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CHARACTERISATION AND QUANTITATION OF MORPHINE IN URINE USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

I. JANE and J. F. TAYLOR
Metropolitan Police Forensic Science Laboratory, 109, Lambeth Road, London, S.E.1 (Great Britain)
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SUMMARY

A simple and rapid method for the identification and quantitation of morphine in urine samples is described. The procedure, which involves conversion of the drug to a fluorescent product followed by liquid chromatography, is shown to be highly sensitive and specific. Levels down to 0.01 μg/ml of morphine can be quantitatively detected in urine. A large number of drugs have been tested and shown not to interfere.

INTRODUCTION

The detection and quantitation of morphine in biological fluids is a problem commonly encountered in forensic science. Since the levels observed and the samples submitted are often small, high sensitivity and reliability are required. In addition, the legal implications require that the method be specific.

Many methods have been proposed for the determination of morphine in biological samples: these include thin-layer and gas–liquid chromatography, UV spectroscopy, fluorimetry[1] and radioimmunoassay[2]. In certain instances these methods may be considered lacking in either sensitivity, specificity, reproducibility or convenience of analysis. In terms of these parameters the method described herein represents an improvement of the analysis of morphine in urine. The method has been in routine use in this laboratory for several months.

EXPERIMENTAL

Apparatus

A Waters Ass. Model 6000M high-pressure reciprocating pump was used. The column used was of stainless steel, 25 cm × 4.6 mm I.D., slurry-packed with Partisil porous silica (H. Reeve Angel, Clifton, N.J., U.S.A.; 7-μm average particle size). The end fitting was a $\frac{1}{4}$: $\frac{1}{16}$ in. reducing union (Swagelok) fitted with a plug of porous PTFE (Phase Separations, Queensferry, Great Britain). The top of the packing was covered with a layer of glass-fibre filter paper, on top of which were placed three layers of 400-mesh stainless steel. Injections were made on to the top of the mesh.

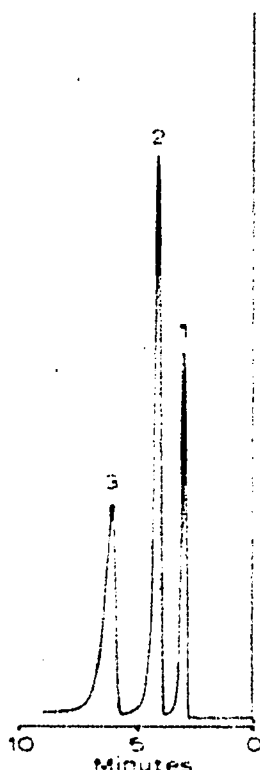

Fig. 1. Liquid chromatographic separation of morphine dimer (1), morphine–dihydromorphine "mixed" dimer (2), and dihydromorphine dimer (3) under the conditions described in the text.

The three products are separated by liquid chromatography (Fig. 1). Morphine is present in the urine predominately as the glucuronide: acid hydrolysis breaks the glucuronide bond and thus increases the chance of detecting morphine.

Procedure

Transfer 5 ml urine to a 15-ml test tube, add 10 μl of a solution of 2.5 μg/μl dihydromorphine (as hydrochloride) in water, and adjust to pH 9–10 with a few drops of 2 *N* NaOH solution. Buffer with 1.5 ml of 1 *M* K_2HPO_4 solution, saturate with NaCl (approximately 1 g), and extract twice with 3 ml of chloroform–isopropyl alcohol (9:1), shaking vigorously each time and centrifuging to separate the phases. Transfer the organic extract to a 10-ml centrifuge tube, add two antibumping granules, and evaporate down to low volume (approximately 20 μl) on a water-bath. Inject 2 μl of this extract on to the column to check for the presence of fluorescent co-extractives. After this injection has eluted withdraw a further 2 μl of the extract in a syringe followed by 2 μl $K_3Fe(CN)_6$ solution (0.04 *M*), and inject the mixture on to the column.

To determine the total morphine content, acidify 1 ml urine with 1 ml 10% hydrochloric acid, heat in a water-bath for 30 min, neutralize with 10% NaOH solution, and treat as for 5 ml unhydrolysed urine.

