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Journal of Chromatography A, 797 (1998) 245–250

JOURNAL OF CHROMATOGRAPHY A

Short communication

Determination of morphine in urine by solid-phase immunoextraction and high-performance liquid chromatography with electrochemical detection

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Abstract

The analysis of morphine in biological fluids is of vital interest in monitoring opiate abuse and in drug abuse research. Although methods for analysis of morphine and its metabolites are well established, studies are still being carried out to improve sample preparation procedures as well as detection levels of morphine in biological samples. In this study, morphine-specific immunosorbents were developed to concentrate morphine prior to HPLC analysis. Urine (0.1 ml) was diluted 10-fold with phosphate-buffered saline, pH 7.4 (PBS), loaded onto a solid-phase immunoextraction column and washed with 15 ml PBS followed by elution with 2 ml of elution buffer (40% ethanol in PBS, pH 4). The eluted fraction was analysed for morphine by HPLC–electrochemical detection using a cyanopropyl (CN) analytical column with 25% acetonitrile in phosphate buffer–sodium lauryl sulphate, pH 2.4 as the mobile phase. Duration of the extraction procedure was approximately 40 min. Calibration graphs were linear from 100 ng ml^{-1} to 500 ng ml^{-1} in urine. The inter-assay R.S.D. was $<10\%$ and the recovery of morphine from urine was $>98\%$. Immunocolumns demonstrated remarkably high specificity towards morphine showing minimal binding with other opiate metabolites such as codeine, normorphine, norcodeine, morphine-3-glucuronide, morphine-6-glucuronide.

Keywords: Morphine

1. Introduction

The determination of drugs and metabolites in biological fluids such as plasma and urine is still a very challenging task. The area of largest difficulty 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. We have recently been investigating the use of immunoextraction as a procedure to give highly specific capture of analyte during the sample preparation process, particularly for environmental analysis [1,2]. This work has been extended to investigate the feasibility of developing immunoextraction protocols to selectively extract drugs from biological samples, using morphine as a model compound.

The purposes of morphine analysis in biological samples are mainly to monitor therapeutic levels in patients, drug concentrations in human and animal pharmacokinetic studies, investigation of opiate

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0021-9673/98/$19.00
PII S0021-9673(97)01185-0

abuse for epidemiological purposes or drug abuse control as well as to identify causes of intoxication or death in cases of clinical, pathological or forensic interest [3].

Urine is the sample of choice for drug-abuse testing and the main metabolite of heroin used to identify opiate abuse is free morphine. Other metabolites such as 6-acetyl morphine are also used as markers to identify recent heroin use [4]. Morphine is extensively conjugated to glucuronide to form the active morphine-6-glucuronide as well as the inactive metabolite morphine-3-glucuronide [5,6]. There are other opiate metabolites present in the urine of heroin users but in much lower proportions. Recently the analysis of morphine-6-glucuronide has also been carried out [6]. Liquid–liquid extraction and solid-phase extraction are currently widely used as sample preparation procedures for the analysis of morphine in biological fluids.

Antibody-based solid-phase extraction is a relatively new technique for the selective concentration and clean-up of low-molecular-mass compounds in biological and environmental matrices. In our study we used immunosorbents, comprising morphine-specific antibodies immobilised onto silica. The immunoextraction procedure for morphine was previously developed and optimised [7,8] and in this study, we demonstrated its application to the analysis of morphine in urine. Because of its sensitivity HPLC with electrochemical detection was used throughout our study.

2. Experimental

2.1. Chemicals

Morphine sulphate and sodium lauryl sulphate were obtained from Sigma, UK. Ethanol, disodium hydrogenphosphate, sodium chloride, sodium dihydrogenphosphate, potassium dihydrogenphosphate and potassium chloride were from BDH-Merck, Poole, UK. All reagents were analytical-reagent grade or equivalent. Phosphate-buffered saline (PBS), pH 7.2–7.4 was prepared by dissolving sodium chloride (8 g), potassium dihydrogenphosphate (0.2 g), potassium chloride (0.2 g) and disodium hydrogenphosphate (2.9 g) in water (1 l).

