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Solid Phase Extraction of Morphine from Whole Blood by Means of Bond Elut Certify Columns

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ABSTRACT: The use of Bond Elut Certify columns for the isolation of morphine from whole blood was evaluated. In order to monitor possible losses and the elution profile of morphine, a small amount of the tritiated analogue was added to the samples. Four sample pretreatment methods, three protein precipitation methods and one sonication/dilution method, were tested. The latter one gave the best results. The blood sample was applied onto the column at pH 3.3 after sonication and dilution with 0.1 M phosphate buffer (pH 3.3). The retention of morphine was affected by the pH of the samples, and the loss of morphine during sample application was minimized at low pH (3.3). The interferences were removed by washing the column with the phosphate buffer, 0.01 M acetic acid (pH 3.3), and methanol, sequentially. Ammoniated methanol, 2 mL at 2%, was selected to elute morphine. As a result, more than 80% of ^{3}H-morphine was recovered for concentrations of morphine ranging from 5 to 4000 ng/mL.

KEYWORDS: toxicology, extraction, morphine, blood, solid-phase extraction, Bond Elut Certify column

Determination of morphine in human blood is important for forensic toxicology because of its frequent misuse. Also, it is one of the main metabolites of heroin. Morphine is an amphoteric drug that may exist in different forms depending on the pH of the medium.

For the determination of morphine in biological samples several gas chromatographic (GC) methods were applied [*1–3*]. Many of these methods require derivatization to improve the chromatographic properties of morphine. Another common technique is high-performance liquid chromatography (HPLC). In recent years HPLC with electrochemical detection has been applied [*4,5*] with limits of quantitation as low as 1 ng/mL.

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However, both GC and HPLC methods generally require sample clean-up prior to the final determination.

The use of solid-phase extraction (SPE) for the isolation of drugs from biological samples has increased in recent years [*6,7*] as SPE offers several distinct advantages over traditional liquid-liquid extraction [*8*]. The application of SPE for isolating morphine from a biological matrix by using C_{18} reversed phase SPE columns has also been reported [*9–11*].

Bone Elut Certify columns contain a mixed-mode bonded silica gel, which consists of hydrophobic and cation exchange functional groups. The columns have successfully been applied for the extraction of various classes of drugs from plasma and urine [*12*], as well as from whole blood [*13*].

Because of the amphoteric character of morphine, the use of Bond Elut Certify columns for the isolation of morphine from whole blood was evaluated in this project. Mixtures of tritiated morphine and varying amounts of unlabeled morphine were used for spiking whole blood. All fractions collected during the extraction were measured in order to assess the behavior of morphine on the column and to trace losses of the drug.

Materials and Methods

Materials

Morphine hydrochloride was obtained from Brocacef (Maarsen, The Netherlands) and was of pharmacopoeial quality. Tritium-labeled morphine solution (80.0 curies (Ci)/mmol) was obtained from NEN (Boston, Massachusetts). This solution was diluted 100-fold with methanol and contained 370 Bq ^{3}H-morphine per μL. A stock solution of unlabeled morphine (1.07 mg/mL) was prepared in methanol. The working solutions contained unlabeled morphine in concentrations ranging from 107 ng/mL to 80.25 μg/mL and 18.5 Bq/μL of ^{3}H-morphine and were prepared by combining appropriate amounts of unlabeled morphine with 50 μL of ^{3}H-morphine stock solution and were further diluted to 1 mL with methanol. The latter solutions were used for spiking blank blood samples. The concentration of unlabeled morphine in the spiked blood were in the range of 5.37 to 4012.5 ng/mL and the concentration of ^{3}H-morphine with 0.09 ng/mL.

Bond Elut Certify columns (130 mg sorbent mass/2.8 mL column volume or 130 mg sorbent mass/10 mL column volume) were supplied by Varian Sample Preparation Products (Harbor City, CA). A Baker-10 vacuum manifold system was purchased from J. T. Baker (Phillipsburg, NJ). A model SC-152 sonic bath was purchased from Sonicor Instrument Corporation (Copiague, NY). A Centaur 2 bench-top centifuge was obtained from MSE Scientific Instruments (Crawley, Sussex, England).

All reagents were of analytical grade (Merck, Darmstadt, FRG). RIA LUMA was used as scintillation liquid and obtained from LUMA*LSC (Olen, Belgium). Outdated blank human blood was obtained from the local blood bank. Autopsy blood was obtained from a traffic-accident victim. The blood samples were stored in a freezer at −20°C until use.

