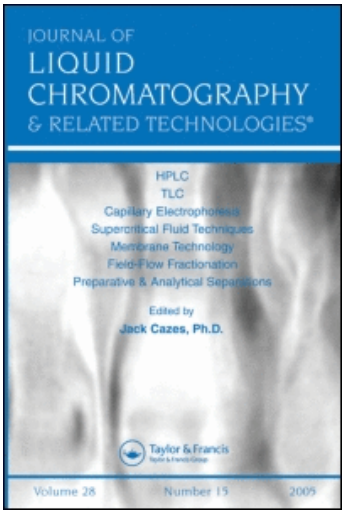

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COMPARATIVE STUDY OF DIFFERENT EXTRACTIVE PROCEDURES TO QUANTIFY MORPHINE IN URINE BY HPLC-UV

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ABSTRACT

A comparative study to extract and purify total morphine in urine is described using several procedure treatments of biological samples. Sep-Pak C18 cartridges, Extrelut columns and liquid-liquid conventional extraction with sequencial purification through Extrelut column were assayed in order to establish the best procedure to quantitatively extract morphine from urine free of endogenous interferents.

Analysis of the drug was made by HPLC-UV with a C18 column and methanol: acetate buffer (pH6.9) (70+30) as eluent.

Recovery of morphine from spiked urine was 88.0% and 79.8% for concentrations of 1µg/ml and 5µg/ml, respectively. The detection limit of the method was 0.2µg/ml and coeficient of variation was 1.8% (n=4).

INTRODUCTION

The recent increase in demand for drug testing in urine, namely to detect morphine resulting from heroine use, has made it imperative to develop a correct procedure to extract and quantify that drug in biological fluids. Urine, being the choice biological

sample to detect morphine, also contains many other compounds that make it difficult to separate the drug from those interferences. So, the main step in preparation of biological extracts is its purification, free of interferent compounds and without loss of morphine. Several methods of extraction of morphine have been published using conventional liquid-liquid procedures (1-6) and pre-packed columns (7-9). In this study we evaluated the best procedure to purify urine extracts for further analysis. We compared urine extracts from heroine treated guinea-pigs which had been prepared by using Sep-Pak C18 cartridges, Extrelut columns and liquid-liquid conventional extraction followed by Extrelut purification. The latter method gave the best results, because the extracts obtained were free of interferents for liquid chromatography analysis.

Although GC/MS has been described as the best method for unequivocal identification and quantification of drugs of abuse (5,6,8,9-11), the preparation of the extracts is further complicated by the need for an additional derivatization step for morphine. Moreover, GC/MS equipment is very expensive and not commonly available in many laboratories. In this study, we present an HPLC-UV method which is sensitive and accurate for the analysis of total morphine in urine at therapeutic and toxic levels.

MATERIAL AND METHODS

Apparatus and Chromatographic Conditions

Varian Liquid Chromatograph, Model 5000, with a variable wavelength UV Detector. Perkin-Elmer stainless-steel RP-18 column, 25cm×4.6mm, 10um spherical particle, equipped with a C18 Alltech guard column, Cat. No. 9251.

The separation was carried out isocratically with a 0.1% sodium acetate solution (pH6.9):methanol (30+70) at room

temperature. The flow rate was 0.7ml/min, the detector wavelength was set at 212nm and the injection volume was 20ul.

Materials

All reagents were of analytical grade. The methanol used in the preparation of standard solutions and in the mobile phase was Lichrosolv, Merck. Morphine was obtained from Alltech Applied Science and naloxone from Sigma Chemical Company. Sep-Pak C18 cartridges were obtained from Waters Assoc. and Extrelut columns from Merck Darmstadt (diatomaceous earth stationary phases pre-packed columns for liquid-liquid extraction).

Standard Solutions

Stock solutions of morphine and naloxone were prepared in methanol to obtain concentrations of 100µg/ml each. From these, working solutions of 1µg/ml and 5µg/ml of morphine and 2µg/ml and 5µg/ml of naloxone were made by dilution.

Samples for Analysis

Guinea-pigs were given, subcutaneously, a single dose (0.07mg) of heroine, were maintained in individual cages during 24h and urine was collected. Samples were stored at -20°C until used for the assay. Urine of a control animal was similarly collected. Known amounts of morphine were added to drug-free human urine for estimation of recovery of the drug.

Sample Purification

Urine, 1ml, was mixed with 100µl of conc. HCl and spiked with 50µl of a naloxone solution (100µg/ml), as internal standard, and heated at 100°C for one hour in a water bath. After cooling, the hydrolysed urine was made alkaline with 15M NaOH.