2.2. Chromatographic conditions

The pump was a Beckman 110B (Beckman Instruments, High Wycombe, UK) operated at 1 ml min^{-1}. Electrochemical detection of morphine was performed with a Coulochem ESA model 5100A (ESA, Bedford, USA) set at a potential of +0.45 V. The HPLC mobile phase was 13% acetonitrile in pH 2.5 phosphate buffer (0.065 *M*) containing 0.0015 *M* sodium lauryl sulphate. Injection volumes up to 100 μl were delivered using a WISP 710A (Waters Associates, Northwich, UK). The column was a 5-μm Hypersil CPS, 25 cm×5 mm I.D. (Jones Chromatography, Hengoed, UK).

2.3. Antisera

Polyclonal antibodies were raised in sheep in response to an N-succinyl-normorphine-bovine serum albumin conjugate [9]. This was obtained from the School of Biological Science, University of Surrey. The antiserum dilution (that gave rise to 50% binding of [H^3]morphine label) was 1:1000 in assay buffer [9].

2.4. Preparation of morphine immunocolumns

A polypropylene disposable separation column, 11×1 cm with support frits was packed with 0.83 g of aldehyde-activated silica, (Clifmar Associates, Guildford, UK). The column was washed with 50 ml PBS to remove remaining traces of gluteraldehyde on the solid-phase. Next 5 ml of PBS was dispensed into the column followed by the addition of 500 μl of unpurified antisera. The column was then closed at both ends and placed on a rotamixer for 2 h at room temperature. The column was washed further with 10 ml of PBS. A 5-ml aliquot of 1 *M* glycine buffer, pH 6, was carefully added and the column rotated overnight. The following day the sorbents was washed with 10 ml of 0.3% hydrochloric acid followed by 20 ml PBS.

2.5. Extraction protocol

A working standard of morphine was made by a 1000-fold dilution of stock standard (1 mg ml^{-1}); 0.01 ml of stock standard was added and diluted to 10 ml with fresh blank urine for a 1 $\mu g\,ml^{-1}$ concentration. The working standard was further diluted to make concentrations of 100, 200, 300, 400 and 500 ng ml^{-1} in urine for the standard curve and 50, 250 and 450 ng ml^{-1} in urine for spiked quality-control samples.

0.1 ml of each sample was placed into tubes and diluted with 0.9 ml of PBS. A 1 ml volume of each diluted sample was loaded onto immunocolumns and washed with 15–20 ml of PBS. Morphine was eluted using 2×1 ml of 40% ethanol in PBS, pH 4. The second eluted fraction was collected and analysed using HPLC with electrochemical detection.

To determine the effects of other opiates in urine on recovery of morphine, the same extraction protocol was followed. Urine was spiked with morphine and one other opiate to determine whether the presence of other opiates in the urine will affect morphine recovery. In this study normorphine, norcodeine, codeine, morphine-6-glucuronide and morphine-3-glucuronide were used in various molar ratios to morphine.

3. Results and discussion

The use of HPLC with electrochemical detection for the analysis of morphine and its metabolites is well established [3,6,10–12]. In a review of different detectors (UV, fluorescence, chemiluminescence and electrochemical) electrochemical detection was recommended for the determination of morphine in biological fluids [3]. The method is useful for detecting low levels of morphine in biological fluids. In our study, HPLC-electrochemical detection was used to monitor recoveries of morphine from the immunoextraction columns. The chromatograms for the analysis of morphine are shown in Fig. 1.

Our previous work had shown that quantitative recovery from the immunocolumn could be obtained in a 1×1-ml fraction by lowering the elution solvent pH and incorporating at least 40% ethanol into the elution solvent [7,8]. Table 1 shows the effect of urine pH on recovery indicating that urine needs to be pH 5–8.5 for quantitative recovery. Although in this work only 0.1 ml of urine was used, samples diluted 10, 50 and 100 times were extracted through the column. In all cases it was still possible to obtain quantitative recovery of morphine (Table 2) which emphasises the ability of these columns to concentrate samples from a large volume should that prove necessary for other analytes.

The extraction time was approximately 30 min. The urine volume used was 0.1 ml diluted to 1 ml using PBS. Because the capacity of the immunocolumn was known to be less that 100 ng morphine, the amount of morphine standard on column was set between 10 and 50 ng. The HPLC traces showed no interference at the position for morphine in the urine blank. Reproducibility of extraction using immunoaffinity columns is given in Table 3. The extraction efficiency was >98% at levels 50, 250 and 450 ng ml^{-1} morphine in urine. Between-day relative standard deviations (R.S.D.s) were <10%. Within-day R.S.D.s (where columns were reused) were not as good, suggesting that the antibody columns took some time to recover following regeneration with PBS, thereby giving better agreement between-day rather than within-day.