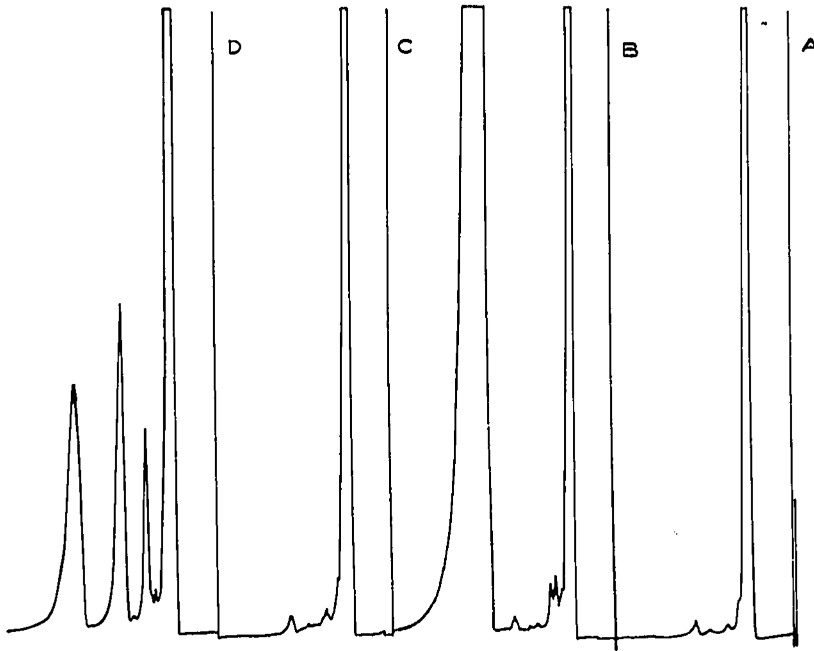

Fig. 2. Liquid chromatograms of blank urine and morphinised urine (2 μg/ml) under the conditions described in the text. (A) 2 μl blank urine extract; (B) 2 μl blank urine extract plus 2 μl ferricyanide reagent; (C) 2 μl morphinised urine extract; (D) 2 μl morphinised urine extract plus 2 μl ferricyanide reagent.

RESULTS

Identification

A typical chromatogram of the reaction products is shown in Fig. 1. Typical results from two urine samples are shown in Fig. 2. A large number of drugs have been examined *in vitro* (Table I), none of these gave a positive result for morphine by our method. The drugs which also undergo the oxidative coupling reaction can be distinguished from morphine by the relative retention times of the peaks produced by oxidation (Table II). These compounds would give a false positive result for some of the other fluorescent methods described in the literature[5,6]. No false positive results have been obtained from samples so far examined by our method.

Calibration

When a given amount of dihydromorphine standard is added to the urine sample, and the described procedure carried out, there is a linear relationship between the amount of morphine in solution and the peak height ratio of the morphine–dihydromorphine dimer to the dihydromorphine dimer. The method is calibrated with a series of standard morphine-in-urine samples and thus the amount of morphine in the unknown can be determined from the peak height ratio by reference to the calibration graph. Under the conditions used the method is able to quantitate the amount of morphine in the urine from 0.1–10 μg/ml. Levels higher than this require

TABLE I

DRUGS TESTED *in vitro* FOR POSSIBLE INTERFERENCES

Amitriptyline	Dihydrohydroxymorphine	6-Monoacetylmorphine	Phenazocine
Amphetamine	Diphenhydramine	Narcotine	Phenbutrazate
Amylobarbitone	Ephedrine	Nicotine	Phencyclidene
Atropine	Hydromorphone	Nitrazepam	Phendimetrazine
Benzocaine	Lignocaine	Normorphine	Phenmetrazine
Benzphetamine	Meprobamate	Nortriptyline	Phenylpropanolamine
Caffeine	Meratran	Oxymorphone	Procaine
Chlordiazepoxide	Methadone	Papaverine	Prolintane
Chlorpheniramine	Methapyrilene	Paracetamol	Quinidine
Cocaine	Methaqualone	Pemoline	Quinine
Cycloserine	Methylamphetamine	Pentazocine	Salicylamide
Dextromoramide	6-Methyldihydromorphine	Pethidine	Seconal
Dextropropoxyphene	Methylphenidate	Phenacetin	Strychnine
Diazepam			

TABLE II

RETENTION TIMES OF THE PEAKS PRODUCED BY OXIDATION, RELATIVE TO THAT OF THE DIHYDROMORPHINE DIMER (6.1 min)

Conditions are as described in text.