A) Sep-Pak cartridge purification

3ml 0.2M phosphate buffer (pH9.2) was added to the sample and

mixed by vortexing. Before use, the Sep-Pak C18 cartridge was washed with 5ml methanol, 10ml of water and 3ml of 2mM phosphate buffer (pH9.2). The sample was passed through the Sep-Pak C18 cartridge, then washed with 20ml of water. Morphine and naloxone were eluted with 2ml methanol as it was comproved by appliing the same procedure in urine fortified with known quantities of standards.

B) Extrelut column purification

1ml 0.2M phosphate buffer (pH9.2) was added to the sample and mixed by vortexing, poured into an Extrelut column and the drugs eluted with 15ml dichloromethane:isopropyl alcohol (85:15).

C) Liquid-liquid extraction with additional purification through the Extrelut column

3ml 0.2M phosphate buffer (pH9.2) was added to the alkaline-hydrolysed sample and extracted twice with 3ml ethylacetate/isopropyl alcohol (90:10) by vortexing. After centrifuging, the aqueous phase was discarded, the organic layers were combined and the drugs were re-extracted using 2×1ml HCl (1 5). The organic layer was discarded, the supernatant was alkalinised with 15M NaOH and 1ml 0.2M phosphate buffer (pH9.2) was added, mixed and poured into an Extrelut column. The drugs were eluted with 15ml dichloromethane:isopropyl alcohol (85:15).

All the eluates were evaporated to dryness, residues were dissolved in 1ml methanol and passed through a 0.22μm Millipore filter.

Chromatographic Analysis of Extracts

The extracts obtained by the different procedures described above were analysed by liquid chromatography at cited conditions. Standard solutions of 1μg/ml and 5μg/ml of morphine and 2μg/ml and 5μg/ml of naloxone were also analysed.

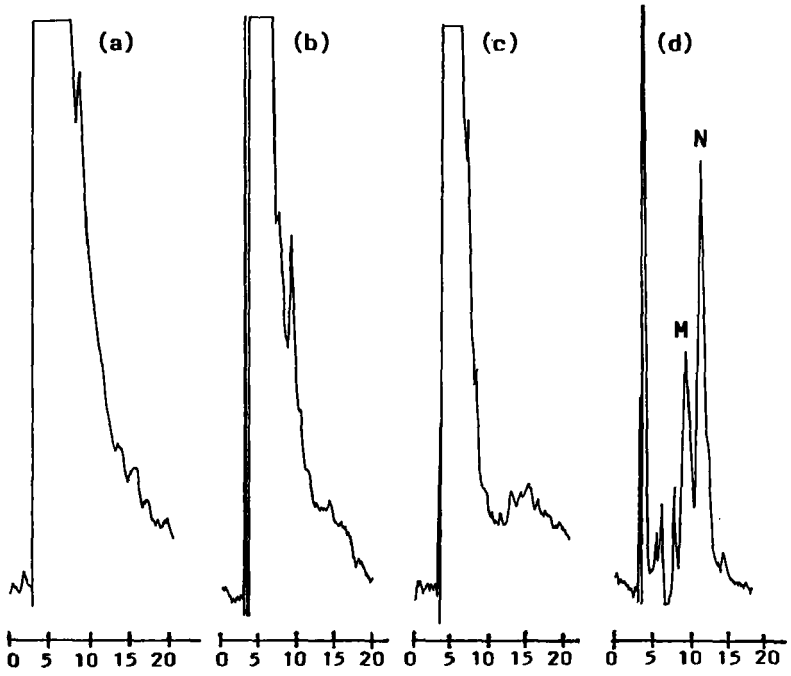

Figure 1. Chromatograms of extracts of blank human urine: Sep-Pak cartridge purification (a); Extrelut column purification (b); Liquid/liquid extraction with additional purification through Extrelut column (c). Chromatogram of a standard solution (d). Morphine (M); Naloxone (N).

Quantification of morphine was made by comparing the ratio of chromatographic peak heights of morphine and naloxone in biological extracts and in standard solutions.

RESULTS AND DISCUSSION

Figure 1 shows the chromatograms referring to the analysis of extracts of blank human urine obtained with the different purification procedures and that of the standard solution.

