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Short Communication

Simultaneous determination of 6-monoacetylmorphine, morphine and codeine in urine using high-performance liquid chromatography with combined ultraviolet and electrochemical detection

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ABSTRACT

A method is described for the simultaneous determination of 6-monoacetylmorphine (6-MAM), morphine and codeine in post-mortem urine specimens using reversed-phase high-performance liquid chromatography with dual ultraviolet spectrophotometric and electrochemical detection. The limits of detection for a 1-ml urine sample were 0.04 mg/l for 6-MAM and 0.05 mg/l for both morphine and codeine. The presence of 6-MAM in urine indicates prior use of heroin and enables differentiation between morphine- and heroin-related deaths.

INTRODUCTION

The detection of 6-monoacetylmorphine (6-MAM) in urine has become of great importance in forensic and clinical drug testing where the use of heroin (diamorphine) is suspected [1]. Heroin is rapidly converted to 6-MAM in blood as a result of both spontaneous hydrolysis [2] and enzymic activity [3]. 6-MAM is then more slowly metabolised to the main active metabolite, morphine, which is then conjugated by glucuronic acid to morphine-3-glucuronide (M3G) and to a lesser extent morphine-6-glucuronide (M6G). Up to 80% of the heroin dose is excreted in urine within 24 h mainly as M3G, 5–7% as morphine and 1% as 6-MAM [4]. Consequently the presence of 6-MAM in urine helps distinguish between heroin or morphine administration [5].

High-performance liquid chromatographic (HPLC) methods have been reported for the determination of 6-MAM. Two methods which have been reported have relied on fluorescence detection [6,7]. No HPLC methods have been published which allow the simultaneous detection of 6-MAM, morphine and codeine. We describe the simultaneous detection of these three opiates in post-mortem urine using a liquid ex-

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traction technique and reversed-phase HPLC with dual ultraviolet spectrophotometric (UV) and electrochemical detection (ED).

EXPERIMENTAL

Materials

Acetonitrile (Mallinckrodt, Melbourne, Australia), isopropanol (Millipore, Melbourne, Australia) and chloroform (AnalaR, BDH Chemicals, Melbourne, Australia) were all of HPLC grade. All other chemicals were of analytical reagent grade (Ajax, Melbourne, Australia). Pure standards of 6-MAM, morphine alkaloid, codeine base and nalorphine hydrobromide were all obtained from the Australian Government Analytical Laboratories (Sydney, Australia).

Reagents and standards

Stock solutions of drugs (1 mg/ml) were prepared in methanol. These were prepared fresh for each assay. Working standards were prepared in deionized water from methanolic stock solutions to give concentrations from 0.25 to 10 mg/l for morphine and codeine and from 0.05 to 2.50 mg/l for 6-MAM.

Borate buffer was prepared by mixing 0.05 *M* boric acid with 0.043 *M* sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$), and making up to 1 l with deionized water and adjusting the pH to 9.9.

UTAK urine controls (UTAK Labs., Canyon Country, CA, USA) which were included in each assay were purchased from Lab Services (Melbourne, Australia).

Chromatographic conditions

The chromatographic system consisted of an LC-6AD pump, an SIL-6B autoinjector, an SPD-6AV UV–VIS variable-wavelength spectrophotometric detector (Shimadzu Oceania, Melbourne, Australia), and an ESA Coulochem electrochemical detector (ESA, Bedford, MA, USA) equipped with a Model 5010 analytical cell and a Model 5020 guard cell. The UV absorbance detector was operated at a wavelength of 210 nm and a sensitivity of 0.02 a.u.f.s. The working parameters for the electrochemical detector were +0.20 V for detector cell 1, +0.55 V for detector cell 2 and +0.75 V for the guard cell. A Shimadzu Chromatopac C-R4A data recorder was used to process the chromatograms.

