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APPLICATION OF THE EXTRACTIVE ALKYLATION TECHNIQUE TO THE PENTAFLUOROBENZYLATION OF MORPHINE (A HEROIN METABOLITE) AND SURROGATES, WITH SPECIAL REFERENCE TO THE QUANTITATIVE DETERMINATION OF PLASMA MORPHINE LEVELS USING MASS FRAGMENTOGRAPHY

W. J. COLE, J. PARKHOUSE and Y. Y. YOUSEF
University Department of Anaesthetics, University Hospital of South Manchester, Manchester M20 8LR (Great Britain)
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SUMMARY

The pentafluorobenzylation of morphine and related phenolic alkaloids by extractive alkylation is described. The alkylation is performed using tetrabutylammonium as counter ion and ethyl acetate as solvent. Optimum reaction conditions are presented together with the gas chromatographic properties of the derivatives formed.

The technique is applied to the quantitation of plasma morphine levels. Using morphine-d_3 as internal standard mass fragmentographic analysis of morphine as its pentafluorobenzyl- and pentafluorobenzyl, mono-trifluoroacetyl derivatives is demonstrated, and a case report is presented. Quantitation to a plasma morphine level of 5 ng/ml is readily attainable.

INTRODUCTION

Biological and forensic samples frequently contain low concentrations of drugs and related metabolites. Drugs of the morphine alkaloid type are highly polar. Their hydrophilic nature necessitates rigorous extraction conditions and often derivatization prior to analysis as exemplified by the submicrogram quantitation of morphine by gas chromatography (GC) using flame ionization detection[1], electron capture detection[2,3], and mass spectrometry (MS)[4]. Extractive alkylation affords a method of isolating polar compounds with simultaneous derivatization; alkylation of phenolic compounds using pentafluorobenzyl (PFB) bromide has been reported[5,6].

This investigation evaluates the use of PFB bromide as a reagent for the extractive alkylation of morphine and related phenolic alkaloids. The conditions for derivatization and their GC properties are presented together with the application of the technique to the quantitation of plasma morphine concentrations using mass fragmentography.

EXPERIMENTAL

Reagents and chemicals

PFB bromide and trifluoroacetic (TFA) anhydride were supplied by Pierce-Warriner (Chester, Great Britain). All solvents (AnalaR grade and redistilled prior to use) and tetrabutylammonium (TBA) hydroxide were obtained from BDH (Poole, Great Britain). Levallorphan and levorphanal tartrate were supplied by Roche Products (Welwyn Garden City, Great Britain), pentazocine by Winthrop Labs. (Newcastle-upon-Tyne, Great Britain), nalorphine hydrobromide by Burroughs Wellcome & Co., (London, Great Britain), and morphine base by MacFarlan Smith (Edinburgh, Great Britain). Morphine-d_3 was synthesized from morphine as previously described[7]. Monoacetylmorphine (MAM) was obtained as a gift from Dr. S. J. Mulé (Narcotic Addiction Control Commission, New York, N.Y., U.S.A.).

Glass equipment

All test-tubes, pipettes, flasks and reactivials (Pierce-Warriner) were washed with concentrated hydrochloric acid distilled water and dried. The glassware was subsequently silanized by treatment with a 4% (v/v) solution of dimethyldichlorosilane in toluene, washed with methanol and dried at 110°.

Gas chromatography

A Pye Unicam GVC gas chromatograph equipped with a pulse modulated electron capture detector (ECD) of the ^{63}Ni type and a flame ionization detector (FID) was used. The detectors were maintained at 300°. Borosilicate glass columns (213 × 0.4 cm I.D.) were packed with 2% OV-17 coated on 100–120 mesh Diatomite C (Pye Unicam, Cambridge, Great Britain) and conditioned for 24 h prior to use. The columns and support material were deactivated by silanization as previously described[8]. The nitrogen carrier gas was freed from contaminants by molecular sieve 13X and used at a flow-rate of 50 ml /min. The FID was operated with hydrogen and air flow-rates of 40 and 500 ml/min, respectively.