Instrumentation

A Tri-Carb 4000 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) was used for the determination of the radioactivity in the samples. The absolute recoveries were obtained by comparing the radioactivities of the spiked samples with that of the standard solutions.

The analysis of morphine was performed on a HPLC system (Waters Chromatography Div., Millipore Corp., Milford Massachusetts) equipped with an electrochemical detector

(Antec, Leiden, The Netherlands), and a Chromsep 100 mm × 3 mm ID column packed with 5 μm Spherisorb cyanopropyl (Chrompack, Middleburg, The Netherlands).

The mobile phase consisted of a mixture of 8.6 volumes of tetramethylammonium hydroxide-citrate buffer (4 mmol/L, pH 5.7), 1.3 volumes of methanol and 0.1 volumes of 1,4-dioxane; the flow rate was 0.5 mL/min.

Sample Pretreatment

Method A—While rotating on a vortex mixer, 2 mL of zinc sulfate-methanol solution (70/30) was added to 1 mL of blood. After centrifuging at 1500 g for 15 min, the supernatant was transferred into a polyethylene tube which contained 2 mL of 0.1 M sodium acetate buffer (pH 6.0). The mixture was vortexed for 30 sec.

Method B—One mL of blood was sonicated in a sonic bath for 15 min at room temperature. 1.5 mL of acetonitrile was added to the blood sample and then it was vortexed. The sample was then centrifuged at 1500 g for 15 min, and the supernatant

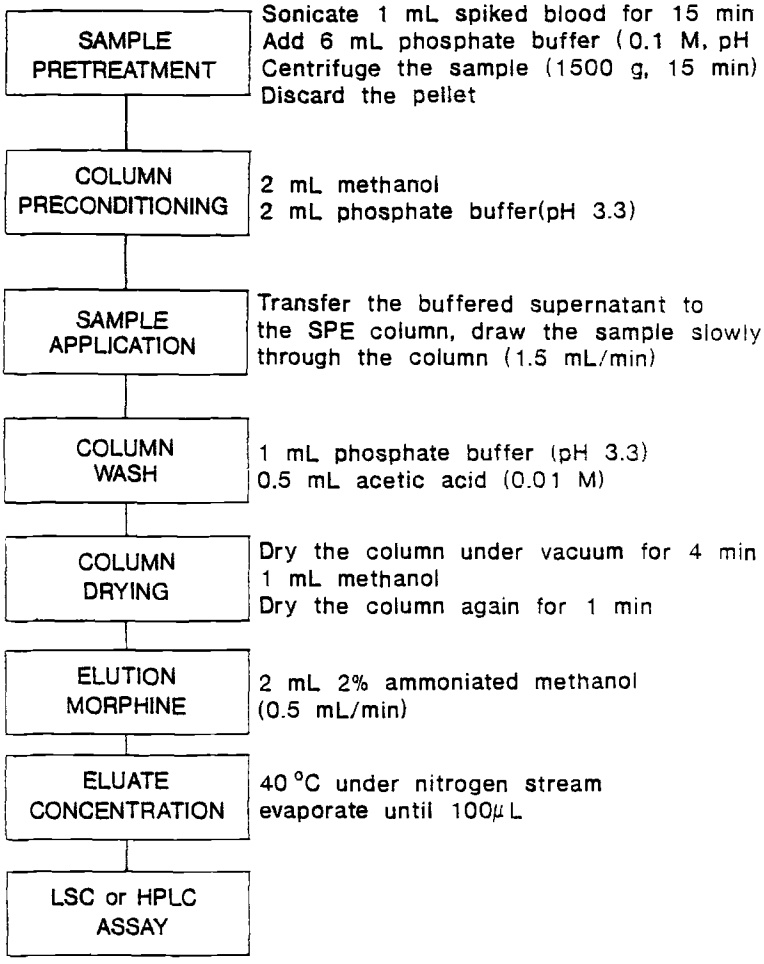

FIG. 1—*Extraction scheme for the isolation of morphine from whole blood using Bond Elut Certify columns.*

was transferred into a polyethylene tube which contained 6 mL of 0.1 M phosphate buffer (pH 6.0). The mixture was vortexed for 30 s.

Method C—Same as Method B, except that 2 mL of methanol was used as a precipitant instead of acetonitrile.

Method D—One mL of blood was sonicated in a sonic bath for 15 min at room temperature. The sample was vortexed for 30 s after the addition of 6 mL of 0.1 M phosphate buffer (pH 6.0 or 3.3). The diluted blood was centrifuged at 1500 g for 15 min, and the supernatant was transferred directly onto the SPE column. This procedure is a modification of the one described by Tebbett [*11*].

