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Determination of Morphine in Urine by HPLC Using Ion-Pair Extraction

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A new method was developed for the analysis of morphine in human urine by high performance liquid chromatography (HPLC) using ion-pair extraction. By adding a kind of hyamine in organic phase, simultaneous extraction and enrichment of morphine were achieved. Effects of the types of organic solvents and carriers, the concentration of carrier and organic, the pH, the stirring rate and the extraction time on the recovery factor of analytes were investigated. Under the optimal experimental conditions, the linear detection ranges were 0.1-10 mg L^{-1} for the studied drugs. The limits of detection at signal-to-noise ratio of 3 were 0.03 mg L^{-1} for morphine. The relative recoveries ranged over 85.5-92.1 %, y = 31.276x - 8.7618, R^2 = 0.9969, the relative standard deviations were 0.3-4.4 % (n = 5). This method was successfully applied to analysis of morphine in real urine specimens, revealing that the determination of morphine in urine was feasible.

Key Words: Urine, Morphine, Ion-pair extraction, HPLC.

INTRODUCTION

Morphine is a μ-opioid agonist traditionally used for the treatment of moderate to severe pain. Morphine is the main alkaloid in poppy seeds having pharmacological and toxicological activity. They are usually used as narcotic analgesic and antitussive drug[1-3]. What's more, codeine is metabolized by O-demethylation to its active metabolite morphine[4]. Following morphine administration, morphine is only present in low concentrations in plasma and urine. Determination of morphine in biological samples is a common practice in many laboratories. The determination of morphine and its metabolites in biological fluids is still a challenging task. One of the largest difficulties is often the sample pretreatment step. Common procedures include liquid-liquid extraction, solid phase extraction and protein precipitation often in combination with evaporation to dryness to preconcentrate samples. In order to cope with this crisis, determination of morphine in the biological specimen becomes the most important critical factor[5]. Several human specimens including urine, blood, hair, saliva and sweat have been used in determining the residues of illicit drugs. Among them, urine, hair and blood in drug testing have frequently been reported. The testing on saliva reveals a short-window of drug abused record. But the drug testing from human saliva is easily to lead imprecise results[6]. Hair can be easily obtained and difficult to adulterate and which could be stored and transported without specific precaution so wing to its stability[7]. But in contrast to saliva, hair specimen supplies alonger detection window. The urine testing is the most popular method to determinate the drug abuse, which provides a short-term historical record of the drug exposure (less than 7 days)[8]. The collection of urine is perceived as less invasive to adulteration as compared to blood specimen. However, the amount of drug and its metabolites are lower in urine than in blood, which therefore demands a much higher sensitivity of the analytic apparatus. A number of methods have been reported for determining morphine in urine by biological assays and chromatographic technique. These methods are often affordability, easy maneuverability and high throughput. However, they are limited for cross-reactivity and poor inter-laboratory reproducibility. Some reports are available in literature about different chromatographic techniques such as HPLC with fluorescence detection and HPLC with dual electrochemical and spectrophotometric detection[9-11]. More recently, various papers have described the quantification of morphine by HPLC-MS, which offers more sensitivity and specificity[12-20]. And HPLC and GC methods have used various extraction techniques (*e.g.* liquid-liquid or solid-phase extraction) to quantify morphine and its glucuronides in urine, plasma and blood.

Ion-pair extraction use a complexation reagent and a counter ion. The higher selective reagent is one of the most important factors to realize their mutual separation[21]. In this work we studied the ability of the morphine anion to form ion-pairs in aqueous solution in the presence of organic and organic cations: ion-pairs have a hydrophobicity and polarity more suitable to the partition than each ion considered separately and can be extracted by a organic phase.