Preparation of derivatives

Pentafluorobenzyl derivatives. An aqueous solution (1 ml) containing 0.4 *M* TBA hydroxide, 0.2 *M* sodium hydroxide and 2 mg of alkaloid was added to ethyl acetate (1 ml) containing PFB bromide (20 μl). The reaction tube was stoppered and shaken at 22° until the amount of derivative formed was constant, and minimal or no underivatized alkaloid could be detected by GC–FID.

Pentafluorobenzyl,mono-trifluoroacetyl (PFB,TFA) derivatives. Ethyl acetate solutions of morphine, morphine-d_3, and nalorphine pentafluorobenzyl derivatives prepared similarly to those above were transferred to a reactivial (1 ml) heated at 75° and evaporated to dryness with a stream of nitrogen. Benzene–methanol (1:4, v/v) (50 μl) was added to the vial, and again taken to dryness to remove last traces of water. After addition of benzenes (50 μl) and TFA anhydride (25 μl) the vial was capped and heated for 15 min at 75°.

Plasma extraction

To plasma (1 ml) containing morphine as standards or unknown concentra-

tions were added morphine-d_3 (60 ng from a stock solution), 4 *M* sodium hydroxide (50 μl), TBA hydroxide (250 μl), ethyl acetate (1 μl) and PFB bromide (20 μl). The capped reaction tube was then shaken for 30 min at 22°. After centrifugation (3000 *g* for 5 min) the ethyl acetate layer was aspirated into a clean tube, to which was added 0.05 *M* sulphuric acid (1 ml). The mixture was shaken for 10 min and following centrifugation (3000 *g* for 5 min) the acid layer was removed, brought to pH 14 with 4 *M* sodium hydroxide, and the solution re-extracted with ethyl acetate (1 ml) for 15 min. The organic phase was washed with a little water and evaporated to dryness in a re-activial heated at 75° using a stream of nitrogen.

Mass spectrometry

An MS30 gas chromatograph–mass spectrometer (AEI, Manchester, Great Britain) was used with a coiled glass column (91.4 × 0.4 cm I.D.) packed with 2% OV-17 coated on 100–120 mesh Diatomite "C". The column was maintained at a temperature of 265° and perfused with helium at 40 ml/min. The silicone membrane separator was maintained at 200°. The mass spectrometer was operated with an ion-source temperature of 250°, a trap current of 300 μA, an ionizing voltage of 20 eV and an accelerating voltage of 4 kV. Spectra were recorded with an ultraviolet oscillograph (Bryans Southern Inst., Croyden, Great Britain) using a chart speed of 3 cm/min. Molecular ion and principal fragment ions of the alkaloid derivatives are listed in Table I.

TABLE I

MOLECULAR IONS AND PRINCIPAL FRAGMENT IONS OF THE ALKALOID DERIVATIVES STUDIED

The values in parentheses are relative abundancies.

Derivative	M^+	*M* – *PFB*	*M* – *OTFA*
Morphine PFB	465 (16)	284 (100)	—
Morphine-d_3 PFB	468 (14)	287 (100)	—
Nalorphine PFB	491 (13)	310 (100)	—
Pentazocine PFB	465 (23)	284 (100)	—
Levorphanol PFB	437 (61)	256 (100)	—
Levallorphan PFB	463 (87)	282 (100)	—
Morphine PFB,TFA	561 (24)	380 (100)	448 (30)
Morphine-d_3 PFB,TFA	564 (25)	383 (100)	451 (29)
Nalorphine PFB,TFA	587 (23)	406 (100)	474 (19)

Mass fragmentography

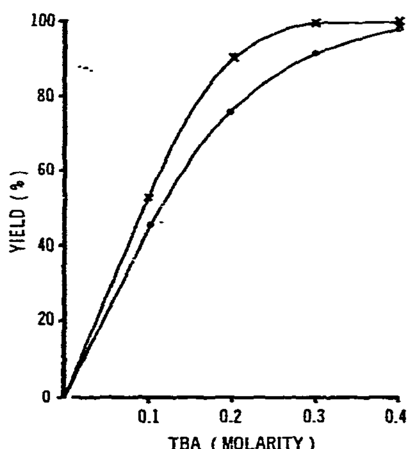
Mass fragmentography studies were performed using the gas chromatograph–mass spectrometer described above equipped with a six-channel multiple peak monitor with sample hold unit. Separations were made using a glass column (45 cm × 0.4 cm I.D.) packed with 2% of OV-17 perfused with helium at 40 ml/min. The column temperature was maintained at 265° for elution of PFB derivatives of morphine and morphine-d_3 with the peak monitor continuously recording the generation of ions at *m/e* 284 and *m/e* 287. The corresponding PFB,TFA derivatives were eluted with a column temperature of 245° and the ions at *m/e* 380 and *m/e* 383 being continuously monitored.