Extraction Procedure

The extraction was performed with Bond Elut Certify columns, which were installed on a Baker-10 vacuum manifold.

The initial extraction procedure according to Varian [*14*] was as follows:

1. The column was preconditioned with 2 mL of methanol and 2 mL of 0.1 M sodium acetate buffer (pH 6.0), sequentially.
2. The blood sample, which was pretreated according to method A and was applied onto the column and passed through the column at a flow rate of 1.5 mL/min.
3. The column was then washed with 2 mL of deionized water, 1 mL of 0.1 M of acetate buffer (pH 4.0), and 2 mL of methanol, sequentially.
4. The column was dried under vacuum for 2 min.
5. The drug was eluted by passing 2 mL of 2% ammoniated methylene chloride-isopropanol (80:20) at a flow rate of 0.5 mL/min.
6. The eluate was evaporated at 40°C in a water bath under a stream of nitrogen until approximately 100 μL of solvent remained in the tube.
7. This residue was mixed with 3 mL of scintillation liquid for the determination of the radioactivity, or 20 μL was injected into the HPLC.

The final extraction procedure as obtained after the optimization is described in Fig. 1.

Results and Discussion

Pretreatment of Whole Blood

Whole blood directly applied onto SPE column tends to cause blockage and low yields of drug in the final extract. Therefore a pretreatment step of whole blood is necessary. Initial experiments were carried out by utilizing the extraction procedure according to Varian [*14*]. As shown in Table 1 only 31% of ^{3}H-morphine was recovered in the final extract. Pretreatment of blood samples with acetonitrile (Method B) or methanol (Method C) were also tested. The recoveries of ^{3}H-morphine were 23% and 11%, respectively. The low recoveries were attributed to co-precipitation of morphine with the blood proteins. Method D, which had been successfully applied for the general screening of drugs [*13*] resulted in a 74% yield of ^{3}H-morphine. Under these conditions still 26% of morphine was lost.

Optimization of the SPE Procedure

In order to improve the recovery of morphine, the influence of pH of the sample and the column system on the retention of the drug, the clean-up step, and the elution efficiency of various eluents were tested by monitoring ^{3}H-morphine in all collected

TABLE 1—*Recovery of ^{3}H-morphine from spiked human blood[a] after various precipitation methods.*

	Method A (%)	Method B (%)	Method C (%)	Method D (%)
	29.5	23.4	12.4	69.3
	32.5	21.3	8.4	77.2
	32.0	23.8	11.4	76.3
Average	31.3	23.0	10.8	74.4

[a]Spiked with of 925 Bq ^{3}H-morphine/mL and 160.5 ng/mL of morphine.

fractions. Because of the color quenching of the sample obtained in the sample application step and the column wash step, which interfered in the radioactivity counting, the initial optimizations were done using spiked phosphate buffer instead of spiked whole blood.

-pH of sample application—It can be seen from the bar called 'buffer' in Fig. 2 that about 7% of ^{3}H-morphine in spiked buffer was not retained during sample application at pH 6. An attempt to minimize the loss of morphine in this step was made by adjusting the pH of the applied sample and column. It was observed that the loss was reduced to 3% at pH 3.3. Hence, the pH of sample application should be around 3. Data are presented in Fig. 3. More acidic pH values were less effective.

Column wash—An efficient clean-up process was obtained by washing the column with 1 mL of phosphate buffer (pH 3.3), 0.5 mL of acetic acid (pH 3.3), and 1 mL of methanol, sequentially. The loss of ^{3}H-morphine, as shown in Fig. 3, was less than 1%

