Morphine [(5α,6α),7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol], (CAS: 52-26-6) in human plasma. The analyte was extracted from plasma samples with chloroform-isopropyl alcohol (90:10, v/v) and analyzed on a Bondapak C18 column. The calibration curves were linear within the range of 10–150 ng/mL. The lower limit of quantitation was 10 ng/mL with 0.5 mL plasma sample. The mean recovery of the drug from plasma samples was 83.39%. The results from analysis of quality-control samples at concentrations of 30, 75, and 150 ng/mL were indicative of good accuracy and precision. This method was successfully used to analyze morphine in plasma samples of patients after abdominal hysterectomy.

Keywords: high performance liquid chromatography, UV detector, plasma samples, morphine, hysterectomy, pain

Many different chromatographic assays for the determination of morphine in biological fluids can be found in the literature (1–40).

It is a well-known fact that morphine measurements can be best performed by using mass spectrometry (HPLC-MS) (2–12), tandem mass spectrometry (HPLC-MS/MS) (11–24) and GC-MS (4, 25–27) detection. In many cases these techniques have achieved both the desired sensitivity and fast run time, which is important in drug analysis. Unfortunately, these combinations are very expensive and therefore not routinely used for monitoring of morphine in laboratories.

Chromatographic procedures, described previously, were based also on fluorescence (1, 4, 10, 28–30), electrochemical (ECD) (10, 31–37) or diode array (DAD) detection (10, 38–40).

The combined ECD detection with UV was also described by some authors (4, 10).

Opiate analgesics may also be monitored by immunoassay or radioimmunoassay (RIA), which are very sensitive tests, but cross-reactions of analytes with the antisera complicate very often the assay and provide false results (10).
Capillary zone electrophoresis (CZE) does not have sufficient sensitivity for pharmacokinetic studies (10).

For these reasons, high performance liquid chromatography is the most popular choice for the determination of morphine and its metabolites in biological fluids (4, 10).

In the present study, a sensitive and inexpensive HPLC-UV assay for the determination of morphine in human plasma is described. During development of a modified HPLC method with fluorescence detection, adequate parameters for the extraction and UV detection of morphine were found. It offers an alternative to the previously described procedures available for pharmacokinetic and monitoring studies of morphine.

Using this HPLC-UV assay, plasma concentrations of morphine in patients after hysterectomy were analyzed.

MATERIALS AND METHODS

Materials
Morphine sulfate, CAS: 52-26-6 (pure for analysis) was obtained from Sigma-Aldrich (Steinheim, Germany). Morphine sulfate – solution
for injections, 20 mg/mL, batch no: 05BDO107 was supplied by Polfa Warszawa, Poland. Acetonitrile, n-hexane, methanol (HPLC grade) and sodium hydroxide (analytical grade, pure for analysis) were purchased from Merck (Darmstadt, Germany). All other chemicals (chloroform, isopropyl alcohol, ethylene diamine tetraacetic acid sodium salt, boric acid, sodium borate, monopotassium phosphate, hydrochloric acid ) were of analytical reagent grade (Merck, Germany). Human plasma was obtained from the Laboratory of the Blood Donation Centre (Poznań, Poland).

Standard solutions
Standard stock solutions of morphine sulfate were prepared by dissolving accurately weighed substances (100 mg) in bidistilled water (100 mL) to obtain final concentration of 1 mg/mL. Stock solutions were stored at 4°C and remained stable for at least 1 month.

Working solutions (used with the purpose of validation and calibration) were prepared ex tempore by further dilution of stock solution with bidistilled water to provide final concentrations of 10, 20, 30, 50, 75, 100, and 150 ng/mL for morphine sulfate in plasma.

For preparing calibration curves, a volume of 10 mL of an adequate working solutions were spiked to 0.5 mL of blank heparinized plasma, and then extracted, according to procedure described below.

Extraction of morphine sulfate from plasma samples:
To 0.5 mL of plasma sample with morphine, 0.5 mL of borate buffer solution (pH 8.9) and 6 mL of chloroform – isopropyl alcohol solution (90:10, v/v) were added. The mixture was vortexed for 20 min and centrifuged (2000 × g, 10 min). The organic layer was transferred to another tube, to which 2 mL of 5 mM ammonium phosphate buffer pH 9.3 was added and the mixture was vortexed for 1 min and centrifuged (2000 × g, 5 min). The supernatant was discarded and the organic layer containing morphine was transferred into a glass vial, 150 mL of 0.1 M hydrochloric acid was added and the mixture was vortexed for 2 min and centrifuged (2000 × g, 5 min). The separated acid layer was transferred to another glass tube, 15 mL of 1 M sodium hydroxide and 1 mL of hexane were added and the mixture was vortexed for 20 s and centrifuged (1500 × g, 2 min). The organic layer was transferred to 1.5 mL polypropylene Eppendorf tube and 50 µL was injected into the chromatographic system.

HPLC analysis
First of all, in order to find optimum wavelength for ultraviolet detection of morphine, the UV spectrum of this drug was determined (Fig. 2). Using Photodiode Array Detector (Waters 474) the wavelength of morphine was set at 226 nm.

