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High-performance liquid chromatographic determination of morphine and its 3- and 6-glucuronide metabolites by two-step solid-phase extraction

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Abstract

To provide more accurate measurement of morphine and its metabolites for a study of the genetic differences on morphine response, a method for the analysis of morphine and its metabolites is described which has the advantages of increased sensitivity and specificity by using a cleaner extraction. The new extraction method involves both the hydrophobic isolation on a carbon cartridge and ion-exchange isolation on ion-exchange resin which has not preliminary been described for morphine analysis. The combination of these two steps successfully purified drugs from human plasma with maximum removal of interfering substance comparing with a conventional C_{18} cartridge alone. The analytes are quantified by high-performance liquid chromatography on a reversed-phase C_{18} column employing a mobile phase consisting of 25% acetonitrile in 0.05 *M* phosphate buffer (pH 2.1), and 2.5 m*M* sodium dodecyl sulfate as the pairing ion with a combination of electrochemical and fluorometric detections. The recoveries for morphine (M), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and hydromorphone after the SPE procedure were 86±7.1%, 82±6.9%, 79±6.0% and 85±6.0%, respectively. Limits of detection for this method are 0.1 ng/ml for M, and 0.18 ng/ml for M3G and M6G. Limits of quantitation were approximately 0.25 ng/ml for M, and 0.45 ng/ml for M3G and M6G. The present assay was applied to measure M, M3G and M6G content in human plasma to test the applicability and suitability of this method for clinical and research use.

Keywords: Morphine; Morphine glucuronides

1. Introduction

Morphine, a traditional opioid analgesic, plays an important role in the treatment of acute and chronic pain. The metabolism of morphine has been of particular pharmacological interest and has been investigated extensively by many researchers [1,2]. Glucuronidation is the primary metabolic pathway which produces morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) as the major metabolites [3], both of which have been reported to be pharmacologically active [4–12]. M6G has been shown to be a more potent analgesic than morphine (M) when administered to experimental animals [6–10] or human subjects [11,12]. M3G has no analge-

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sic function, but is thought to account for some morphine side effects, including myoclonus and seizures.

Many assays of morphine have been reported in the literature. These include gas–liquid chromatography (GLC) [13,14], radioimmunoassay [15–17], thin-layer chromatography (TLC) [18,19], gas chromatography (GC) particularly coupled to mass spectrometric detection [20–23]. High-performance liquid chromatography (HPLC) allows simultaneous separation and detection of morphine and its metabolites [24–32]. Recently, HPLC tandem mass spectrometric assays (LC–MS) have been developed for the analysis of morphine and morphine glucuronide metabolites in urine and serum samples [33–36]. Such techniques may give reasonable results, but are extremely expensive and hence are not commonly available. Of these HPLC has the advantage of increased direct sensitivity without any need of derivatization. Various detection methods for HPLC measurement have been used in recent years, including ultraviolet (UV) [24–26], fluorometric [27,28], and electrochemical detection (ED) [29,30], or combination of detection methods, e.g., ED and UV [29,31] or fluorometric [32] detection. On the basis of these methods, C_{18} solid-phase extraction (SPE) has been used to extract M and its metabolites. However, experience in our own laboratory and others [25,30] have indicated that accurate assessment of M, M3G and M6G content in plasma is still remained problematic. SPE is prone to interferences from various substances present in biological samples, which may cause substantial variability in HPLC analysis. Further development of this technique seemed essential since preliminary modifications of C_{18} SPE methods to remove interfering substances failed to achieve the expected improvements.

Accordingly, the aims of this study were (1) to develop and validate methods for the accurate and consistent quantitation of M, M3G and M6G content in plasma, with particular emphasis on the potential problem associated with the variability of drug level determinations, and (2) to apply this method to measure M, M3G and M6G content in human plasma to test the applicability and suitability of this method for clinical and research use.