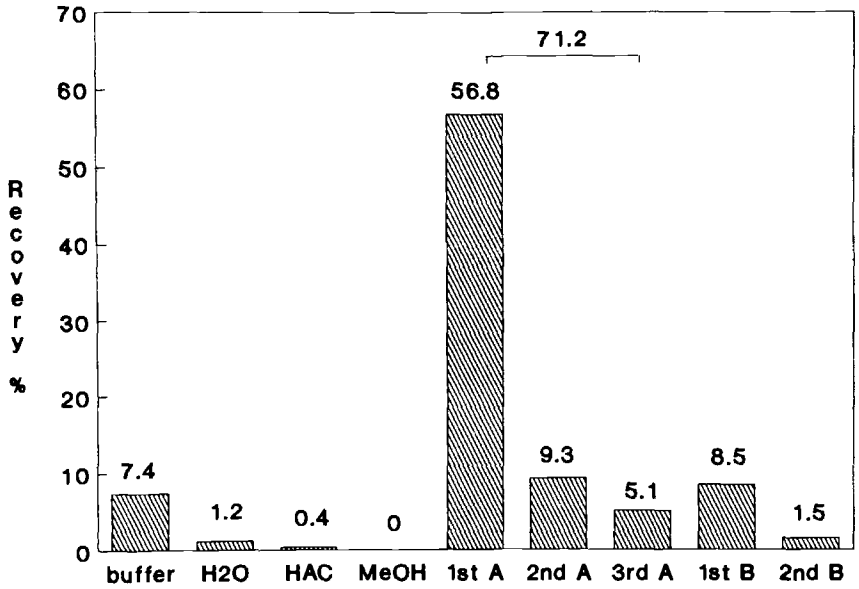

FIG. 2—*Recovery of ^{3}H-morphine from phosphate buffer spiked with of 925 Bq ^{3}H-morphine/mL and 401.3 ng/mL of morphine at pH 6. The bars represent the mean recoveries of ^{3}H-morphine of triplicate determinations of the fractions obtained from the sequential steps in the extraction procedure. Eluent: A = methylene chloride-isopropanol (80/20) with 2% ammonium hydroxide 2 mL; and B = 2% ammoniated methanol 2 mL.*

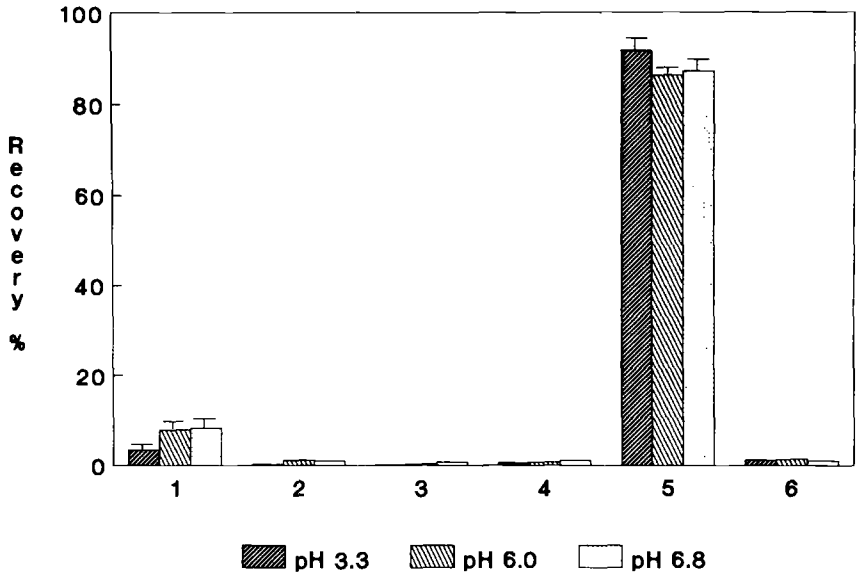

FIG. 3—*Recoveries of ^{3}H-morphine from spiked phosphate buffer samples at various pH values. The concentration of morphine was 401.3 ng/mL combining with of 925 Bq ^{3}H-morphine/mL. The groups of bars represent the mean recoveries of triplicate determinations of the fractions obtained from the sequential steps in the extraction procedure where 1 = spiked buffer; 2 = 1 mL washing buffer pH 3.3; 3 = 0.5 mL acetic acid pH 3.3; 4 = 1 mL washing methanol; 5 = first 2 mL 2% ammoniated methanol; and 6 = second 2 mL 2% ammoniated methanol.*

