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IDENTIFICATION OF OPIATES IN URINE BY CAPILLARY COLUMN GAS CHROMATOGRAPHY OF TWO DIFFERENT DERIVATIVES

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SUMMARY

A capillary gas chromatographic method is described for the identification and confirmation of morphine, codeine, ethylmorphine and 6-monoacetylmorphine in urine. The method was useful for forensic purposes, as morphine and 6-monoacetylmorphine could be measured together with the legal drugs codeine and ethylmorphine. The legal non-prescription drug pholcodine could be detected together with the metabolites normorphine and norcodeine. After extraction and evaporation the opiates were derivatized with either N,O-bis(trimethylsilyl)trifluoroacetamide or pentafluoropropionic anhydride, and both types of derivative were chromatographed on a non-polar capillary column with a nitrogen–phosphorus selective detector. The use of two chemically different derivatives was found to be necessary for the unequivocal identification of all opiates of interest, which were not all separated as a single derivative. If a mixture of opiates was subjected to the two different derivatization agents, the derivatives were eluted in a different order. This improved considerably the selectivity of opiate analysis in urine. Particularly difficult samples would require mass spectrometric confirmation.

INTRODUCTION

Sensitive and selective determination of the different legal and illegal opiates is necessary to distinguish legal use of prescribed drugs from illegal use of banned drugs. The widespread use of immunological methods in screening for opiate abuse has introduced the need for sensitive, simple and specific methods for identification of drugs that give positive screening reactions for opiates. The legal drugs codeine and ethylmorphine are both transformed by oxidative O-dealkylation to morphine [1,2]. The illegal drug heroin is hydrolysed in vivo to 6-monoacetylmorphine (6-MAM) and further to morphine. Thus, no firm conclusions can be

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drawn from the mere detection of morphine in biological samples. It is necessary to establish whether morphine has been taken as such (by exclusion of precursor intake) or as an illegal or legal precursor, by detection of precursors or specific metabolites. A method for this purpose should be able to detect in a single run morphine, 6-MAM, ethylmorphine and codeine, together with other metabolites (e.g. norcodeine and normorphine) and identify other opiates that give positive opiate reactions by immunological methods [e.g. pholcodine and O^3-(2-morpholinoethyl)morphine, which is in clinical use as an antitussive]. The method reported in this paper meets these criteria. Because of the instability of 6-MAM, the method is most useful in unhydrolysed urine.

EXPERIMENTAL

Instruments

An HP 5890 gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was used. It was equipped with a split/splitless capillary injector, an HP cross-linked methylsilicone capillary column (12.5 m×0.2 mm I.D., film thickness 0.33 μm) and a nitrogen–phosphorus detector operated in the nitrogen mode. Signals were received on an HP 3392 integrator. A VG 12-250 mass spectrometer (Manchester, U.K.) with a HP 5790 gas chromatograph was used for identification. Electron-impact ionization was used: 70 eV, 200°C ion source; full scan in the mass range 40–720 or operated in the selected-ion monitoring (SIM) mode.

Chromatographic conditions

These were as follows: detector temperature, 300°C; injection port temperature, 250°C; column temperature programme, starting temperature 120°C, 0.5 min isothermic, 30°C/min to 220°C, 5°C/min to 240°C, 40°C/min to 300°C (this programme may be varied slightly to achieve optimal separation); carrier gas, helium; flow-rate, 1.3 ml/min; hydrogen flow-rate, ca. 3.8 ml/min (adjusted to achieve optimal conditions for the collector in use); air flow-rate, 80 ml/min; make-up gas, helium at a flow-rate of 30 ml/min. The samples were injected splitless, and the splitter was reopened after 30 s to a split ratio of 1:40.

Chemicals

Morphine, codeine, nalorphine and ethylmorphine were purchased from Norsk Medisinaldepot (Oslo, Norway). Norcodeine and pholcodine were generous gifts from Weiders Farmasøytiske (Oslo, Norway). 6-Monoacetylnalorphine (6-MAN) and 6-MAM were synthesized locally. Normorphine and ampoules (1 ml) of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) were purchased from Supelco (Bellefonte, PA, U.S.A.).

Standards

Stock solutions were prepared in methanol; working solutions were prepared by diluting the stock solutions with water. In all series, standards were prepared

in an appropriate medium to final concentrations in the range 0.5–5.0 μmol/l. The internal standard (I.S.) was 6-MAN or nalorphine.