2. Experimental

2.1. HPLC apparatus

The HPLC system consisted of a Model-10AD pump (Shimadzu, Kyoto, Japan), a SIL-10A autoinjector, SCL-10A system controller, RF-551 spectrofluorometric detector (all from Shimadzu), a Coulochem II electrochemical detector (ESA, Bedford, MA, USA), equipped with a Model 5010 analytical cell and a Model 5020 guard cell, an Ultrasphere ODS analytical column (250 mm×4.6 mm I.D., 5 μm particle size) (Beckman, Palo Alto, CA, USA) protected by a μBondapak C_{18} Guard-Pak (Waters, Milford, MA, USA) and a CH-30 column heater (Alltech, Deerfield, IL, USA).

2.2. Chemical and reagents

Morphine hydrochloride, M3G, M6G, hydromorphone (the internal standard) and human plasma were purchased from Sigma (St. Louis, MO, USA). C_{18} SPE cartridges (1-ml), carboxylic acid (COOH, 3-ml) extraction cartridges and SPE column processors were obtained from J.T. Baker (Phillipsburg, NJ, USA). All solvents used were of HPLC-grade and all other chemicals used were of analytical grade (Sigma).

2.3. Clinical study design

To investigate the potential genetic differences in morphine metabolism, a study was designed by researchers at the Javeriana University in Bogota, Columbia, in collaboration with the University of Pennsylvania, to measure both physiological and pharmacological responses of normal healthy volunteers in an indigenous Indian population and the population of European descendants. Formal approval was obtained from the Institutional Review Board at both institutions [37]. The need for a more accurate measurement of the blood level of morphine, M6G and M3G prompted the development of the technique described below. After obtaining full written informed consent, healthy non-smoker male volunteers 18 through 40 years old (N=46) with no pain complaints were recruited. To avoid the vari-

ability that can accompany chronic diseases, subjects with a history or laboratory evidence of renal or hepatic disorders and evidence of regular drug or alcohol use were excluded. A catheter was placed in an antecubital vein, and subjects were monitored with a continuous EKG and an automatic blood pressure measuring device. After a baseline period of 30 min, a loading dose of 0.08 mg/kg of morphine was given i.v. push followed by a constant infusion of 0.002 mg/kg/min for 30 min. Blood samples were drawn at 0, 5, 15, 25, 35, 40, 50, 65 and 95 min, then hourly until 6 h after the morphine injection for measurement of serum levels of morphine, M3G and M6G. Blood was collected in tubes with EDTA, then centrifuged and the separated plasma was frozen immediately until the assay could be performed.

2.4. Solid-phase extraction

An internal standard consisting of 10 μl of $2.5 \cdot 10^{-5}$ *M* hydromorphone (100 ng) in water was added to 1 ml of each plasma. Plasma samples were then vortex-mixed for 30 s and transferred to C_{18} cartridges that had been conditioned with 2 ml of methanol and 2 ml of 10 m*M* phosphate buffer (pH 9.5). The C_{18} cartridges were washed with 2 ml of 10 m*M* phosphate buffer (pH 9.5) and finally was eluted with 1 ml of methanol containing 0.5% triethylamine (TEA). The eluate was dried by evaporation in a speed vacuum concentrator.

Samples were reconstituted with 1 ml of 80% acetonitrile in water and transferred onto carboxylic acid cartridges that had been conditioned with 4 ml of acetonitrile. The cartridges were washed with 4 ml of acetonitrile, then eluted with 1.5 ml of 80% methanol containing 0.05 *M* HCl. The eluate was evaporated as described above. Samples were reconstituted in 200 μl of the HPLC mobile phase. A 150-μl aliquot of each sample was injected into the HPLC system for analysis.

2.5. Standard solutions

A mixture of stock solution of M, M3G and M6G was prepared in water at a concentration of 1 mg/ml and stored at 4°C. On each day the assay was performed, six mixtures of standard aqueous solutions for calibration containing 10, 20, 50, 100, 200 and 600 ng/ml of morphine, M3G and M6G were prepared by serial dilution of the stock solution. Calibration curves were constructed by plotting the height of the drug peaks against the known concentrations of drugs in the standards and fitted by linear regression analysis.