RESULTS AND DISCUSSION

Reaction conditions

Pentafluorobenzylation of the alkaloids was performed by an adaptation of the extractive alkylation technique used by Ehrsson[9] to prepare PFB derivatives of phenols and carboxylic acids. The method has been successfully employed to study chlorthalidone[10] and sulphonamides[11]. The PFB derivatives are normally prepared using methylene dichloride as the organic phase. However, when used with plasma as the aqueous phase, separation of the two components proved impossible owing to protein precipitation. Ethyl acetate was selected as an acceptable alternative, however, when after separation of the two phases by centrifugation, emulsions were either absent or of acceptable minimal proportions.

Quantitative PFB derivatization was dependent on the molarity of the TBA hydroxide used (Fig. 1). Morphine proved to be the most difficult alkaloid to extract from aqueous solution, 0.4 *M* TBA being required to effect a 98% yield as determined by GC–FID. The reaction was also time dependent (Fig. 2). Most of the alkaloids were fully derivatized within 20 min; morphine, however, required a reaction time of 30 min, in the presence of 0.4 *M* TBA, before the GC–FID peak heights of the PFB derivative became constant.



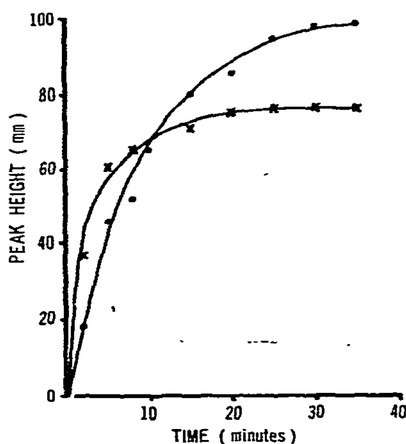


Fig. 1. Influence of TBA on the pentafluorobenzylation of morphine (●) and nalorphine (×). Temperature: 22°. The yields were determined by GC–FID.

Fig. 2. Influence of time on the PFB alkylation of morphine (●) and nalorphine (×). Aqueous phase: 0.4 *M* TBA in 0.2 *M* sodium hydroxide; organic phase: ethyl acetate containing PFB bromide (25 μl); temperature: 22°. Peaks heights determined by GC–FID.

The PFB derivatives of morphine and nalorphine were found to be stable in solution for at least 48 h at 4°. The stability of PFB compounds has previously been demonstrated[5].

All derivatives when examined by GC–MS demonstrated molecular and fragment ions consistent with the addition of one PFB group to the phenolic hydroxyl

group present. Only morphine and nalorphine possess an additional alcoholic function capable of incorporating a TFA group.

Gas chromatographic properties

The derivatives exhibited good peak symmetry. Retention data for the alkaloids studied are given in Table II. Incorporation into the molecule of one PFB group causes less than a three fold increase in retention time; the large increase in molecular weight being partly overcome by the degree of volatility imparted by the PFB group. Reaction of morphine and nalorphine to form the PFB,TFA derivatives further increased the volatility, such that their retention times were intermediate between that of the free bases and their corresponding PFB derivatives. Morphine-d_3 derivatives possessed the same retention data to the corresponding morphine compounds.

TABLE II

RETENTION TIMES RELATIVE TO CODEINE = 1 OF THE ALKALOIDS AND THEIR DERIVATIVES

Column: 2% OV-17 operated at 265° and 245° for PFB and PFB,TFA derivatives, respectively.

Compound	t_{rel}		
	Underivatized	*PFB*	*PFB,TFA*
Pentazocine	0.56	1.16	
Levorphanol	0.56	1.11	
Levallorphan	0.73	1.59	
Morphine	1.19	2.78	1.95
MAM	1.44	2.75	
Nalorphine	1.56	3.70	2.43

The ECD response for morphine and nalorphine PFB derivatives were measured; the minimum detectable quantities[12] were estimated to be $1.2 \cdot 10^{-17}$ and $2.3 \cdot 10^{-17}$ moles/sec, respectively. The high ECD response is in accord with that of other PFB compounds[11,13].