Chromatographic separation was achieved using a Nova-Pak phenyl column (5 μm particle size, 150 mm × 3.9 mm I.D.), protected with a Nova-Pak phenyl guard column (Waters Millipore, Melbourne, Australia). The mobile phase consisted of acetonitrile and 10 m*M* sodium dihydrogenphosphate, pH 6.6 (10:90). The column temperature was ambient and the run time was 40 min with a flow-rate of 1.2 ml/min under isocratic conditions. The volume injected onto the column was 50 μl.

Extraction procedure

All glassware used was silanized to prevent the adsorption of opioids onto the glassware. This was accomplished by immersing the glassware in a 5% solution of Surfasil (Pierce, Rockford, IL, USA) in toluene for 2 h and then rinsing the glassware twice in methanol. The glassware was dried before use.

For total morphine, codeine and 6-MAM analysis, 1 ml of urine standards and unknowns were added to 10-ml silanized glass extraction tubes. The internal standard nalorphine (10 μl of a 1:10 dilution of stock) was then added to each tube.

Urine standards (0.05–10.0 mg/l), urine controls and unknown urines were hydrolysed by adding 3000–3500 U of *Patella vulgata* glucuronidase (Sigma, St. Louis, MO, USA) to all tubes followed by 300 μl of 1.1 *M* sodium acetate buffer (pH 5). All samples were then incubated overnight at 55°C. Following hydrolysis, 0.5 ml of borate buffer was added to each tube to achieve a pH of 8.3–8.5.

To each tube 8 ml of extraction solvent (chloroform–isopropanol, 90:10) were added and gently rotated for 30 min. The tubes were then centrifuged at 2500 *g* for 10 min and the aqueous layer was discarded. The organic layer was washed twice with 3 ml of sodium dihydrogenphosphate buffer (NaH_2PO_4) pH 9.9, the tubes were gently rotated for 10 min after each wash

and centrifuged, and the aqueous layer was discarded. The organic layer was then transferred to a clean silanized glass tube containing 200 μl of 0.2% phosphoric acid and then gently rotated for 30 min. The organic layer was removed and a 50-μl aliquot of the remaining acid layer was injected directly onto the chromatographic system.

Calculations

Relative retention times (RRT) to internal standard were calculated for 6-MAM, morphine and codeine, as well as UV absorbance and electrochemical peak-height ratios.

Calibration curves (five-point) were constructed by dividing drug-peak height response of 6-MAM, morphine and codeine by internal standard peak height and relating these to the concentrations. Concentrations of drugs for post-mortem samples were calculated by comparing their peak-height ratios to those of the corresponding drug standards. Weighted linear regression was used to calculate standard curves [8].

RESULTS AND DISCUSSION

Previous HPLC methods have relied upon fluorescence detection for the analysis of 6-MAM in plasma or urine [6,7]. Our initial attempts using fluorescence detection for the determination of 6-MAM in post-mortem urine specimens showed that 6-MAM (unless derivatized) had relatively poor fluoresence, which ultimately limited sensitivity and detection. The use of ED enabled a nine times greater response in comparison to UV detection. Similarly, the ED response of morphine was nearly seven times more sensitive than UV detection. When using ED some compounds require greater oxidising–reducing potentials than others; such is the case for codeine. At the same voltage setting used to oxidise 6-MAM and morphine, codeine was found to be less sensitive. An increase in potential suitable for the detection of both analytes caused a large increase in background noise severely limiting detection of morphine and 6-MAM. The use of dual UV–ED allowed codeine to be detected in the same extracts as well as enabling other possible co-eluting substances to be observed in the chromatograms.

The extraction method described previously by us for the detection of morphine and codeine in blood and bile was adapted to urine studies [9]. Post-mortem urine samples were assayed in batches incorporating five standards for 6-MAM, morphine and codeine. Each assay also included controls to provide a measure of quality assurance. The external control used was a UTAK urine control containing 1.2 mg/l morphine and 2.0 mg/l codeine. Internal urine controls were prepared from drug-free urine at two levels, 0.05 and 0.5 mg/l, and were also run with each assay.