Table 4 demonstrated that the immobilised antibodies are highly specific to morphine. The presence of normorphine, norcodeine, codeine, morphine-3-glucuronide and morphine-6-glucuronide showed minimal antibody binding and showed no significant effect on the recovery of 25 ng morphine at various molar ratios of the opiates. This also indicated that high molar ratio of the opiates to morphine did not significantly affect the retention of morphine in the immunocolumns. Table 5 shows the extraction of morphine from the morphine antibody column compared with extraction from a column containing a nonimmune serum and a column containing antibodies to clenbuterol. The results clearly show that quantitative recovery in such a small (1 ml) fraction of elution buffer is only possible on the column containing the morphine antibodies, emphasising that nonspecific binding plays a very minimal role compared with the binding due to antibody–antigen interactions.

In our study, the analysis of morphine in urine using immunoaffinity sample preparation was suc-

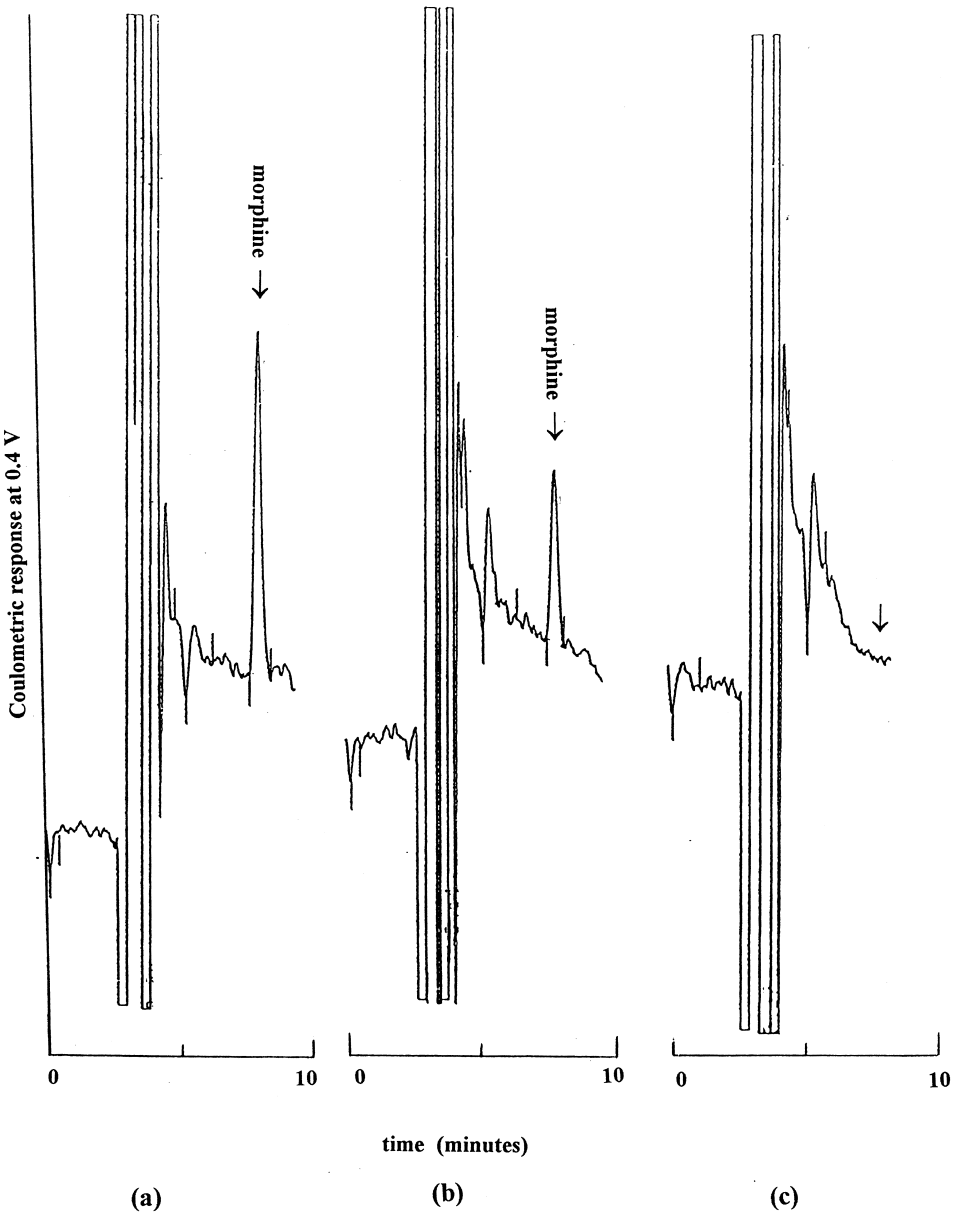

Fig. 1. HPLC chromatograms of morphine (a) morphine standard in PBS, 100 ng ml^{-1} (b) morphine, 450 ng ml^{-1} (extracted from 0.1 ml urine) (c) urine blank. HPLC conditions as Section 2.2.

cessfully carried out. The method was fast and simple, using a single step procedure comparable to traditional solid-phase extraction. The immunoaffinity sorbents showed retention capacity of up to 45 ng of morphine. Our results indicated the possibility of analysing free morphine in urine with no interference from glucuronide conjugates. However the analysis of morphine would be improved with the use of a suitable internal standard. The specificity of the immunocolumn makes this difficult for the extraction step, but an internal standard added after the immunoextraction might help improve precision fur-

Table 1
The effect of urine pH on morphine recovery (0.1 ml of 250 ng ml^{-1})

	pH					
	8.5	7	6	5	4	2.5
% Recovery of morphine in E2 fraction (1st analysis)	100	100	100	100	67	50
% Recovery of morphine in E2 fraction (2nd analysis)	100	100	100	100	67	50

Table 2
Recovery of morphine from diluted urine (0.1 ml of 400 ng ml^{-1} added)