Mass fragmentography

Quantitative determination of plasma morphine levels was studied initially by monitoring the ions at m/e 284 and m/e 287 generated by the loss of the PFB group from morphine PFB and morphine-d_3 PFB, respectively. Pentazocine is the only narcotic likely to cause ion interference (m/e 284), it is, however, easily separated from morphine as indicated by the GC data.

Using a column temperature of 265° and a helium flow-rate of 40 ml/min, morphine eluted with a retention time of 2.25 min. The slight degree of tailing observed (similar to Fig. 3) in the ion intensity peaks recorded was probably due to the short length of column (45 cm) used and/or the relatively low temperature (200°) of the silicone membrane separator. Nevertheless a good linearity graph (analogous to Fig. 4) of the ratio of ions m/e 284 to m/e 287 *versus* morphine concentration was obtained, and proved applicable to the determination of unknown plasma morphine levels.

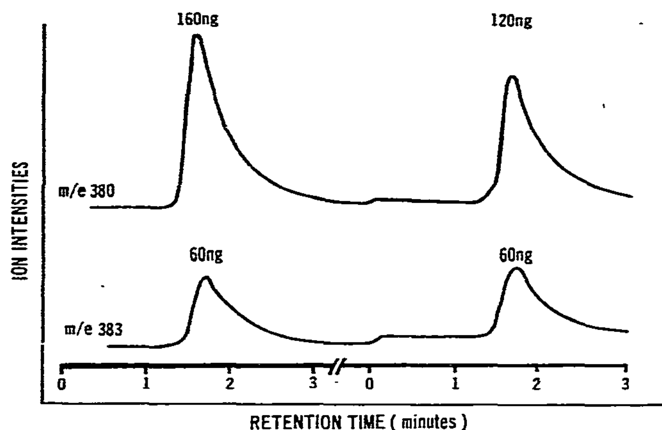


Fig. 3. Mass fragmentogram obtained by continuously monitoring the generation of ions at *m/e* 380 and *m/e* 383. The morphine standards (160 ng and 120 ng) were isolated by the extractive alkylation technique from plasma, containing morphine-d_3 (60 ng) as internal standard, and converted to their corresponding PFB,TFA derivatives. Column: 2% OV-17; column temperature: 245°; helium flow-rate: 40 ml/min.

The method has a number of advantages over that reported for plasma morphine quantitation employing mass fragmentography of the di-TFA derivatives[4]. In our hands, although the di-TFA derivatives gave excellent peak symmetry, long retention time impurities always interfered with subsequent analyses unless given sufficient time to clear the instrument. Also for low plasma morphine level detection the spectrometer multiplier had to be used with the highest setting possible that allowed a permissible signal to noise ratio. Using the PFB derivatives no impurities with long retention times were encountered; this allowed the continuous analysis of samples. Also a lower multiplier setting could be used, presumably owing to the presence of increased morphine levels resulting from its more efficient isolation using the extractive alkylation technique; it was estimated that the method gave a five fold in-

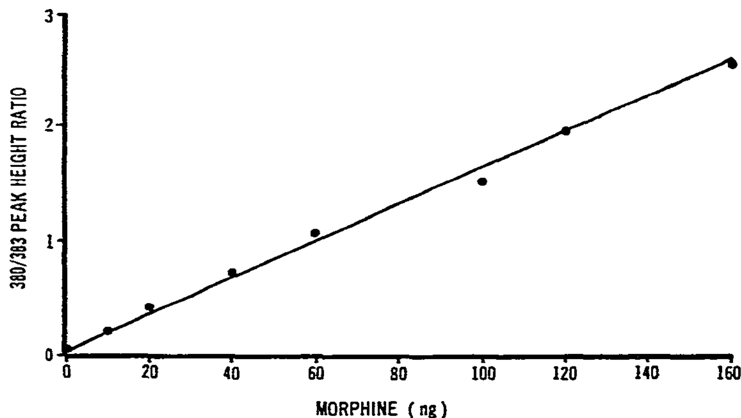


Fig. 4. Standard curve for plasma morphine concentrations analysed as the PFB,TFA derivatives by mass fragmentography (*M* — PFB ions). Internal standard: morphine-d_3 (60 ng); column: 2% OV-17; column temperature: 245°; helium flow-rate: 40 ml/min.

crease in sensitivity over that experienced with the di-TFA derivatives. The method should be generally applicable to the other phenolic alkaloids mentioned in the GC section with the exception of MAM, which undergoes deacetylation with subsequent conversion to morphine PFB.