Solvents

Dichloromethane was of HPLC grade from Fisons (Loughborough, U.K.). Acetonitrile, *n*-butyl acetate and *n*-butanol were of glass-distilled grade from Rathburn (Walkerburn, U.K.). Methanol was p.a. grade from Merck (Darmstadt, F.R.G.).

Buffers

Buffer A: 1 *M* ammonia was titrated with 2 *M* hydrochloric acid to pH 9.0. Buffer B: 5 *M* ammonia was added to buffer A until a mixture with an equal volume of 0.05 *M* sulphuric acid gave a pH of 9.0 (pH meter) [3].

Extraction

To 1 ml of urine were added 1 ml of buffer A, 30 μl of 6-MAN (0.1 mmol/l) and 8 ml of dichloromethane–butanol (19:1). The mixture was shaken for 10 min in a reciprocating mechanical shaker. Phase separation was accomplished by keeping the mixture standing on the bench for 10 min. The organic phase was transferred to a new tube containing 1 ml of 0.05 *M* sulphuric acid. The mixture was shaken for 10 min. The acid phase was transferred to a new tube containing 1 ml of buffer B and 8 ml of dichloromethane–butanol. The mixture was shaken for 10 min. The organic phase was transferred to a conical tube and evaporated under nitrogen (60 °C, in an electrical heating block). The dry residue was derivatized immediately or was kept frozen in tightly capped containers until derivatization.

Derivatization

To the dry extracts were added 80 μl of BSTFA–acetonitrile (1:2) or 80 μl of pure PFPA or HFBA. The capped tubes were heated at 60 °C for 15 min. The reaction mixtures were evaporated to dryness under nitrogen (60 °C) and dissolved in 100 μl of butyl acetate. The volume injected into the gas chromatograph was 1–2 μl.

RESULTS

Preliminary experiments and experience from our routine analyses indicated that underivatized opiates showed poor chromatographic properties and besides gave impractically low sensitivity.

Derivatization

HFBA derivatization. This derivatization agent gave more than one peak for most of the opiates: one main peak and one smaller one. Increasing the temperature (80 °C) or the reaction time (30 min) did not change the formation of reaction products. This suggests that at least two different derivatives were formed,

TABLE I

CHROMATOGRAPHIC SEPARATION OF OPIATE DERIVATIVES

Order of retention and relative retention times (RRT) to 6-MAN are shown.

Compound	PFPA derivatives		TMS derivatives	
	Order of retention	RRT	Order of retention	RRT
Morphine	1	0.75	4	0.83
Codeine	2	0.79	2	0.77
Normorphine	3	0.82	1	0.75
Ethylmorphine	4	0.84	3	0.80
6-MAM	5	0.87	5	0.87
Norcodeine	6	0.89	2	0.77
Nalorphine	7	0.92	6	0.95
6-MAN	8	1.00	7	1.00
Pholcodine	9	1.5	8	1.56

and this is in accordance with the results of Paul et al. [4]. This rendered the agent useless for our purposes.

BSTFA and PFPA derivatization. Derivatization was quantitative judged by the chromatograms of pure opiates. The yield of derivatives was not increased by increasing the reaction time, temperature or amount of derivatization reagent. No underivatized opiates were detected. The time and temperature allowed appeared to be sufficient for quantitative derivatization. The peak heights of the derivatives on chromatography were proportional to the amounts of opiates added. Each opiate gave only one derivative on the gas chromatogram, and this was verified by gas chromatography–mass spectrometry (GC–MS) of the PFPA derivatives, again in agreement with the results of Paul et al. [4]. The order of retention and the relative retention times (relative to 6-MAN) of the different opiate derivatives after GC separation are shown in Table I. It should be noted that the trimethylsilyl (TMS) derivatives of codeine and norcodeine eluted in the same peak. Fig. 1 shows a chromatogram of a BSTFA derivatized opiate mixture.

Purity of the chromatograms

The TMS derivatives obtained by derivatization with BSTFA gave the cleanest chromatograms with fewer extra peaks than the PFPA derivatives. Fig. 2A and B show chromatograms from the same authentic sample after BSTFA and PFPA derivatization, respectively. The number of extra peaks in the chromatograms of PFPA derivatives was lower for standards and blanks prepared from urine samples provided by the staff of the Institute than in authentic samples. Fig. 3 shows chromatograms of blank urine derivatized with BSTFA (Fig. 3A) and PFPA (Fig. 3B).

