Original Article

Development of a High Performance Liquid Chromatography with Coulometric Electrochemical Detector Method for the Analysis of Morphine in Human Plasma

การพัฒนาวิธีวิเคราะห์มอร์ฟีนในพลาสมามนุษย์โดยการใช้เทคนิกโครมาโทกราฟีของเหลวสมรรถนะสูงร่วมกับเครื่องตรวจวัดแบบเคมีไฟฟ้าชนิดคูลอมเมตริก

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Received: 2 June 2009 **Accepted**: 25 July 2009

Abstract

A HPLC method using reversed-phase chromatography coupled with a coulometric electrochemical detector (ECD) was developed for the determination of morphine in human plasma. Hydromorphone was selected as an internal standard. The compounds were extracted using solid-phase extraction with C_{18} cartridges and separated on a reversed-phase C^{18} column with the mobile phase consisting of 69% v/v phosphate buffer pH 2.2 (5 mM sodium phosphate monobasic and 0.7 mM sodium dodecyl sulfate) and 31% v/v acetonitrile. The working electrode of the ECD was set at +450 mV for analytical purposes. Under this condition, the limit of detection for morphine was 0.33 ng/mL, whereas the limit of quantitation was 1.88 ng/mL. The percent recovery, intra-day and inter-day variation of morphine determinations were in the percentage range of 85.07-93.41, 2.86-6.41 and 3.76-11.39, respectively. The frozen samples kept at -20°C proved to be stable for at least one month prior to extraction. Also, the repeated use of cartridges for extraction was found to generate reliable results.

Keywords: Morphine, HPLC, Electrochemical detector

IJPS 2009; 5(2): 123-130

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บทคัดย่อ

การพัฒนาวิธีวิเคราะห์มอร์ฟีนในพลาสมามนุษย์ โดยใช้โครมาโทกราฟีของเหลวสมรรถนะสูง ร่วมกับเครื่องตรวจวัดแบบเคมีไฟฟ้าชนิดคูลอมเมตริก ใช้ไฮโดรมอร์โฟนเป็นสารมาตรฐานภายใน สารตัวอย่างถูกสกัดผ่านคาร์ทริด C_{18} และถูกแยกในระบบโครมาโทกราฟีโดยคอลัมน์ ชนิด C_{18} สารขับดันที่ใช้ประกอบด้วยสารฟอสเฟตบัฟเฟอร์ค่าความเป็นกรด/ด่าง 2.2 ซึ่งประกอบด้วย โซเดียมฟอสเฟต 5 mM และ โซเดียมโดเดคซิลซัลเฟต 0.7 mM จำนวนร้อยละ 69 ผสมกับอาซิโตรไนไตรอีกร้อยละ 31 อิเล็กโทรดที่ใช้วัดกำหนดไว้ที่ 450 mV จากการทดลองพบว่าความเข้มข้นต่ำสุดของมอร์ฟีนที่สามารถวัดได้คือ 0.33 ng/mL และความเข้มข้นต่ำสุดของมอร์ฟีนที่วิเคราะห์ได้คือ 1.88 ng/mL การกลับคืน ค่าเบี่ยงเบนความเที่ยงตรงภายในวันเดียว และค่าเบี่ยงเบนความเที่ยงตรงระหว่างวันในการวิเคราะห์มีค่าร้อยละ 85.07-93.41, 2.86-6.41 และ 3.76-11.39 ตามลำดับ ตัวอย่างที่เก็บไว้ที่อุณหภูมิ -20°C มีความคงตัวอย่างน้อยหนึ่งเดือนก่อนนำไปสกัดเพื่อวิเคราะห์ การทดสอบการนำคาร์ทริดกลับมาใช้ซ้ำเพื่อการสกัดพบว่าได้ผลที่น่าเชื่อถือสามารถนำมาใช้ซ้ำได้