The main disadvantage of the method was that the *m/e* 284 and *m/e* 287 ion intensity traces did contain ill-defined short retention time (1–2 min) impurities which produced an initial base line drift. Thus the corresponding PFB,TFA derivatives were examined by monitoring the ions at *m/e* 380 and *m/e* 383 generated by the loss of the PFB group from morphine PFB,TFA and morphine-d_3 PFB,TFA, respectively. Using a column temperature of 245° and a helium flow-rate of 40 ml/min morphine eluted with a retention time of 1.25 min. Although the peak shape still exhibited some tailing (Fig. 3), no short or long retention time impurities were discernable, thus allowing continuous sample injection. From a duplicate series of morphine standards extracted from plasma incorporating morphine-d_3 (60 ng) as internal standard, the calibration curve (Fig. 4) was constructed. The curve was used to determine the decay of plasma morphine levels (Fig. 5) resulting from the intramuscular administration of morphine to a 69-kg man. The curves are typical for the dose administered (0.15 mg/kg) and the morphine is readily detectable at the 5 ng/ml level. It is estimated that, if the area of the ion intensity traces were determined by computer linked integration, the overall sensitivity for the total method could be an order of magnitude greater, *i.e.*, quantitation at the picogram level, than those of similar GC–MS methods. Work relating analgesia to plasma morphine levels in patients is currently in preparation.

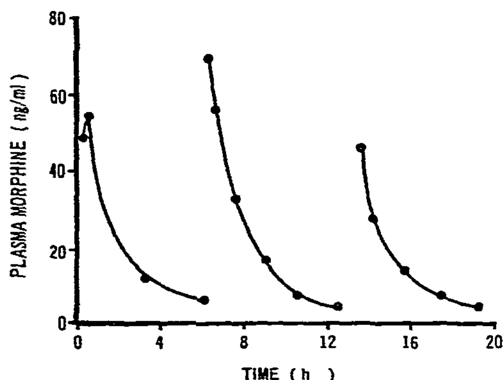


Fig. 5. Time–concentration curves illustrating the decay of plasma morphine concentrations. The morphine (0.15 mg/kg) was administered to a 69-kg man, once preoperatively and twice postoperatively by intramuscular injection.

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REFERENCES

1 G. R. Wilkinson and E. Leong Way, *Biochem. Pharmacol.*, 18 (1969) 1435.
2 J. E. Wallace, H. E. Hamilton, K. Blum and C. Petty, *Anal. Chem.*, 46 (1974) 2107.
3 B. Dahlaström and L. Paalzow, *J. Pharm. Pharmacol.*, 27 (1975) 172.
4 W. O. R. Ebbighausen, J. H. Mowat, H. Stearns and P. Vestergaard, *Biomed. Mass Spectrom.*, 1 (1974) 305.
5 F. K. Kawahara, *Anal. Chem.*, 40 (1968) 1009.
6 H. Brötell, H. Ehrsson and O. Gyllenhaal, *J. Chromatogr.*, 78 (1973) 293.
7 M. M. Abdel-Monem and P. S. Portoghese, *J. Med. Chem.*, 15 (1972) 208.
8 K. B. Eik-Nes and E. C. Horning, *Gas Phase Chromatography of Steroids*, Springer, New York, 1968, p. 11.
9 H. Ehrsson, *Acta Pharm. Suecica*, 8 (1971) 113.
10 M. Ervik and K. Gustavii, *Anal. Chem.*, 46 (1974) 39.
11 O. Gyllenhaal and H. Ehrsson, *J. Chromatogr.*, 107 (1975) 327.
12 T. Walle and H. Ehrsson, *Acta Pharm. Suecica*, 7 (1970) 389.
13 T. Walle, *J. Chromatogr.*, 114 (1975) 345.