EXPERIMENTAL

Morphine was obtained from Yunnan province tumor hospital. All reagents used were HPLC grade and purified water from a Milli Q system was used throughout the experiments. Standard stock solutions containing these compounds were prepared in methanol at a concentration of 500 μg mL^{-1}. Working solutions were prepared daily by an appropriate dilution of the stock solutions. Cetyltrimethylammonium bromide (CTAB), 12 alkyl 2-methyl benzylammonium bromide, tetraoctylammonum bromide Na_2SO_4, $ZnSO_4$, $MgSO_4$, $ZnCl_2$, ammonium acetate, *n*-butyl alcohol, NaOH, *iso*butanol, CH_2Cl_2 and cyclohexane were prepared immediately before each experiment.

The high performance liquid chromatography (HPLC) system included four Agilent 1200 series LC-20AT pumps, an SPD-M20A DAD detector and an auto injector. An ultrasonic cleaner with temperature control (Shanghai, China) was used for ultrasonic extraction. A centrifuge with calibrated centrifugal tubes (Shanghai, China) was used for the phase separation process.

HPLC conditions: The separations were performed on an Agilent TC-C_{18} column (150 mm × 4.6 mm, i.d, 5 μm). An Agilent Chemstation for LC system was utilized to control the system and for the acquisition and analysis of the chromatographic data. Quantification was done by the evaluation of peak areas. The mobile phase was methanol: 0.05 % acetic acid (5:95, v/v) at a flow-rate of 1.0 mL/min. The injection volume was 20 μL and the DAD detector was chosen at 240 nm. The column temperature was 25 ºC.

Preparation of urine samples: Urine samples were collected from 10 health volunteers aged 21-29 (including 5 men and 5 women) at early morning time. All samples were stored and frozen at -18 ºC until analysis. 50 mL urine was transferred into a 100 mL volumetric flask and diluted to the mark with distilled water. The 10 mL aliquots of urine were subjected to the ion-pair extraction procedure.

Iron-pair extraction procedure: For the extraction and preconcentration of morphine, 1.5 mL *iso*butanol, 0.025 mol cetyltrimethyl ammonium bromide was added to 8 mL purified water spiked with morphine. The pH of the samples was adjusted to pH 9 by adding 1 mol/L NaOH. 30 min ultrasound was turned on to promote mass transport. After extraction for a certain time, centrifuged once (4,000 rpm for 5 min). After phase separation. The organic phase was diluted in 2.4 mL of mobile phase and 20 μL were injected into the HPLC system for subsequent analysis.

Liquid-liquid extraction procedure: For comparison with ion-pair extraction, the conventional liquid-liquid extraction method was performed with the spiked urine under study. In liquid-liquid extraction procedure, the extract solvents were commonly selected as organic solvents such as $CHCl_3$ and *iso*butanol 10 mL prepared urine mixed with 1.5 mL extract solvents (*e.g. iso*butanol) was placed in an ultrasonic water bath at 35 kHz of ultrasonication frequency for 30 min. Following centrifugation, the organic phase was diluted to 2 mL with acetonitrile and 20.0 μL was injected into the HPLC system.

Protein precipitation procedure: For comparison with ion-pair extraction, the conventional protein precipitation method was also applied to prepare the spiked urine under study. Usually, inorganic salt such as $ZnSO_4$, $MgSO_4$ and $Pb(CH_3COO)_2$ was applied for protein precipitation in biological samples. 10 mL prepared urine mixed with 26 mg precipitants (*e.g.* $ZnSO_4$, $MgSO_4$ and $Pb(CH_3COO)_2$) was centrifuged at 4000 rpm for 3 min. The supernatant was removed to a screw cap glass centrifuge tube with conical bottom by a syringe, following the ion-pair extraction procedure.

RESULTS AND DISCUSSION

The purity of morphine was confirmed by HPLC and ion-pair extraction. The HPLC chromatography of morphine showed one single peak. The purity of morphine was confirmed by peak area. The influences of effective parameters such as salt (type and amount), water-miscible organic solvent, phase volume, equilibration time, centrifugation time and pH of the sample solution were studied and optimized.

Effect of complexation reagent type and amount: The effect of ionic strength was extensively evaluated in traditional liquid-liquid extraction; because addition of a hyamine is often used to form complex, so decrease the solubility of hydrophilic compounds in the aqueous phase through extraction and consequently increase the partition of analytes into the organic phase.