Quality control samples of M with a concentration of 100 ng/ml were prepared by adding the drug to commercial plasma and storing at −80°C. For each validation run, duplicate quality control samples were assayed using the same procedure employed for the clinical samples.

2.6. Recovery

The recovery of analytes and internal standard from the SPE step was estimated by measuring the amount of each compound recovered after the extraction step in standard solution containing known molar quantities of M, M3G, M6G and hydromorphone. Standard solutions containing of 10, 50, 100 ng/ml of each drug in plasma were used to determine the recovery. Blank samples were prepared by adding the same volume of solvent without the drug to 1.0 ml of the control plasma samples. These samples were analyzed as controls to verify that no peak appeared near to the retention times of either M, M3G, and M6G or internal standard hydromorphone in the final HPLC experiments. The recovery of each drug was determined by comparing the height of the analyte peak measured in the extracted standard to the height of the analyte peak measured directly in spiked unextracted aqueous sample containing the original amount of the analyte.

2.7. HPLC conditions

A conventional C_{18} reversed-phase column was chosen and protected by a C_{18} guard cartridge for the whole study. All HPLC separations were carried out at a temperature of 35°C at a flow-rate of 1.0 ml/min with a mobile phase consisting of 25% acetonitrile, 2.5 m*M* sodium dodecyl sulfate (SDS), and 50 m*M* phosphate buffer (pH 2.1). Based on the current/voltage curves [38], detection was performed with

the electrochemical detector having 0.2 V for detector cell 1, 0.35 V for detector 2 and 0.4 V for the guard cell for M, M6G and hydromorphone. A fluorescence detector was combined using an excitation wavelength of 275 nm and emission at 345 nm for M3G.

2.8. Accuracy and precision

Chromatographic peaks for drugs were identified by retention time and standard addition protocols [39,40]. All of the detected species were assayed by measuring the chromatographic peak heights and drug concentrations determined from the standard calibration curve that was prepared daily. For each validation run, the recovery of each drug in plasma was determined using hydromorphone as the internal standard. Quality control samples with morphine concentrations of 100 ng/ml were used to determined the inter- and intra-assay variability. Variability was expressed as standard error of the mean (±S.E.M.). Relative standard deviations (RSDs) were determined for inter- and intra-assay variation.

3. Results

The mean concentration (±S.E.M.) recoveries for M, M3G, M6G and hydromorphone (containing 100 ng/ml of each drug) after the C_{18} and carboxylic acid SPE procedure were 86±7.1, 82±6.9, 79±6.0 and 85±6.0% (n=6), respectively. There were still a number of interfering substances following C_{18} cartridge extraction, but the addition of carboxylic acid SPE eliminated most of the remaining interfering chromatographic peaks in the HPLC separation.

Plasma samples spiked with drug standards resulted in an increased height of the peaks corresponding to M, M3G, M6G and hydromorphone, respectively, confirming the identification of these compounds. The mean concentrations (±S.E.M.) measured in six plasma samples containing 100 ng/ml of M, M3G and M6G extracted by the SPE procedure and injected into the HPLC system for analysis were 97±5.3, 96±6.7 and 94±6.0 ng/ml, respectively. Thus, the intra-assay RSD was less than 7.0%. The mean concentrations (±S.E.M.) measured in six plasma samples containing 100 ng/ml of M, M3G and M6G assayed on 6 different days using the same method were 95±8.0, 95±6.9 and 94±7.1 ng/ml, respectively, indicating that the inter-assay RSD was less than 8.5%. The same experiments with a concentration of 20 ng/ml for the analytes of M, M3G and M6G were also performed and the results demonstrated the consistency of the precision. The chromatographic peak heights for M, M3G and M6G were linear with the plasma drug concentration ranges of 5–600 ng/ml. The linear regression equations for morphine, M3G and M6G standard curves were $y=0.0764x+0.121$ ($r=0.999$), $y=0.4031x+0.2048$ ($r=0.9999$) and $y=0.1444x+0.1245$ ($r=0.9998$), respectively.