Coefficients of variation (C.V.) for intra-assay and inter-assay variability are shown in Table I. C.V. data were less than 20% for both day-to-day and within-run reproduciblity for the lowest standard (0.05 mg/l) and less then 5% for the highest standard (0.5 mg/l). Calibration curves (from 0.05 to 10.0 mg/l) were analysed by weighted linear regression analysis and found to be linear with $r^2 \geq 0.99$ for 6-MAM, morphine and codeine.

The limit of detection was determined by estimating the minimum concentration equivalent to

TABLE I

INTRA- AND INTER-ASSAY REPRODUCIBILITY

Drug	Concentration added (mg/l)	Concentration found (mean ± s.d.) (mg/l)	C.V. (%)
Intra-assay (n = 8)			
Morphine	0.050	0.041 ± 0.012	16
	0.50	0.46 ± 0.02	4.3
Codeine	0.050	0.049 ± 0.011	20
	0.50	0.52 ± 0.02	3.8
6-MAM	0.050	0.049 ± 0.008	16
	0.50	0.49 ± 0.02	4.3
Inter-assay (n = 8)			
Morphine	0.50	0.42 ± 0.04	8.5
Codeine	0.50	0.52 ± 0.08	16
6-MAM	0.50	0.43 ± 0.05	11

or greater than three times the background noise whilst still allowing detection on both detectors. The detection limit for a 1.0-ml urine specimen was 0.04 mg/l for 6-MAM and 0.05 mg/l for morphine and codeine. Although the confirmable detection limit for 6-MAM was 0.04 mg/l when using ED/UV response ratio, the actual limit using ED alone was 0.01 mg/l. The value of using ED/UV ratio in reducing the possibility of peak contamination, however, outweighs the small loss of sensitivity.

Recoveries of 6-MAM, morphine and codeine in urine samples were calculated by measuring peak heights of a known extracted amount of drug and comparing this to the peak height obtained from a standard containing the same concentration of 6-MAM, morphine and codeine. The average extraction recovery for 6-MAM was 85%, morphine 59%, codeine 75% and the internal standard, nalorphine, 80%.

The relative retention times were reproducible showing assay variability on average less than 0.5%. Table II shows relative retention times together with ED/UV response ratios of compounds which run under these chromatographic conditions. ED/UV ratio variability for 6-MAM, morphine and codeine throughout an assay was less than 1% and between-assay values varied from 5 to 8% (n = 15). No drugs were found to interfere with the assay for these three analytes including those substances listed in Table III.

TABLE II

RETENTION TIMES AND RELATIVE RETENTION TIMES AND ED/UV RATIOS FOR A NUMBER OF OPIATES

t_R = Retention time; RRT = relative retention time to internal standard, nalorphine.

Drug	t_R (min)	RRT	ED/UV ratio
Morphine	5.9	0.233	6.8
Dihydrocodone	14.8	0.568	0.34
Codeine	19.2	0.757	0.52
Oxycodone	23.9	0.912	0.67
Nalorphine (I.S)	25.2	1.000	9.0
6-MAM	30.5	1.190	8.8
Hydrocodone	42.2	1.676	1.09

TABLE III

COMPOUNDS DETECTED BY ROUTINE TOXICOLOGY IN OPIATE-POSITIVE CASES, BUT FOUND NOT TO INTERFERE CHROMATOGRAPHICALLY WITH THE ELUTION OF 6-MAM, MORPHINE AND CODEINE IN THIS ASSAY

7-Aminoclonazepam
7-Aminoflunitrazepam
Amitriptyline/nortriptyline
Diazepam/nordiazepam
Dothiepin
Doxepin
Ephedrine
Methadone
Methamphetamine/amphetamine
Oxazepam
Propoxyphene/norpropoxyphene
Quinidine
Quinine
Sulphamethoxazole
Thioridazine/sulphoridazine/mesoridazine
Trimethoprim

Fig. 1 shows typical chromatograms for an extracted blank urine, a urine standard and a post-mortem case specimen using combined UV–ED. These chromatograms show no interference in the area of the drug standards. The post-mortem urine extract from an intravenous drug user (Fig. 1C) shows both morphine and 6-MAM.