Stability of the derivatives

The TMS derivatives obtained by derivatization with BSTFA were sensitive to moisture, and it was found to be important that the samples were completely

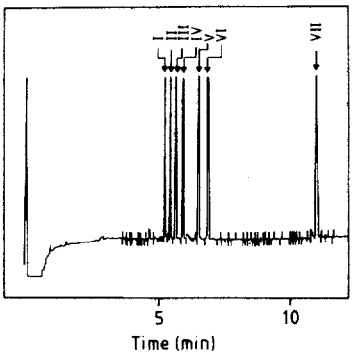

Fig. 1. Chromatogram of a BSTFA-derivatized opiate mixture (20 pmol injected of each opiate) containing codeine (I), ethylmorphine (II), morphine (III), 6-MAM (IV), nalorphine (V), 6-MAN (VI) and pholcodine (VII).

dry prior to addition of BSTFA. In the absence of moisture, the TMS and PFPA derivatives were equally stable at room temperature. The PFPA derivatives could be stored in the freezer overnight without appreciable decomposition. The sam-

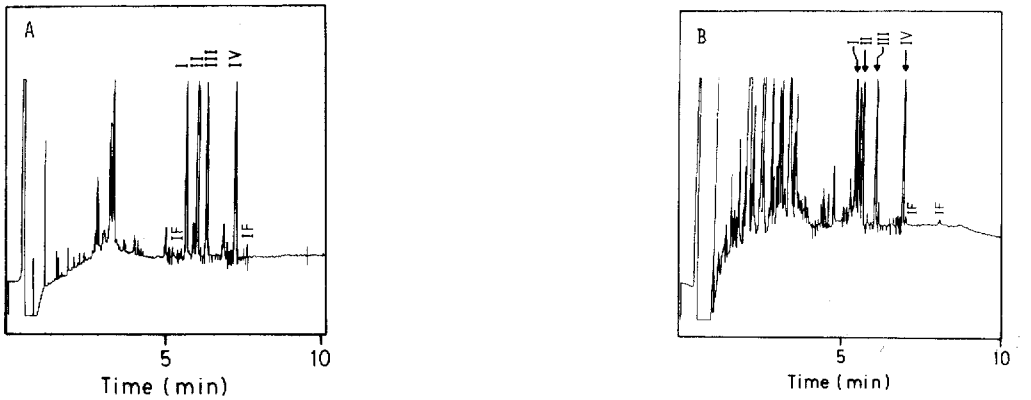

Fig. 2. (A) Chromatogram of a BSTFA-derivatized authentic urine sample containing codeine (I), morphine (II), 6-MAM (III) and 6-MAN (IV). (B) Chromatogram of a PFPA-derivatized authentic urine sample containing morphine (I), codeine (II), 6-MAM (III) and 6-MAN (IV).

Fig. 3. (A) Chromatogram of a BSTFA-derivatized urine blank. (B) Chromatogram of a PFPA-derivatized urine blank.

TABLE II

MOLECULAR IONS, MAJOR FRAGMENT IONS AND AREA ABUNDANCES OF OPIATE PFPA DERIVATIVES USED FOR MS VERIFICATION

Compound	Ions	Abundances (%)
Morphine	577 (M^+)	18.5
	414 (M^+-$OCOC_2F_5$)	100
Codeine	445 (M^+)	56.0
	282 (M^+-$OCOC_2F_5$)	100
Normorphine	355 (M^+-2 $OCOC_2F_5$-CO)	100
Ethylmorphine	459 (M^+)	71.8
	296 (M^+-$OCOC_2F_5$)	100
6-MAM	473 (M^+)	22.0
	414 (M^+-$OCOCH_3$)	75.0
Norcodeine	577 (M^+)	28.0
	355 (M^+-$OCOC_2F_5$-CH_3O-CO)	58.5

ples could be successfully re-derivatized if loss on storage was suspected. Pure 6-MAM gave an additional peak eluting as morphine with both derivatives, from 2 to 5% of the 6-MAM peak area. When the 6-MAM derivatives (both derivatives) were stored at room temperature for more than 3 h, there was a progressive decrease of the 6-MAM peak and a corresponding increase in the morphine peak, corresponding to hydrolysis of the acetyl ester.

Mass spectrometric verification of the derivatives

A full scan of the derivatives of the pure substances was attempted for both derivatives. PFPA derivatives were more suitable for MS and gave fragments that were suitable for the SIM mode; TMS derivatives fragmented too easily to be useful. Molecular ions and major fragment ions with area abundances that may be useful for MS verification are shown in Table II. The values and structures are mostly in accordance with previously published values [4]. The spectrum of the PFPA derivative of normorphine has been published previously [5]. The fragmentation of the PFPA derivative of norcodeine has not been published previously.

Stability

There was no appreciable loss of 6-MAM or morphine in spiked urine on storage at 20°C (room temperature) in polystyrene containers [6] for one week.