Clear separation of standard M, M3G, M6G and hydromorphone peaks was achieved under our HPLC condition (Fig. 1). Fig. 2 shows a typical HPLC elution pattern of the plasma extract from a human sample and the distinct chromatographic peaks for M, M3G, M6G and internal standard hydromorphone. A third guard electrode, placed before the injector and run at higher potential than the analytical cells, can be used to oxidize compounds in the mobile phase. Therefore, the mobile phase was recycled for a long time and no baseline noise was increased. The sample collected during the constant rate infusion of morphine demonstrated that a ''plateau'' level of morphine concentration in the blood was achieved in 100–200 min. Blood levels of M3G and M6G were constantly increasing, indicating the ongoing formation of morphine metabolites after morphine administration had stopped. The blood level of M3G rose faster than that of M6G (Fig. 3). The results of the plasma concentration–time curves for morphine and its metabolites were consistent with the known pharmacokinetic properties of the drug in a clinical setting.

Limits of detection for this method are 0.1 ng/ml for morphine, and 0.18 ng/ml for M3G and M6G (signal-to-noise ratio=5). Assuming a recovery of 80% after the extraction, limits of quantitation of approximately 0.25 ng/ml and 0.45 ng/ml for morphine and M3G, M6G were determined by assuming a concentration of analyte that yielded a signal-to-noise ratio of 10.