คำสำคัญ: มอร์ฟีน นิกโครมาโทกราฟีของเหลวสมรรถนะสูง เครื่องตรวจวัดแบบเคมีไฟฟ้า

วารสารเภสัชศาสตร์อีสาน 2552; 5(2): 123-130

INTRODUCTION

The determination of very low concentration of morphine and other morphine-like compounds in biological fluids is often required for experimental or clinical studies. Several analytical techniques have been employed to determine morphine and its metabolites including HPLC-UV detection, radio-immunoassay (RIA), GC-mass spectrometry, LC-MS, HPLC-MS and Fluorometry (McQuay et al., 1990; Glare et al., 1991 Gerostamoulos et al., 1993; Liu et al., 1997; Meadway et al., 2002; Projean et al., 2003; Kudo et al., 2006). The limit of detection for the compounds in biological fluids ranged from pg/mL to μg/mL. Since the 1970s, HPLC with electrochemical detector (ECD) has been used, and is now a popular technique for analysis of morphine and morphine-like structures (Shoup, 1986). White (1979) was the first to report an HPLC assay of morphine using ECD. This method is sufficiently selective and sensitive; the limit of quantitation was 1 ng/mL with amperometric instrument (Wright et al., 1994) and 200-240 and 500 pg/mL with coulometric method, respectively (Bouquillon et al., 1992; Ary and Rona, 2001). Meng et al. (2000) have demonstrated that two step extractions by solid phase extraction of human plasma give a very low limit of detection of morphine at 100 pg/mL. However, the process is costly and time consuming. In Thailand, although morphine has been used for pain relief for such a long time, there is no report of the assay of morphine concentration in biological fluids, specifically with ECD. This study describes an extraction procedure for morphine in human plasma, involving single solid-phase extraction as the purification method followed by determination of morphine concentration using HPLC coupled with coulometric electrode. The main objective of the study was to develop the HPLC assay in association with electrochemical detection for the determination of morphine concentration in human plasma.

Materials and Methods

HPLC apparatus

The HPLC system consisted of a pump (Perkin Elmer, Model LC- 200, USA), a Coulochem II electrochemical detector equipped with a Model 5011 standard analytical cell (ESA, Coulochen II, USA), and an Integrator (Perkin Elmer, Model 1022, USA). A 250x4.6 mm Zorbax C_{18} column (5 μm partical size, Agilent, USA) protected by a 12.5x4.6 mm precolumn (5 μm particle size, Zorbax C_{18}, USA) was used.

Chemical and reagents

Morphine sulphate, a product of Glaxo Wellcome (B.P/U.S.P, USA) was purchased from the Food and Drug Administration, the Ministry of Public Health, Thailand. Morphine standard and hydromorphone HCL and sodium dodecyl sulphate (AR grade) were the products of Sigma-Aldrich (USA). Other solvents and chemicals were HPLC or AR grade. Sep-pak C_{18} cartridges (3-mL) and SPE column processors were purchased from Water® (Ireland). HPLC grade water was obtained by purifying distilled water in a Milli-Q filtration system (Millipore, USA).

Chromatography and standard solution

The assay for morphine was performed using a mobile phase consisting of 69% v/v buffer containing 5 mM sodium phosphate monobasic and 0.7 mM sodium dodecyl sulfate (pH adjusted to 2.2) and 31% v/v acetronitrile. A flow rate was maintained at 1.0 mL/min. The working potential was set at 450 mV. A stock solution of morphine was prepared in methanol with a concentration of 278 μg/mL. A set of working standard solutions was made by serial dilution. Freshly prepared standard solutions were prepared on a daily basis. A stock solution of hydromorphone HCl (IS) at a concentration of 260 μg/mL was diluted in methanol, and further diluted to approximately 1 μg/mL in water.

Sample preparation

Plasma used in this validation study was drug-free and obtained from the Blood Bank Division, the Faculty of Medicine, Khon Kaen University. It was kept in the freezer (-20°C) before use. Morphine solution (concentration as required) was added into a test tube, then dried with nitrogen gas. Two mL of plasma was added, then mixed for 5 sec. One hundred ng of internal standard was added and mixed for another 5 sec. Three mL of 0.5 M ammonium sulfate (pH 9.3) solution was added and mixed. The mixture was then passed through a pretreated Sep-Pak cartridge which was conditioned with 5 mL methanol followed by 5 mL of de-ionized water. The cartridge was washed with 10 mL of 5 mM ammonium sulfate solution (pH 9.3) and 4 mL of 20% methanol in 5 mM ammonium sulfate solution (pH 9.3). The sample was then eluted with 1.2 mL of 30% acetonitrile in 0.2% phosphoric acid, then evaporated to dryness. The dried residues were redissolved with 0.2 mL of mobile phase and 20 μL samples were injected into the HPLC column.

Assay Validation

Calibration curves and linearity

Calibration curves were prepared from blank plasma samples spiked with morphine to cover the concentration range 1.9 to 47 ng/mL. Morphine solutions were transferred to test tubes then dried under stream of oxygen-free nitrogen. Two mL of plasma and internal standard at a fixed weight of 100 ng were added. Sample extraction was performed as previously described in sample preparation session.

Lower limits of quantity (LLOQ)

Eight determinations of lowest concentrations of standard morphine were analyzed. The LLOQ was established by a signal to noise ratio of 5:1. The mean and relative standard deviation were obtained.