Because of the ability of the morphine anion to form ion-pairs in aqueous solution in the presence of organic and organic cations several salts, including cetyltrimethylammonium bromide, 12 alkyl 2 methyl benzylammonium bromide and tetraoctylammonum bromide were tested (Fig. 1). Therefore, the polarity of the morphine was reduce with the increase of hyamine's carbon chain. So cetyltrimethyl ammonium bromide was selected for further experiments. In order to form more ion-pairs, the different amount of cetyltrimethyl ammonium bromide was tested (Figs. 2 and 3). The results demonstrated that the recovery was increased when the concentration of cetyltrimethyl ammonium bromide was increased from 0 to 0.034 mol, and 0.025 mol was the best choice.

Effect of extraction solvent: The selection of an appropriate extraction solvent is essential for the ion-pair extraction. The extraction solvent has to meet certain requirements such as miscibility with aqueous phase and extraction capability of analytes. Based on these considerations CH_2Cl_2, cyclohexane,

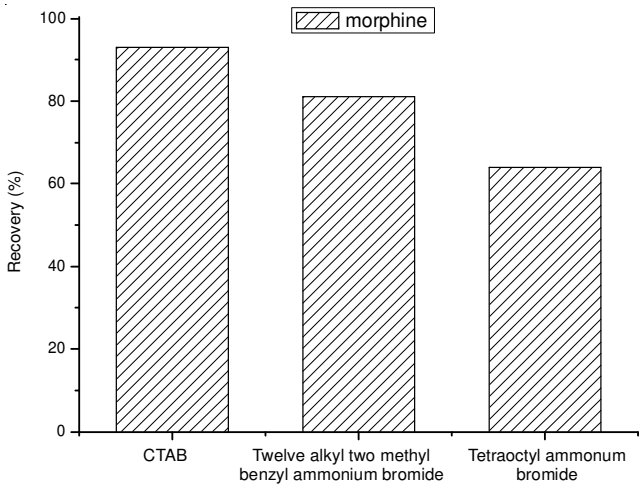

Fig. 1. Effect of complexation reagent nature

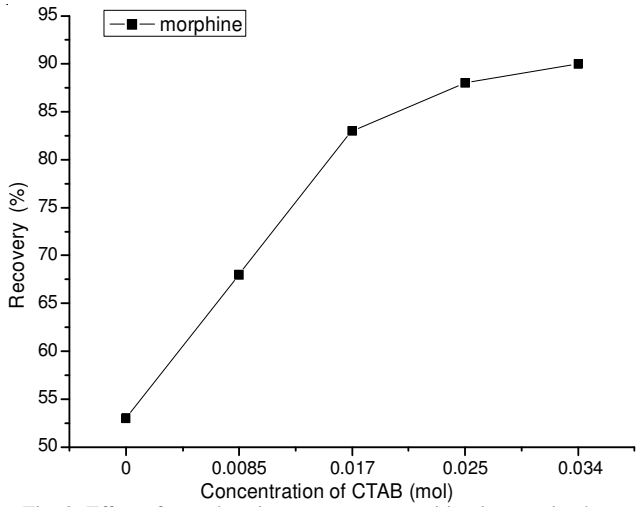

Fig. 2. Effect of complexation reagent concentrition in organic phase

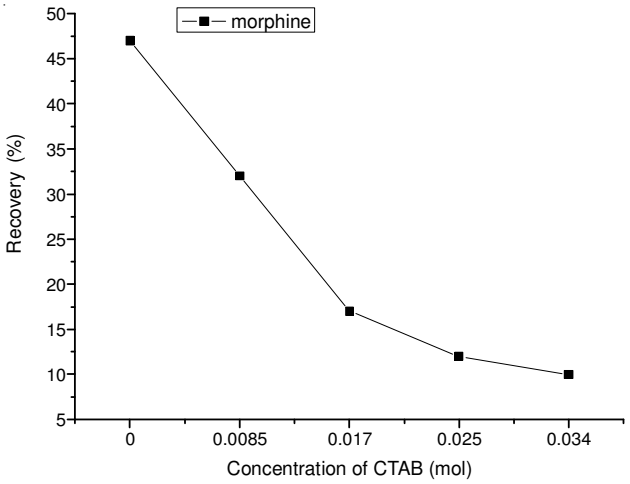

Fig. 3. Effect of complexation reagent amount in aqueous phase

n-butyl alcohol and *iso*butanol were tested (Fig. 4). The results show that *iso*butanol exhibited the highest extraction efficiency when compared with the other solvents. Therefore, *iso*butanol was selected as the extraction solvents for subsequent experiments.