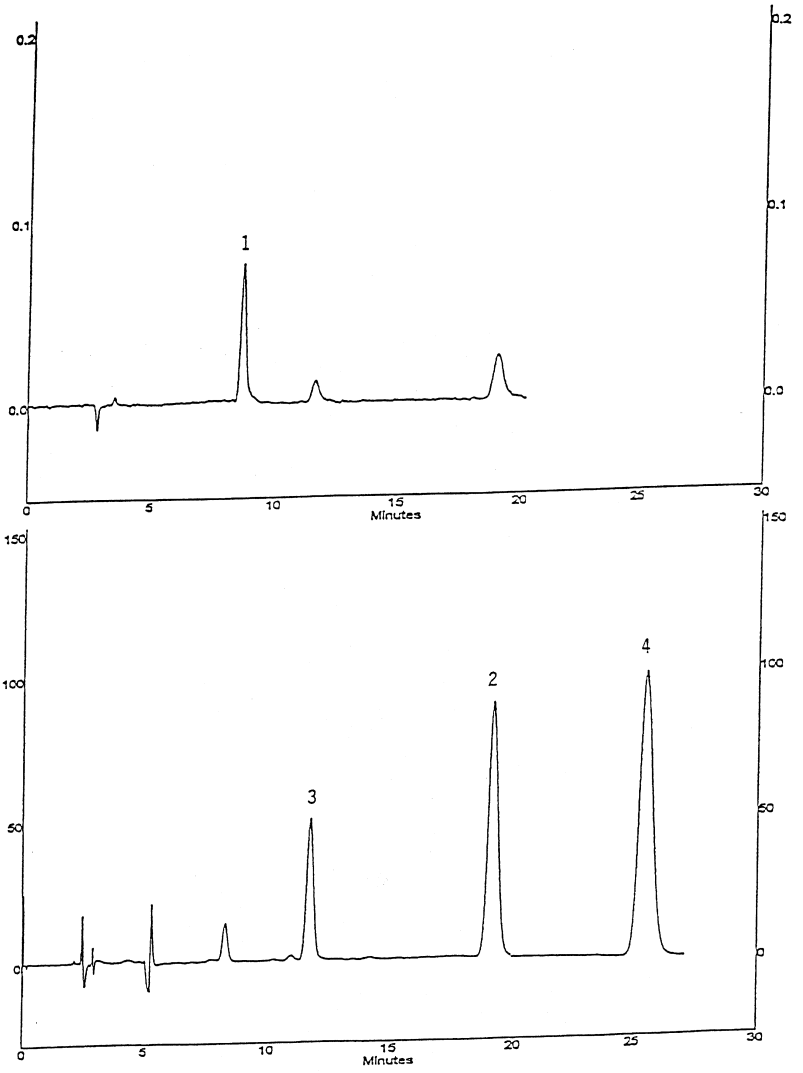

Fig. 1. HPLC separation of morphine, M3G, M6G and hydromorphone on a Ultrasphere ODS column (250 mm×4.6 mm I.D., 5 μm particle size) under the isocratic conditions described in the text. (Top) Chromatogram of standard M3G (1) from fluorescence detection. (Low) Chromatogram of standard morphine (2), M6G (3), and hydromorphone (4) from electrochemical detection (all 5 ng).

4. Discussion

In the first stage procedure of SPE extraction, a hydrophobic C_{18} cartridge was used as the conventional method of extraction. Water-soluble material was removed from the extraction column by washing with buffer. Analytes were collected using an aqueous organic mixture along with all endogenous compounds of similar hydrophobicity. Experience has shown that presence of interfering substances that persist after this extraction procedure remained problematic for HPLC analysis. Also, it was observed an additional Sep-Pak related impurity peak derived from the solid-phase sorbent with a retention time in the 90–100 min range [30]. To overcome this problem, the procedure was modified by either using two C_{18} cartridges [29,32] or different elution for C_{18} cartridge [25], but there were no important