This method was based on the HPLC methods with fluorescence detection (1, 10, 29, 30).

The HPLC system was constructed from the following components: HPLC Waters 2695 Separations Module with autosampler, Waters 2487 Dual l Absorbance Detector. Analytes were separated by an analytical column Bondapak C18 (150 mm × 4 mm, 10 µm) from Waters. Temperature of column was maintained at 25°C. Total analysis time was 7 min and the flow rate was set at 0.8 mL/min. Samples were eluted with a mobile phase: acetonitrile : bidistilled water : methanol (60:840:100, v/v/v) with addition of 0.012 M monopotassium phosphate, and 0.5 mM ethylenediaminetetraacetic acid disodium salt.

Data collection and processing was carried out by Empower Pro software.

Clinical study
Consent for the study
The study was approved by the Ethics Committee at the Poznan University of Medical Sciences. A day before the surgery, patients received written information with a consent for participation in the studies.

Characterization of the investigated population
Subjected were 15 patients (women, 54.3 ± 4.6 years of age, weight 64.5 ± 9.4 kg) after hysterectomy, whom applied intravenous bolus (i.v.) of morphine (Morphini sulfas – solution for injections, 20 mg/mL) with dose titration to achieve the effective morphine dose (2 mg of morphine given every 2–3 min, until the pain was eliminated or lessened – VAS < 4), then constant infusion was maintained after calculating the infusion rate based on the biological morphine half-life (t1/2 = 3 h); breakthrough pains were treated with administration of an additional bolus dose of 2 mg i.v. After the surgery, the patients additionally received 100 mg of ketoprofen (Ketonal – solution for injections, 100 mg/2 mL, i.v.).

Patients were qualified to ASA risk groups I (n = 12) and II (n = 3), without history of liver and kidney diseases, with normal laboratory results, according to standards of premedication care, not taking analgesics on a chronic basis and without contraindications to morphine analgesia. Surgeries were carried out under general anesthesia with intratra-
cheal intubation, after induction with propofol, muscle relaxation with rocuronium.

Course of the study

The patients had 5 mL of venous blood sampled 7 times, from a peripheral vein, in aseptic conditions; the first sample to exclude the patient’s taking morphine, second – after the surgery, but before administration of morphine – sample 0, with subsequent samples 1, 2, 4, 8 and 12 h after administration of morphine. Blood was sampled to syringes, transferred to test tubes with lithium heparin, and then centrifuged at temperature of 20°C for 10 min and frozen at temperature of −70°C. These samples were used for determination of plasma concentrations of morphine (HPLC-UV).

The patients were monitored for 12 h after the surgery. Pain intensity was evaluated according to a VAS scale before administration of morphine, and 1, 2, 4, 8 and 12 h after administration. During this period, arterial blood pressure, heart rate, diuresis, SpO₂ and breath rate per minute were monitored. Depth of sedation was also estimated with an Aldrete scoring. All cases of undesirable effects were recorded.

RESULTS

The HPLC method was validated in accordance with the published guidelines (41, 42).

Specificity

The specificity of the method was examined by comparing the chromatogram of the drug-free plasma sample with that of the plasma sample spiked with morphine. Representative chromatograms of morphine are shown in Fig. 3.

Retention time of morphine was approximately 5.037 min. The total run time for each sample injection was about 7 min. There were no interfering peaks in the blank plasma at the retention time of morphine. No carry-over (memory effect) problem was observed in this assay as 50 µL of mobile phase was injected into the HPLC system after analyzing plasma samples containing morphine.

Linearity

Calibration curves (n = 6) were prepared by spiking 10 µL of the appropriate working solution to 0.5 mL of blank human plasma. Final concentrations of morphine in plasma samples were: 10, 20, 30, 50, 75, 100 and 150 ng/mL. Three spiked plasma samples were analyzed at each concentration level. Calibration curves for the plasma assay developed with peak area of morphine (y) versus drug concentration (x) were found to be linear in the examined concentration range.

Coefficient of variation (CV) for slope (a = 274.04 ± 25.76; n = 6) and intercept (b = 82.17 ± 13.24; n = 6) were 9.4 and −16.11%, respectively. Assay linearity in the range of the morphine concentrations to be expected was concluded from the mean correlation coefficient of r = 0.994 ± 0.003 (n = 6); CV was 0.34%.

Recovery

The recovery of morphine was assessed by comparing the response of five replicates of extracted QC samples (30, 75 and 150 ng/mL) to the response of the pure standard at the same concentration level.

The standard specimens were dissolved in water assigned for injection and injected directly into the HPLC chromatograph. The recovery of morphine was greater than 90% for all tested concentrations. The results are shown in Table 1.