Recovery, accuracy and precision

Six determinations between three concentrations of standard morphine (1.9, 20, and 47 ng/mL) in plasma and in water were analyzed. Recovery determination was calculated by comparing the peak area of extracted plasma samples at each concentration with unextracted standards. Accuracy was measured at three concentration levels as recovery study. These samples were analyzed in six replicates for the drug content by computing the ratio of estimated concentration obtained from standard calibration of morphine in plasma to the known concentrations of morphine in plasma multiplied by one hundred. Intra-day and inter-day assay precision were evaluated by the determination of the percent relative standard deviation (%RDS). For the intra-day variation, sets of six replicates were analyzed on the same day of the samples at the three concentrations. For the inter-day validation, sets of six replicates of the same concentrations on the three different days were determined.

Short and long term stability studies

For short term stability study, six determinations of three concentrations of standard morphine (3, 20, and 40 ng/mL) in plasma were studied. Three aliquots of these samples were analyzed immediately and the other three aliquots were stored at -20°C for 24 hrs. After that, samples were thawed at ambient temperature for 4 hrs, then the samples were extracted and analyzed with HPLC. The resulting data were compared between before and after storage. For long term stability study, three aliquots of the samples at the same concentrations were analyzed after stored at -20°C for 1 month. The study process and evaluation were similar to the short term study.

Repeatability of using of cartridges

This study was designed to determine whether the reuse of the cartridge for extraction was reliable. Three concentrations of standard morphine in plasma, similar to those previously described, were used. Each of the cartridges was used to extract sample twice. The deviation of the mean estimated concentrations was compared between the first and the second extraction.

Results and Discussion

HPLC-ECD system development and sample preparation

Before commencing the HPLC analysis, passivation of the HPLC pump was necessary to minimize erosion of metallic substances from the HPLC unit. In brief, the HPLC pump (column and detector disconnected) was run with 30 mL of 6 N nitric acid, followed by 30 mL of glacial acetic acid. De-ionized water was then passed through the system until the pH of the waste water was approximately 7. The working electrode was set at 450 mV. The guard cell electrode was selected at 300 mV. To improve retention and separation, 0.7 mM sodium dodecyl sulfate was added as an ion-pairing agent in the mobile phase (Wright et al., 1994). It was found that the background current of the mobile phase ranged 40-80 nA which was acceptably low. The optimization procedure was performed with regard to the best possible separation as well as detection of morphine. For the sample preparation process, it was found in this experiment that water in washing process was a key role of washing off the endogenous substances. A one-step extraction in our study was sufficient to eliminate junk peaks from the serum samples, compared to a two step extraction (Meng et al., 2000).

Detection and validation

Figure 1 illustrates chromatograms for pre-dose (drug-free) plasma and plasma sample. Morphine and IS were eluted as sharp peaks at approximately 11 and 14 min, respectively. The lower limit of detection and lower limit of quantitation were 0.33 and 1.88 ng/mL, respectively, whereas the lower limit of detection reported by Liaw et al. (1998) and Meng et al. (2000) was 0.1 ng/mL. However, in the latter case, their research team used a two-step extraction process which took longer time compared

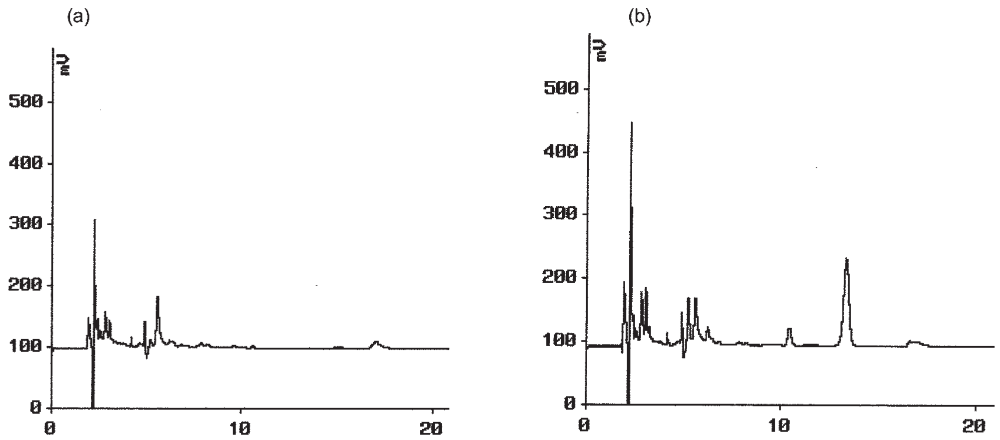

Figure 1 Chromatograms of (a) blank plasma and (b) spike morphine (M) plasma at a concentration of 3 ng/mL and 100 ng/mL hydromorphone (IS)

to our study. Our method also showed higher sensitivity than that used GC-MS in which the lower limit of quantitation was reported at 10 ng/mL and that for lower limit of detection of 2 ng/mL (Meatherall, 2005). Another study using HPLC-MS method reported the lower limit of quantitation at 0.3 ng/mL (Projean et al., 2003). The detection limit obtained here was low enough to determine morphine concentration in plasma as it has been reported at concatenations of 2 ng/mL or higher (Bourgetet al., 1995; Drake et al., 1996; Gourlay et al., 1997).