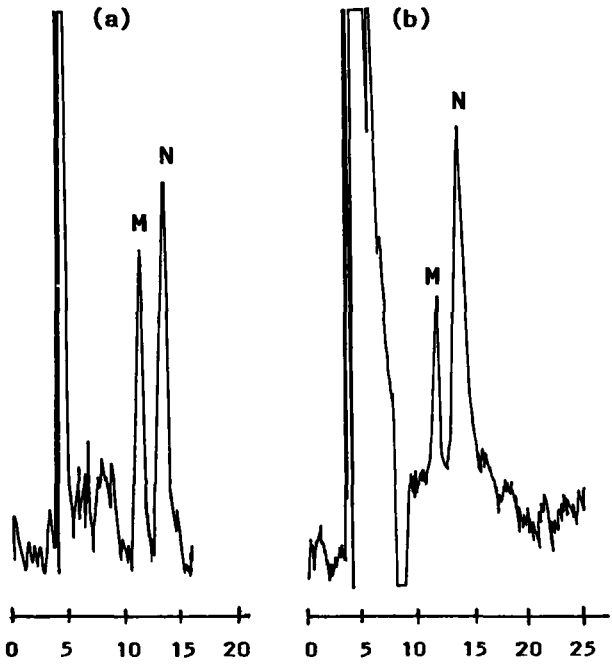

Figure 2. Chromatogram of urine extract of a heroine user (a) and of a standard solution (b).

Although procedures A and B are simple and fast, the obtained extracts have such a vast background that for high sensitivity detection, it interferes with the chromatographic peaks of the drugs. Procedure C is more complicated but the resulting extracts are free of endogenous impurities that could obscure the chromatographic peaks of the internal standard and morphine. The applicability of the method has been confirmed in our laboratory, by analysing urine samples of drug abusers. Figure 2 shows the chromatogram of urine extract of a heroine user.

Figure 3 shows the chromatograms of the analysis of the urine extract of guinea-pigs treated with heroine and of a standard solution of morphine and naloxone. This urine sample

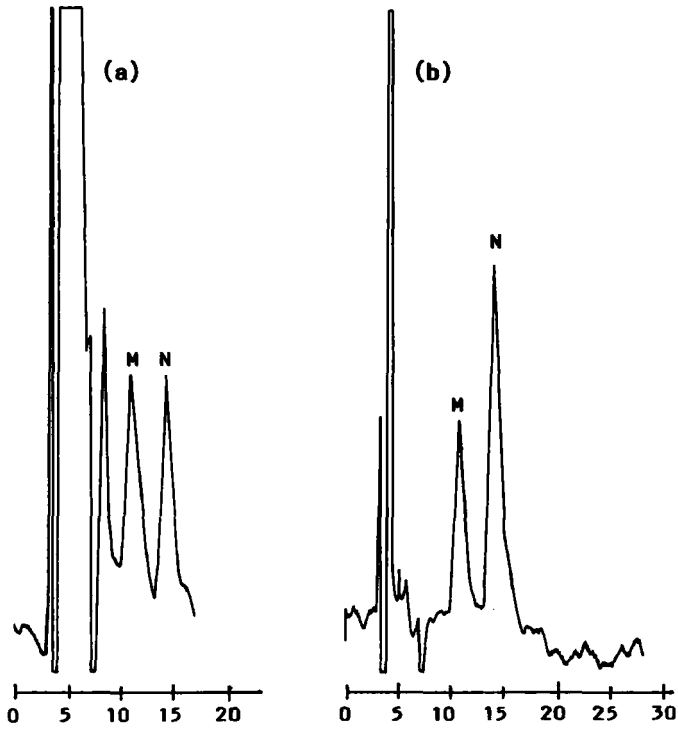

Figure 3. Chromatogram of urine extract of a guinea-pig treated with heroine (a) and of a standard solution (b).

was used to study the reproducibility of the adopted procedure. The coeficient of variation was 1.8% (n=4).

In order to establish the recovery of the procedure, human urine samples were added to known quantities of morphine (1µg/ml and 5µg/ml) along with the appropriate internal standard. After the samples had been processed as described above, the ratios of peak heights of morphine and naloxone obtained in chromatograms of urine extracts were compared to those of chromatograms of pure standards. Results are summarized in Table 1. Recoveries were of 88% and 79.8%,

TABLE 1

Recovery of Morphine from Spiked Human Urine

	drugs added	
	2µg/ml naloxone 1µg/ml morphine	5µg/ml naloxone 5µg/ml morphine
urine	recovery (%)	
1	92	80
2	87	78
3	95	89
4	83	70
5	83	82
	C.V.=6.1%	C.V.=8.6%

respectively with a coeficient of variation of 6.1% and 8.6%. Detection limit of the method was 0.2µg/ml.

Other studies have already been published concerning the analysis of morphine in biological samples by HPLC-UV (2,7). The authors refer purification technics that we experimented (conventional liquid/liquid extraction or packed column purification) but the extracts' background was important, obscuring the chromatographic peaks of the compounds.

The presented method adequately eliminates the impurities and is accurate and efficient for extracting and measuring morphine in urine.

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