X	*X–X*	*X-dihydro*	*Dihydro-dihydro*
Morphine	0.47	0.66	1.00
Normorphine	1.88	1.37	1.00
Nalorphine	0.25	0.42	1.00
6-Monoacetylmorphine	0.31	0.50	1.00
6-Methyldihydromorphine	three peaks which are not resolved		
Dihydrohydroxymorphone	0.54	0.71	1.00
Oxymorphone	retained		1.00
Hydromorphone	retained		1.00
Pentazocine	0.38	0.57	1.00
Phenazocine	0.31	0.40	1.00
Paracetamol	0.32	0.45	1.00

dilution of the sample, lower levels can be detected but for quantitation less dihydromorphine must be added as internal standard.

Twenty replicate analyses of a standard sample of morphine in urine showed a relative standard deviation of 6%. The absolute detection limit under the conditions described is 4 ng injected on the column.

DISCUSSION

Various extraction schemes were investigated; the one chosen gave good reproducibility of analysis and approximately 60–70% recovery of low levels of morphine. Since an internal standard is incorporated, no attempt is made at 100% recoveries. However, it is very important that the pH of extraction is reproduced accurately because the partition coefficients of morphine and dihydromorphine vary in a different manner with pH, leading to variable results if the pH changes. The hydrolysis conditions were those normally used in this laboratory.

The optimum conversion to the fluorescent products is obtained by carrying out the reaction on top of the column with 0.04 *M* $K_3Fe(CN)_6$ solution as oxidising agent. This has the additional advantages of involving no reaction delay and only requiring conversion of part of the extract (the fluorescent products are unstable under the oxidising conditions used).

The chromatographic conditions were chosen to give acceptable resolution between the reaction products and the fluorescent co-extractives from the urine in the shortest possible analysis time. The column had been in use for several months and has not noticeably deteriorated. The use of the fluorimetric detector, with the excitation and emission wavelengths optimised for the dimers, increases both the sensitivity and selectivity of the method.

CONCLUSION

There is wide inter- and intra-subject variation in the rate of excretion of morphine[5]. This prohibits any back-calculation of dosage. However, for toxicological and forensic purposes it is an advantage to be able to quantitate the morphine present in the urine. Most important from the forensic viewpoint is the fact that the method is specific.

The range of calibration used was chosen as being most applicable to the samples received in this laboratory. Should the expected levels be different, the range can be altered by varying the amount of dihydromorphine added as internal standard. The absolute sensitivity of the method is limited by the detection limit of the fluorimeter (4 ng injected), the amount of sample available, and the care and precautions taken in the extraction. In the samples examined so far, the most common ratio between free morphine and the glucuronide is 1:8. Therefore, there is considerably more chance of detecting morphine if the urine sample is hydrolysed.

Although the method was developed to determine morphine in urine samples, it is also applicable to other body fluids or extracts. It would also seem to be potentially useful in following the excretion of morphine. The method has been found useful for the confirmation of small traces of morphine in injection syringes and ampoules received in this laboratory.

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REFERENCES

1 S. J. Mulé, *J. Chromatogr. Sci.*, 10 (1972) 275.
2 R. J. Coumbis and B. Kaul, *J. Forensic Sci.*, 19 (1974) 307.
3 B. B. Wheals, C. G. Vaughan and M. J. Whitehouse, *J. Chromatogr.*, 106 (1975) 109.
4 M. Sansur, A. Buccafuri and S. Morgenstern, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 880.
5 D. E. Fry, P. D. Wills and R. G. Twycross, *Clin. Chim. Acta*, 51 (1974) 183.
6 H. Kupferberg, A. Burkhalter and E. L. Way, *J. Pharmacol. Exp. Ther.*, 145 (1964) 247.