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration founded (ng/mL)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>Recovery (%)</th>
<th>n</th>
</tr>
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<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>30</td>
<td>31.02</td>
<td>3.31</td>
<td>10.66</td>
<td>3.40</td>
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<td>75</td>
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<td>0.63</td>
<td>100.10</td>
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<tr>
<td>150</td>
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<td>9.25</td>
<td>6.24</td>
<td>-1.20</td>
<td>103.78</td>
<td>5</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>28.81</td>
<td>3.04</td>
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<td>-3.97</td>
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<td>15</td>
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<td>10.36</td>
<td>6.74</td>
<td>2.50</td>
<td>96.43</td>
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</table>

SD = standard deviation; CV = coefficient of variation
Blank plasma samples were spiked with morphine at three concentration levels QC (30, 75 and 150 ng/mL). The intra-day accuracy and precision were determined using five replicates of QC samples on the same day; the inter-day accuracy and precision were determined by analyzing three calibration curves with five replicates of QC samples on three separate days.

The intra- and inter-day precision was expressed as the coefficient of variation value: $CV = \frac{SD}{C} \times 100\%$, where SD is the standard deviation of the mean concentration of morphine and C is the mean concentration of morphine (Tab. 1).

The accuracy (bias) for morphine was expressed as the percentage deviation of observed concentration from theoretical concentration.
Bias = \[\frac{\text{found concentration} - \text{added concentration}}{\text{added concentration}} \times 100\%\] (Tab. 1).

Limits of quantitation and detection

The limit of quantitation (LOQ) of morphine, defined as the lowest concentration that could be measured with accuracy and precision, i.e., within ±20% of the actual value, was 10 ng/mL.

The lowest amount of morphine in the sample, which can be detected but not necessarily quantitated under stated experimental conditions (detection limit, LOD), was based on SD of response and slope: \[\text{LOD} = 3.3\delta/S,\] where S is the slope of the calibration curve and δ is SD of blank response (34).

LOD of morphine in this method was 5.23 ng/mL.

Stability

Short-term stability of plasma samples was examined by supplementing blank plasma with appropriate amounts of working solutions of morphine to yield quality-control (QC) samples containing 30, 75, and 150 ng/mL. Each sample was analyzed at room temperature for 48 h (every 4 h), including their residence time in an autosampler. CV values were: 1.84, 1.50 and 1.67% for morphine concentrations of 30, 75 and 150 ng/mL, respectively.

Long-term stability of plasma samples at room temperature was assessed for 30 days. The concentration of the stability QC samples (30, 75, and 150 ng/mL) were compared to the mean of back-calculated values for the standards at the appropriate concentration from the first day of long term stability testing, CV values for analyzed QC samples: 30, 75, and 150 ng/mL were 4.58, 4.76 and 3.98%, respectively.

To determine long-term freezer stability of morphine in plasma, QC samples (30, 75, and 150 ng/mL) were analyzed in triplicate after storage at −80°C for 1, 2, and 30 days. The drug was regarded as stable if more than 90% was intact at the end of the study period. The amount of the initial concentration of morphine remaining after this time was: 98.20 ± 2.14%, 95.15 ± 1.58% and 97.45 ± 2.34% for 30, 75 and 150 ng/mL, respectively.

The results of stability of morphine plasma samples are presented in Table 2.

Clinical study

The mean plasma concentration versus time profiles of morphine in women after hysterectomy are presented in Figure 4.

In research patient group no serious adverse effects were observed. During the research, blood pressure, pulse value, breathing frequency and sedation level were not significantly different from values of these parameters before the morphine was applied.

DISCUSSION

A specific and sensitive HPLC-UV method for measurement of morphine plasma concentrations was described. This method is in accordance with guidelines for validation of analytical methods employed in bioavailability and pharmacokinetic studies in men and animals (41, 42).

The chromatograms of morphine, obtained under this assay conditions, were sharp and symmetrical; total run time was less than 7 min (Fig. 3). The sample volume required is only 0.5 mL, compared with 1.0–2.0 mL for other methods (4, 10).

Extraction recoveries for morphine were consistent, precise and reproducible throughout the validation experiments and were within the acceptance criteria. The intra- and inter-run precision was less than 11%, and the accuracy was within ±3.97% (Tab. 1), which proves the acceptable accuracy and precision of the method developed.

Stability experiments showed that no significant degradation occurred at ambient temperature for 40 h, 30 days and during the three freeze-thaw cycles for morphine plasma samples. The small mean CV values for 30, 75 and 150 ng/mL indicated analyte stability.

Standard solutions of morphine prepared in bidistilled water were stable for at least 1 month at 4°C.

The reagents used in the method are inexpensive and readily available and the procedure does not involve any critical experimental conditions. This HPLC method may be suitable for quantification of morphine sulfate in small samples of plasma. The fact, that our assay of morphine is less sensitive than that using, for example, mass spectrometry detection is evident. However, HPLC-MS or HPLC-MS-MS for quantification of morphine is far more expensive than the HPLC-UV method.

Detection limit of the assay is sufficient for drug monitoring in optimizing of postoperative analgesia. The procedure is sensitive and selective as well as suitable for determining the pharmacokinetics of morphine in clinical studies. It offers a suitable alternative to existing HPLC techniques.

REFERENCES


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