Specificity

No interfering peaks from the container material appeared when control urine was stored under the same conditions for the same duration. All the tested samples gave positive opiate reactions by enzyme multiplied immunoassay.

Extraction yield

The extraction yields of morphine, codeine, ethylmorphine, 6-MAM and 6-MAN (concentration 2 μmol/l) are shown in Table III.

TABLE III

EXTRACTION YIELD OF SOME OPIATES FROM URINE

Initial concentration 2 μmol/l in each case.

	Yield (%)	Relative S.D. (%)
6-MAN	58	4.6
Morphine	74	3.6
Codeine	86	2.1
6-MAM	83	4.7
Ethylmorphine	84	3.1

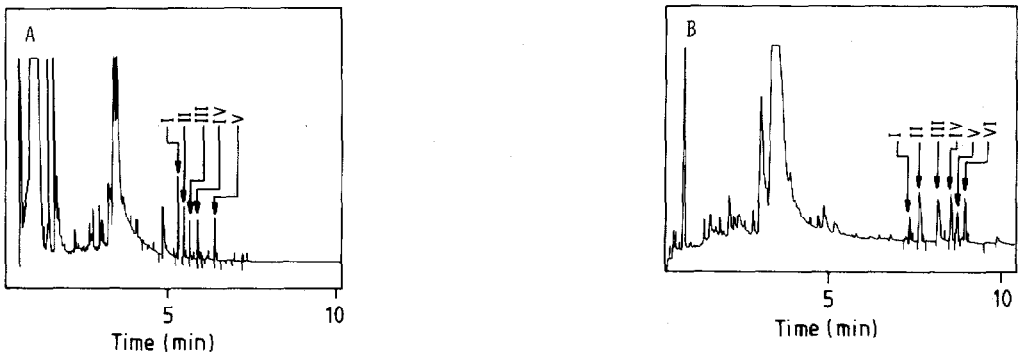

Fig. 4. (A) Chromatogram of a BSTFA-derivatized extract of drug-free urine spiked with 0.25 μmol/l of the following opiates: codeine and norcodeine (I), ethylmorphine (II), morphine (III), 6-MAM (IV), nalorphine (V). (B) Chromatogram of the same spiked urine as in (A) after PFPA derivatization: morphine (I), codeine (II), ethylmorphine (III), 6-MAM (IV), norcodeine (V), nalorphine (VI).

Limit of detection

The limit of detection was better than 0.25 μmol/l for both derivatives. Fig. 4A and B show chromatograms of BSTFA and PFPA derivatives (0.25 μmol/l), respectively of some opiates extracted from spiked urine.

GC–MS in the SIM mode of the PFPA derivatives was useful for special problem samples, for example samples with lower concentrations than 0.25 μmol/l or samples yielding chromatograms with unidentified peaks in the opiate area. These conditions appear to occur in some severe cases of drug addicts.

DISCUSSION

The procedures described here are sensitive and specific for a number of legal and illegal opiate drugs and their metabolites. Additional methods, such as thin-layer chromatography (TLC) or MS, are still recommended for identification of 6-MAM and morphine when illegal drug use is in question. The illegal substances are the primary goal of our opiate analyses, and for this reason the specificity should not be in question. So far, we have not experienced any false-positive results by the present method, compared with GC–MS or TLC.

The ratio of codeine/ethylmorphine to morphine is important to distinguish

between legal and illegal drugs taken simultaneously. Ideally, the presence of codeine in the same concentration range as morphine could indicate that codeine had been taken and that morphine was a metabolite. Codeine is present in opium and will be a contaminant of crude morphine, so the presence of codeine and morphine in the same urine sample cannot exclude intake of crude morphine. The presence of ethylmorphine will be highly suggestive of ethylmorphine intake. Most of the illegal intake of opiates is in the form of heroin, of which 6-MAM is a specific metabolite. The detection of other precursors of morphine, such as codeine and ethylmorphine, in 6-MAM positive samples only indicates that other opiates may have been taken together with the heroin.

We believe that the use of two different derivatives for opiate confirmation is an innovation. Most previous workers have used underivatized samples or only one derivative. By using two different derivatives, most of the opiates chromatographed in a different order. Only codeine and its metabolite norcodeine did not separate as TMS derivatives. Our results demonstrate that a wider than usual spectrum of opiates and metabolites may be detected in the same system. Previous work has concentrated mainly on measuring codeine and morphine together. Admittedly, these two are the most important besides 6-MAM, but it is also important to identify other opiates.

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