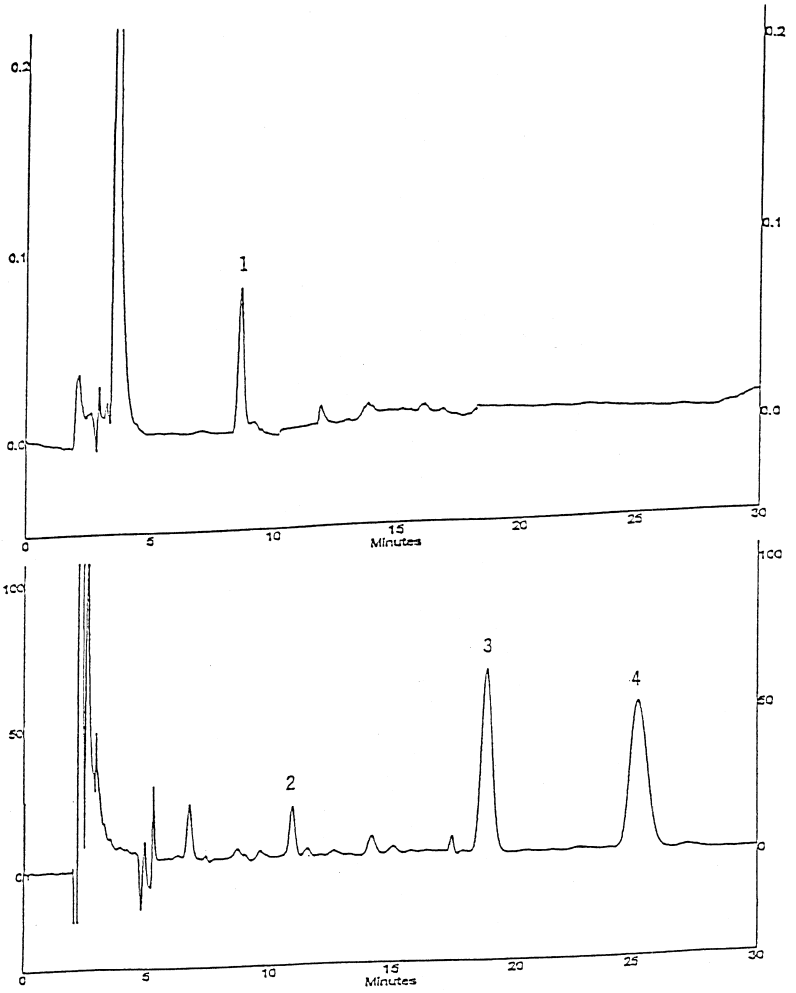

Fig. 2. Typical chromatogram obtained from extracted human plasma sample that had measured (1) M3G (23 ng/ml), (2) morphine (5 ng/ml), and (3) M6G (15 ng/ml). HPLC conditions as described in Fig. 1.

differences in the appearance of the chromatograms between those resulting from these of two Sep-Pak cartridges, as in the original method of Svensson et al. [24]. It is possible that the poor resolution from interfering substances for HPLC measurement may be responsible for the very high assay variation that has been reported.

To overcome these problems we added a cartridge of cation-exchange resin to extract M, M3G and M6G after the C_{18} cartridge extraction. A carboxylic acid cation exchanger was chosen for this work, because, as a weak acid, it will remain charged under a proper range of pH condition and can be easily washed with acidic solution. Due to its high selectivity, it has been used to isolate basic drugs and other compounds from a variety of matrices. Within a moderate pH range (4–9.5), the analytes of M, M3G and M6G had strong affinities to carboxylic acid due to the tertiary amine, which is part of a ring structure with a full positive charge for each compound. By washing with an acidic solution, these alkaloids were easy to replace because of the stronger affinity and higher concentration of hydrogen ions. Therefore, the ion-exchange reaction on carboxylic acid cartridge occurred at different hydrogen ion concentrations and this character was suc-

