission of a dye upon titration with increasing concentrations of morphine & its metabolites. However, several parameters had been taken in consideration to get the best optimum conditions for separation and quantitation of morphine & its metabolites such as dye concentration, pH effect, buffer concentration, phytic acid concentration, applied voltage and capillary ID. The amount of dye available for complexation can affect the CE-LIF response, and it was therefore necessary to optimize the concentration of dye for any given analytical protocol. We studied dye concentration effect through using a fixed morphine sample concentration (5.00 x 10^{-4}M) conducted with three different concentrations of SQ-BA3 added to the ammonium phosphate buffer. When morphine concentration was fixed at 5.00 x 10^{-4}M, the peak

area of the complex didn't increase when the SQBA dye concentration increased from 1.00 x 10^{-6}M to 2.00 x 10^{-6}M, but the peak area of the complex decreased when the dye concentration was further increased to 1.00 x 10^{-5}M due to the high amount of aggregated dye molecules (Figure 2). Thus, 1.00 x 10^{-6}M dye in running buffer provided the best compromise between sensitivity towards added morphine compounds and minimal dye background signal. Regarding pH effect, it was noted that 25mM ammonium phosphate buffer pH 9.50 was the optimum buffer for fluorescence enhancement of SQ-BA3 when complexed with 5.00 x 10^{-4} morphine, M3G or M6G. This buffer was used as the running buffer mixed with 1.00 x 10^{-6}M SQ-BA3 dye but unfortunately, the three analytes comigrated together at 4.16 min as seen in Figure 3a. Different pH ranges of the same buffer were used up to 10.50 without any improvement in separation pattern. 10mM phytic acid dodecasodium salt was used as a buffer additive and separation was resumed again starting from pH 8.00 which didn't show any signs of separation as shown in Figure 3b. However, pH 8.50 started to show an intial differentiation of the three compunds (Figure 3c) and once pH reached 10.50, a complete separation did happen where M6G, morphine and M6G migrated at 2.56, 3.15 and 3.79 min, respectively (Figure 3g). This sequence of migration was the same as the sequence of increasing fluorescence enhancement (M6G then Morphine then M3G). Recently, the use of the dodecasodium salt of inositol-hexaphosphoric acid, better known as phytic acid, has been reported to improve separation efficiency and resolution of proteins [30-33] and peptides [34,35]. Phytic acid is a naturally occurring nontoxic compound containing six hexane-ring bound phosphate groups with pK values ranging from 1.90 to 9.50 [36]. Phytic acid concentrations ranging from 5 to 20mM were studied, and 10mM was chosen as optimal, since it afforded the best separation between morphine, M3G and M6G peaks. Lower concentration of phytic acid 5mM did not afford complete separation of the three compounds while higher concentrations of phytic acid up to 20mM did not improve the separation significantly but did result in increased migration times (Data not shown). Buffer concentration effect was tested also when varied from 10mM to 100mM where 25mM was chosen as optimal due to a combination of the best resolution, peak shape, and fluorescence intensity at this pH (Data not shown).

Voltage comparison studies were also established by using different applied voltages varying from 10kv to 30kv. All applied voltages showed relevant separation of morphine compounds but the voltage of 30kv showed better peaks within short migration time (Figure 4). It should be noted that using lower voltage improved separation of M6G from both morphine and M3G but there were still some issues of peak broadening of M3G peak and splitting of morphine peak. Additionally, capillary Inner Diameter (ID) effect was studied using different dimensions 25, 50 and 75µm and it was found that 50µm was optimal for separation due to peak shape and resolution behavior as 75µm capillary didn't achieve complete resolution of the three compounds while 25µm capillary achieved the sepration but didn't afford good peaks. As a result, the optimal method was established using 30kv applied voltage and 50µm ID capillary filled with 25mM amm. Phosphate running buffer with 10mM phytic acid dodecasodium salt (pH 10.50) mixed with 1.00 x 10^{-6}M SQ-BA3 dye. This mode is called On-column labeling where the dye concentration was essentially constant in the buffer throughout the capillary, and the equilibrium between the free dye and the morphine-bound dye was readily established and maintained stable as morphine compounds mixture migrated through the capillary without any dissociation problems known to be common in the pre-column labeling mode. On-column labeling provides a simpler protocol with no sample pretreatment required, and typically offers an enhancement in sensitivity as demonstrated in previous work with related squarylium dyes [37]. A calibration curve was established using different concentrations of the three analytes where a linear increase in peak area for M3G, morphine and M6G-SQ-BA3 complex was reported with a high correlation coefficient of R2 > 0.996. This method showed a linearity range of 2.50 x 10^{-4}M - 2.50 x 10^{-3}M for morphine, 1.50 x 10^{-4} M - 1.50 x 10^{-3}M for M3G and 5.00 x 10-^{4}M - 2.00 x 10^{-3}M for M6G and a sensitivity of 1.37 x 10^{10}, 4.98 x 10^{10} and 6.87 x 10^{9} for morphine, M3G and M6G, respectively (Table 2).

Conclusions

The new dye, SQ-BA3, has been shown to possess novel useful characteristics for labeling morphine & its metabolites. The spectral properties of SQ-BA3 were explored under a variety of solution conditions in order to determine the best conditions for morphine compounds labeling and analysis by CE-LIF. Optimization of CE-LIF conditions such as pH effect, buffer concentration, phytic acid concentration, dye concentration, applied voltage effect and capillary ID effect was our essential interest. Phytic acid in basic buffer played a very important role in the separation procedure. Also, on-column labeling method had the advantage of reduced sample handling and thus lowered risk of contamination or loss and obviated the need for sample derivatization prior to injection, which holds much promise for analytical method development for clinical, forensic, and environmental samples. By combining the high resolving power of CE with the sensitivity of LIF detection and the convenience of on-column labeling, applications for novel, squarylium dyes as probes for new drugs of interest are expected to grow specially in the clinical field.

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