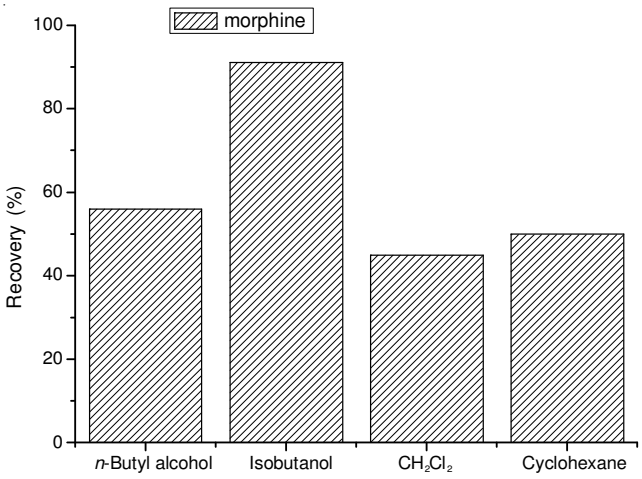

Fig. 4. Effect of extraction solvent

Effect of the volume of *iso*butanol and the concentration of cetyltrimethyl ammonium bromide : It has been well known that the adjustment of solvent amounts used and the concentration of cetyltrimethyl ammonium bromide are important operating variables. For successful recovery of morphine, it is desirable to use a minimum amount of *iso*butanol and cetyltrimethyl ammonium bromide for maximum extraction of morphine. In order to obtain the effect of concentration of *iso*butanol and cetyltrimethyl ammonium bromide on extraction of morphine, different initial concentrations were tested (Fig. 5). It was found that the 1.5 mL of *iso*butanol and 0.025 mol cetyltrimethyl ammonium bromide obtain a good recovery. For all subsequent works, 1.5 mL of *iso*butanol and 0.025 mol cetyltrimethyl ammonium bromide was selected in all later studies.

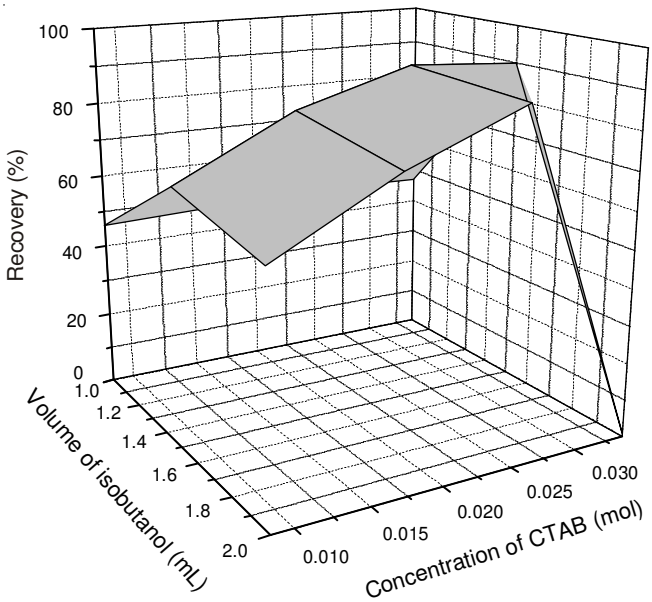

Fig. 5. Effect of the volume of *iso*butanol and the concentration of CTAB

Effect of pH: pH Plays an important role on subsequent extraction. Fig. 6 showes morphine could be almost completely extracted into the organic phase at pH 9. Because all the analytes were primarily in their unprotonated forms and would form ion-pairs with hyamine. Even under the above acidic conditions, the prontonated analytes would not form ion-pairs with the neutral or cationic carrier. And all the carriers markedly enhanced the extraction efficiency, especially for the hydrophilic drugs at pH 9. Therefore, the enhancement of extraction efficiency was due to the formation of ion-pairs.