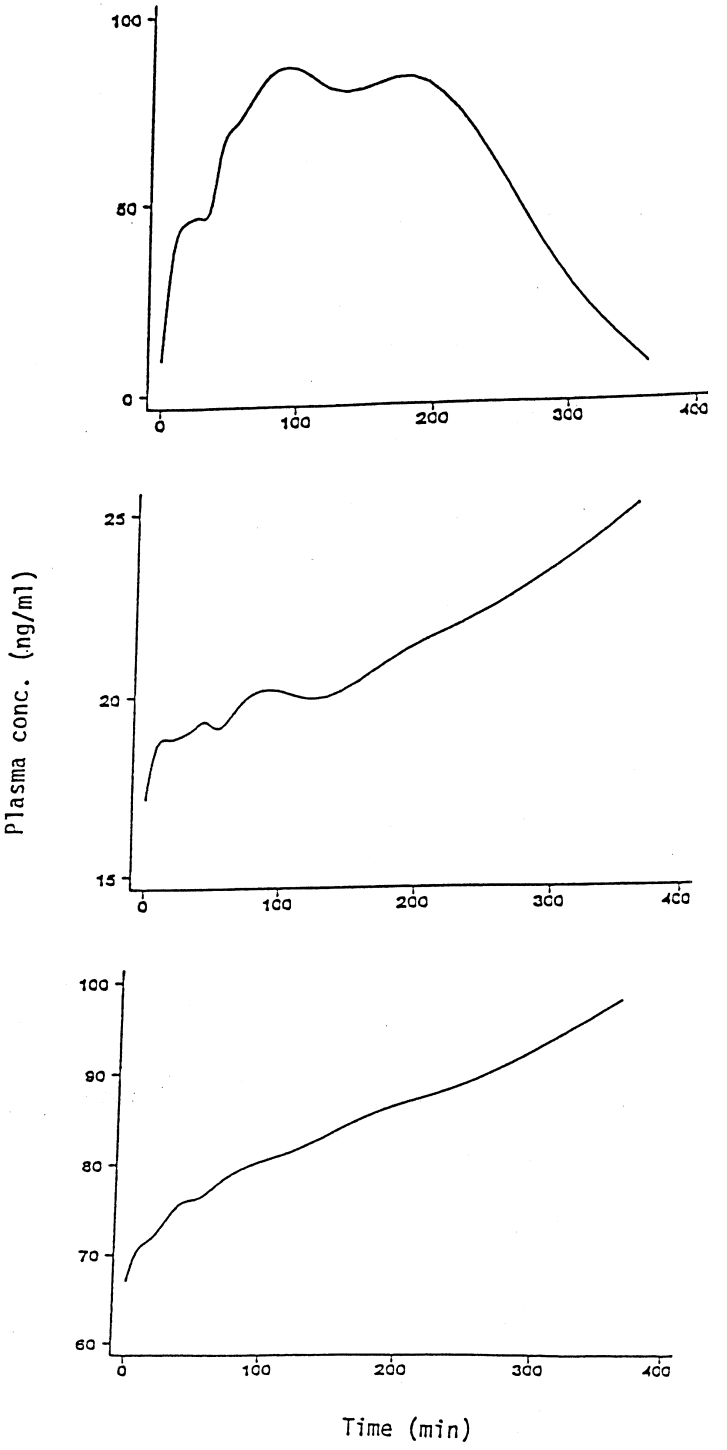

Fig. 3. Plasma concentration of morphine (top), M3G (low), and M6G (middle) following intravenous administration of morphine. HPLC conditions as described in Fig. 1.

cessfully selected for the SPE of M, M3G and M6G. In the late 1980s, mixed-phase SPE cartridges, containing reversed-phase and cation-exchange, sorbents, were commercially introduced [41]. However, it was suggested in some studies that SPE, in spite of its obvious advantages, also had some serious drawbacks. The most important problem, which was raised several times and regard to various commercial products, is the reproducibility of the quality of the packing material. Very different recoveries, up to complete failure of the extraction, were observed for the same or different batches of cartridges [42]. Comparative studies on cation-exchange and mixed-phase columns showed low reproducibility, with morphine recoveries from blood ranging from 12 to 90% [43,44]. The multitude of methods for the isolation of morphine and its metabolites from biological fluids may serve as evidence of these difficulties. The method described in this paper involves both the non-specific hydrophobic isolation on a carbon cartridge and the much more specific ion-exchange isolation on ion-exchange resin. The combination of these two steps successfully purified drugs with high recoveries and maximum removal of interfering substance employing very different isolation mechanisms.

A similar method for the separation of opium alkaloids by reversed-phase ion-pair HPLC was described earlier [45,46], but using a 0.05 *M* phosphate buffer (pH 2.1), 25% acetonitrile, and 2.5 m*M* SDS as the pairing ion, a conventional C_{18} reversed-phase column provided clear separation for M, M3G, M6G and hydromorphone in our study. A higher temperature was chosen to decrease the column pressure and maintain the efficiency of the analytes. Compared with various reported detection methods, a combination of ED and fluorometric detection is an optimal sensitive and specific method for the determination of M, M3G and M6G in plasma, which was used in a similar method to Joel et al. [32]. To improve this method, we used a third guard electrode, running at higher potential than the two analytical cells and placed before the injector, to permit complete removal of the interfering peaks in the mobile phase. In our laboratory, the mobile phase is recycled for several hundreds of sample injections, thus cutting the cost of the assay considerably and keeping the HPLC experiment in the constant condition as well. The hydromorphone was selected as the internal standard, as it is neither a metabolic nor degradation product of morphine. Its structural similarity and compatible recovery of extraction to morphine minimized the experimental variation so that morphine, M3G, and M6G can be measured simultaneously. The autoinjector, SPE column processor, and column heater are helpful to maintain the accuracy and reproducibility of this assay.

This new extraction method for morphine and its metabolites, coupled with the combination of ED and fluorometric detection, the improved removal of interfering substances for the HPLC analysis, all worked together to minimize the variance, and increase the sensitivity and specificity of our assay.

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