Introduction of the extraction process for 6 independent replicated standard curves ranged from 2-50 ng/mL gave good linearity with r^2>0.998, with Y (peak area ratio of morphine/IS) = 0.0243X (plasma morphine concentration) 0.0247. Table 1 and 2 summarize the recovery of morphine after extraction, the accuracy and precision analysis. The percent recovery extraction of morphine and internal standard was found to be high with low %RSD (Table 1). Approximately 85-93% of morphine was extracted from low, medium and high concentrations. The lowest concentration (1.88 ng/mL) showed only 3% of variation. For intra- and inter-assay, variation was less than 12% for the lowest concentration of 1.88 ng/mL. The acceptable variable value for the lowest concentration was normally lower than 20%. In addition, the accuracy of analysis found nearly 100% of drug added (Table 2). The obtained validation results were found in an acceptable range, compared to those reported by Liaw et al. (1998) and Meng et al. (2000).

For stability study, it was found in Table 3 that the ratios of morphine before and after both storage conditions were very close in three concentrations, compared to day 0 where %RSD of these studies was lower than 6. In this study, the repeat of using cartridge was performed to reduce the cost of extraction while the extraction was still efficient. It was found that, between the two extractions that, the average of morphine concentrations found were nearly the same in the three concentrations determined (Table 4). Therefore, the concept of repeated use of the cartridges was reliable and repeatable, similar to that reported by Wright et al. (1997).

Table 1 Recovery of morphine in plasma after single solid phase extraction

Spike value of morphine (ng/mL)	% Recovery	
	Mean±SD	%RSD
1.88 (n=6)	85.07±2.62	3.08
20.00 (n=6)	93.41±1.77	1.90
47.00 (n=6)	91.36±1.84	2.02

Table 2 Precision and accuracy validation of morphine in plasma after single solid phase extraction

Precision	Morphine concentration added (ng/mL)	Morphine concentration found (ng/mL) (Mean±SD)	%RSD
Intra-day (n=6)	1.88	1.94±0.12	6.41
	20.00	20.94±0.98	4.67
	47.00	46.80±1.35	2.86
Inter-day (n=18)	1.88	1.84±0.21	11.39
	20.00	21.03±0.98	4.68
	47.00	46.27±1.74	3.76
	Morphine concentration added (ng/mL)	Morphine concentration found (ng/mL)	%Found±SD
Accuracy (n=6)	1.88	1.94	103.34±6.62
	20.00	20.74	103.69±4.23
	47.00	46.80	99.53±2.87

Table 3 Stability of morphine in plasma samples after 1 day and 1 month of storage in the refrigerator (-20°C)

Known morphine concentration (ng/mL)		Morphine concentration found (ng/mL)			
Day 0 (n=6)		Day 1 (n=6)		Day 30 (n=6)	
Mean	% RSD	Mean	% RSD	Mean	% RSD
2.93	4.22	2.91	3.27	2.83	5.44
20.04	3.55	20.10	2.00	18.50	3.10
39.40	1.01	39.51	2.19	39.00	1.04

Table 4 Repeatability of using cartridges on the extraction of morphine in plasma

Morphine concentration (ng/mL)			
First extraction (n=6)		Repeated extraction (n=6)	
Mean	% RSD	Mean	% RSD
3.21	5.99	3.22	5.88
21.89	4.59	21.04	6.50
37.03	1.77	37.02	3.40

Conclusion

A validated HPLC-ECD assay method for determination of morphine in human plasma with rapid sample preparation was demonstrated. Such a type of detection had not been reported for the determination of morphine in human plasma in the country. This developed method has been validated with good linearity, sensitivity, accuracy, precision, selectivity and stability. Therefore, it can be applied for morphine analysis experimentally and clinically for the purpose of research and services.

Acknowledgements

The authors would like to thank the Narcotics Control Division, the Food and Drug Administration, the Ministry of Public Health, Thailand for the whole financial support. Many thanks go to the Faculty of Pharmaceutical Sciences, Khon Kaen University for supporting the facilities used in the HPLC system development.

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