Effect of equilibration time: The equilibration time is also an important operating variable industrially. For successful recovery of morphine, it is desirable to use a minimum time for maximum extraction of morphine. For the sake of discussing the effect of time on extraction of steroids hormone, different time was tested (Fig. 7). It has been observed that 0.5 h was beneficially chosen for all subsequent experiments, thus 0.5 h was selected in all later studies.

Effect of centrifugation time and rates: It was found that the increase of centrifugation rate has no considerable effect upon the extraction efficiency and analytical signal. The effect of centrifugation time on phase separation of urine was

studied in the range 2-20 min at 4500 rpm. The results show that 5 min was enough to get a complete phase separation. So a centrifugation time of 5 min was selected as optimum.

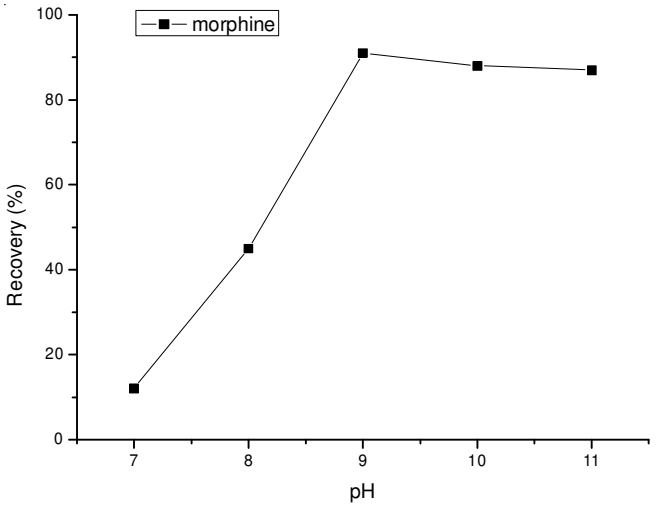

Fig. 6. Effect of pH

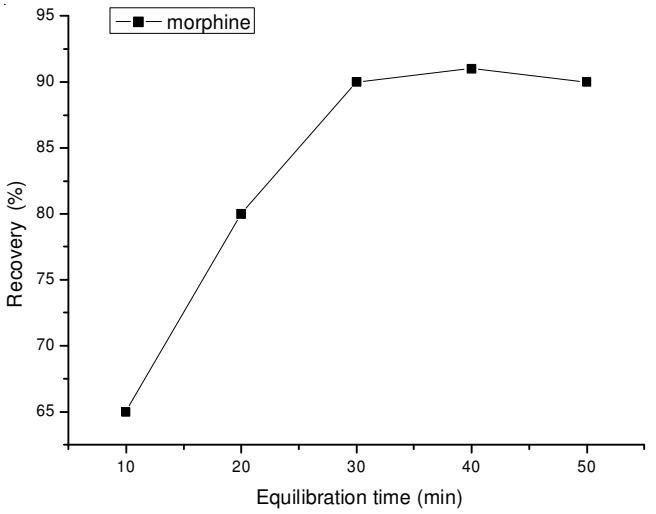

Fig. 7. Effect of equilibration time

Comparison with other methods: In order to compare the proposed method with the previously reported methods for HPLC recovery of morphine in water, the application of the proposed ion-pair extraction and the conventional liquid-liquid extraction were studied (Table-1). As shown in Table-1, the ion-pair method is superior to liquid-liquid extraction. In order to obtain better extraction effect, the repeatedly extraction was studied. And the results show that the repeatedly extraction is slightly better than once extraction.