100 μl of urine diluted	Volume of sample loaded (ml)	Amount of morphine recovered (ng)	
		Column 1	Column 2
10 times	1	40	39
50 times	5	37	39
100 times	10	35	39

Table 3
Immunoextraction and analysis of morphine (0.1 ml of urine) between-day variation (n=6)

Day	50 ng ml^{-1}	250 ng ml^{-1}	450 ng ml^{-1}
1	5.4	21.6	43.6
2	5.8	24.5	43.0
3	5.1	20.5	43.6
4	5.5	20.1	42.0
5	5.5	24.1	42.2
6	5.8	22.8	41.9
Mean	5.51	22.26	42.72
S.D.	0.26	1.838	0.786
R.S.D. (%)	4.7	8.3	1.8

ther. The use of this solid-phase immunoextraction procedure, however, as with all antibody based processes does have its drawbacks. Harsh conditions in the elution buffer and repeated elution processes could physically change the structural configuration of the immobilised antibody. This could be seen in the within-day analysis where the relative standard deviation was high compared to the between-day analysis.

In conclusion, we have developed a single step, solid-phase immunoextraction for morphine in urine which offers a highly specific sample preparation

Table 4
Recovery of morphine from urine spiked with morphine alone, normorphine, norcodeine, codeine, morphine-3-glucuronide and morphine-6-glucuronide

Molar ratio of morphine to:	% Recovery of morphine (250 ng m^{-1}) from urine (n=2 for each molar ratio)				
	Normorphine	Norcodeine	Codeine	Morphine-3-glucouronide	Morphine-6-glucouronide
1:0	100	100	100	100	100
1:1	82	100	96	120	85
1:4	94	90	96	120	110
1:8	82	100	111	100	110
1:16	100	100	100	115	110

Table 5
Recovery of morphine from antibody columns (1 ml of 25 ng ml^{-1} in PBS)

	BT	W1	W2	W3	W4	W5	E1	E2	E3
Nonimmune antibody	0	17	51	34	0	0	0	0	0
Anti-Clenbuterol	0	0	66	29	5	0	0	0	0
Anti-morphine	0	0	0	0	0	0	0	100	0

approach for biological fluids using tailor-made columns. The method was rapid, simple and reliable resulting in a cleaner extract for the HPLC. With a higher capacity immunocolumn, the extraction efficiency and method precision may be improved. The use of morphine antibodies with different cross reactivities with respect to opiate metabolites [9] may also be useful for the extraction of morphine and its metabolites from urine.

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