Over 100 cases analysed for opiates with the method described have been examined. 6-MAM was detected in urine only in those cases in which intravenous use of heroin was suspected. 6-MAM was not found in any cases in which either codeine or morphine were known to be used and where heroin use was not suspected. However, the absence of 6-MAM in urine should not necessarily indicate the absence of heroin use since 6-MAM is metabolically and chemically unstable and may not be found in urine some hours after heroin use [5].

In conclusion, a specific and sensitive HPLC assay using combined UV–ED is described, suit-

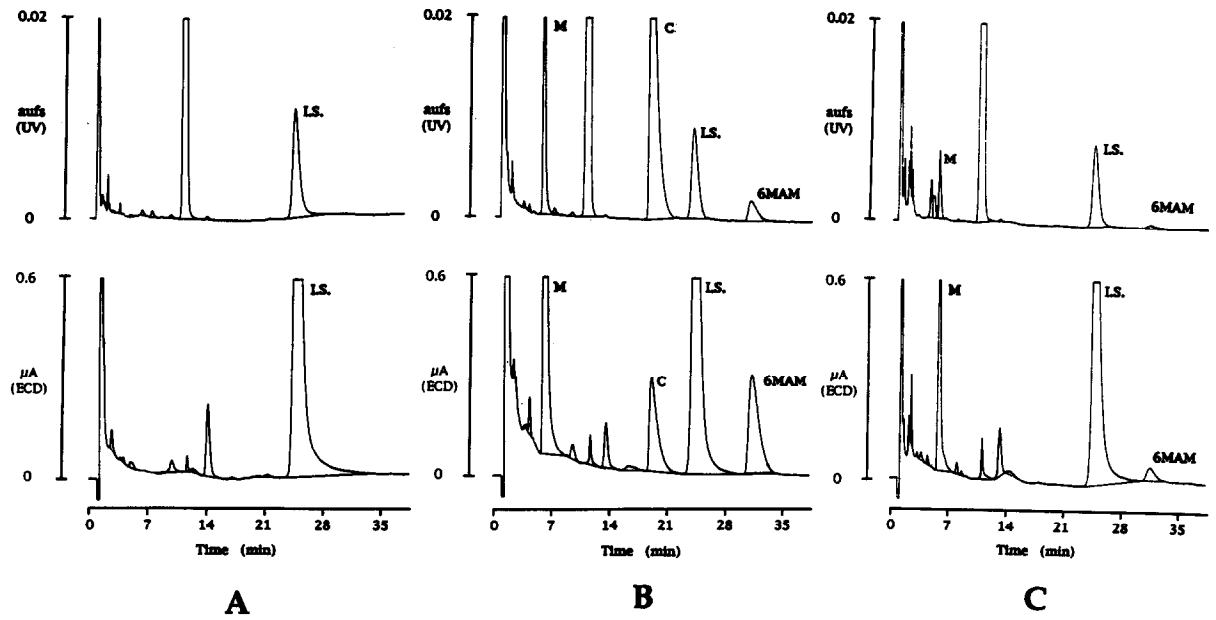

Fig. 1. Chromatograms showing UV (upper) and ED (lower) responses to (A) an extracted blank urine sample with internal standard (nalorphine), (B) an extracted urine standard spiked with morphine (M, 0.25 mg/l), codeine (C, 0.25 mg/l) and 6-MAM (0.25 mg/l), and (C) an extracted post-mortem urine from a case with a concentration of morphine of 0.22 mg/l and 6-MAM of 0.04 mg/l.

able for the simultaneous measurement of 6-MAM, morphine and codeine in urine specimens, applicable not only to forensic toxicology but also to clinical toxicology.

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