TABLE-1
RECOVERY (%) OF THE PROPOSED METHOD AND LLE

Analyte	Isobutanol	$CHCl_3$	Ion-pair
Morphine	53.1	43.3	90.3

Analysis of real samples: In order to validate the accuracy and precision of the proposed method under the selected conditions, spiked samples had been tested. Chromatograms of urine samples spiked at 25 mg L^{-1} are shown in Fig. 8 using the proposed method. The chromatograms of urine samples spiked at 25 mg L^{-1} are shown in Fig. 9 without enrichment. The results were satisfied, showing no obvious interferences.

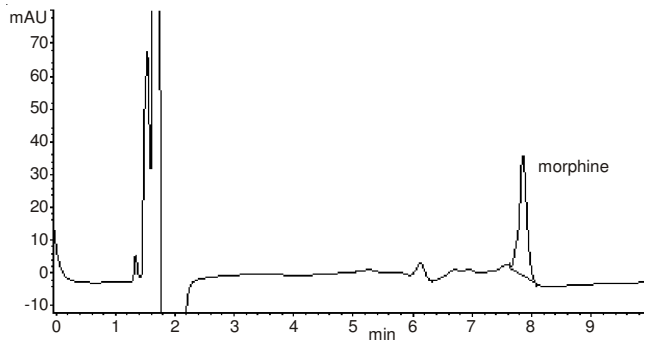

Fig. 8. Chromatograms of urine samples spiked at 25 mg/L morphine

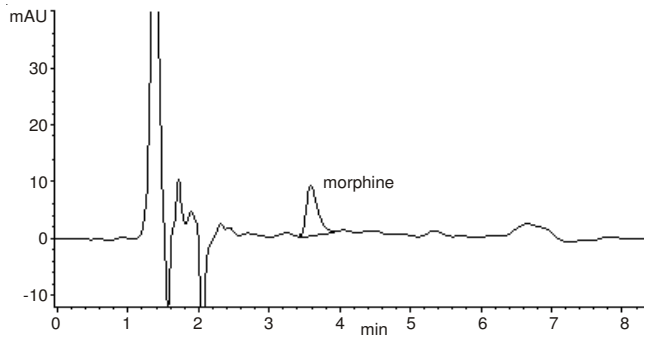

Fig. 9. Chromatograms of urine samples spiked at 25 mg/L of morphine without enrichment

Method evaluation: The relative recoveries ranged over, y = 31.276x - 8.7618, R^2 = 0.9969, the relative standard deviations were 0.3-4.4 % (n = 5). Table-2 showed morphine of the proposed method. The linearity of morphine was in the range 0.1-10 mg L^{-1}. The standard curves for the morphine with a coefficient of determination (R^2) more than 0.9969 (n = 5). The absolute recovery and precision morphine was determined on spiked blank samples at three concentration levels low (0.1 mg L^{-1}), medium (1 mg L^{-1}) and high (5 mg L^{-1}). The mean recoveries were in the range 85.5-92.1 % and the relative standard deviations (RSD, n = 5) were from 0.3 to 4.4 %.

TABLE-2
PERFORMANCE CHARACTERISTICS OF THE PROPOSED METHOD

Analyte regression equation x(mg L^{-1}); y (peak area percentage)	Correlating coefficient	Linear range (mg L^{-1})	Detection limit (mg L^{-1})
Morphine y = 31.276x - 8.7618	R^2 = 0.9969	0.1-10	0.03

Conclusion

The ion-pair extraction was applied as an effective method for the extraction of morphine in aqueous samples. Through the study pH = 9, 0.025 mol cetyltrimethylammonium bromide, 1.5 mL *iso*butanol and extracting 0.5 h were the best choices. Under optimized experimental conditions, calibration plots were found to be linear in the range of 0.1-10 mg L^{-1} for

morphine, with coefficient of determinations more than 0.9969. The relative recoveries ranged over 85.5-92.1 %, the relative standard deviations were 0.3-4.4 % (n = 5). The high recoveries and precision showed the optimal experimental conditions were satisfied. So the proposed method is a simple, rapid and effective method for the simultaneous determination of morphine with their very low concentration in urine.

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