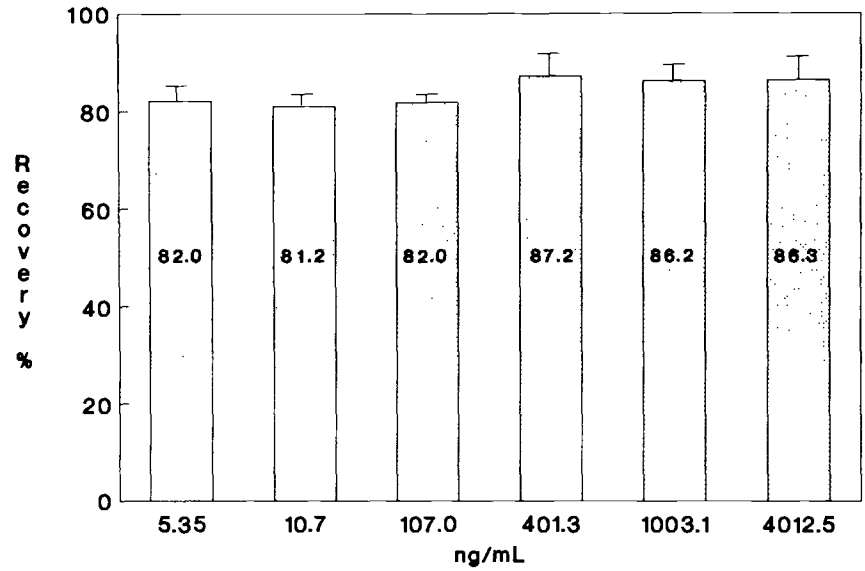

FIG. 4—*Recoveries of ^{3}H-morphine (925 Bq/mL) from spiked autopsy blood samples at variou concentration levels of morphine. The bars represent the mean recoveries of triplicate determinations Eluent: 2 mL of 2% ammoniated methanol.*

at pH 3.3, because morphine is protonated under these conditions and retained by the cation exchange functional groups of the sorbent. The methanol wash was found to remove virtually all colored endogenous interferences, but in order to do this properly it was found essential to dry the column under full vacuum for 4 min after the acetic acid wash. After the methanol, column drying under full vacuum for 1 min is also necessary.

Elution efficiency of various eluents—Although an alkaline methylene chloride-isopropanol mixture solution was recommended by the manufacturer of the columns, it was observed that this eluent was, in fact, not efficient enough to elute morphine completely. This phenomenon was confirmed by the observation that after passing three times 2 mL of ammoniated methylene chloride-isopropanol through the SPE column, a substantial portion of radiolabeled morphine still remained on the column and which could be further eluted by ammoniated methanol (Fig. 2). These results indicated that a polar eluent is necessary for obtaining the highest yield. Therefore, methanol was used to improve the elution efficiency. As illustrated in Fig. 3 more than 90% of ^{3}H-morphine was recovered by passing 2 mL of 2% ammoniated methanol through the column. Further optimization of the elution step was not necessary since only 1% of ^{3}H-morphine remained on the column.

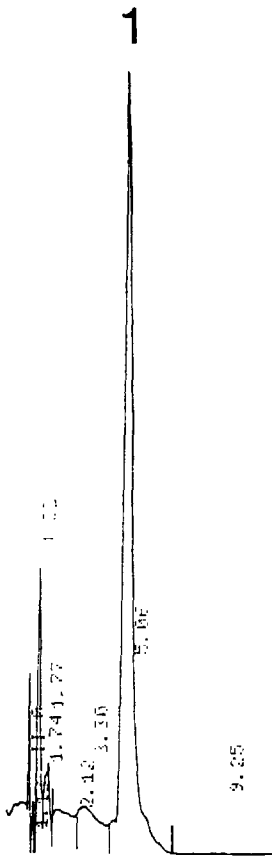

FIG. 5—*Typical HPLC chromatogram of spiked whole blood at a concentration of 160.5 ng/mL after solid phase extraction with a Bond Elut Certify column. 1 = morphine.*

Recovery of ^{3}H-Morphine from Blood Samples

After optimizing each step, the final extraction procedure was obtained as depicted in Fig. 1. This procedure was utilized to extract autopsy blood samples which had been spiked with various amounts of unlabeled morphine containing of 925 Bq ^{3}H-morphine/mL. As illustrated in Fig. 4, the absolute recoveries were between 81% and 87%. The results demonstrate that the absolute recoveries of morphine are constant for concentrations between 5 and 4000 ng/mL. A typical chromatogram of spiked human blood after extraction with a Bond Elut Certify column is shown in Fig. 5.

Conclusions

The pretreatment of whole blood is very important for quantitation of morphine in combination with SPE. The most efficient method is to sonicate and dilute the blood. Passing the thus treated samples through Bond Elut Certify columns at low pH (3.3) can reduce the loss of morphine during the sample application step. Under this pH condition, the column can be washed with various solvents to remove impurities without loss of the drug. An alkaline polar solvent is required for the elution of morphine from the Bond Elut Certify column in a relatively small volume. Methanol containing 2% of ammonia gave absolute overall recoveries >80%, which were independent of the concentrations in the range of 5 to 4000 ng/mL.

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