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DETERMINATION OF OPIATES IN BIOLOGICAL SAMPLES BY GLASS CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic method for the simultaneous determination of morphine, 6-acetylmorphine and codeine in human plasma or blood has been developed. The samples are buffered to pH 9 and extracted on silica columns, cleaned by extraction and finally acylated with pentafluoropropionic anhydride. The derivatives formed are separated on a glass capillary column with falling glass needle injection and electron-capture detection. The choice of the extraction conditions and the preparation of suitable capillary columns are discussed.

INTRODUCTION

There is a great need for sensitive and specific assays of drugs in tissues. A large number of analyses are often necessary, so that long-term reproducibility and time of analysis are important. It is also of interest to be able to detect metabolites simultaneously. Codeine is metabolized to morphine, which is of interest in pharmacokinetic investigations. The determination of 6-acetylmorphine and morphine in *post mortem* blood specimens after acute heroin poisoning is another example.

Liquid chromatographic assays for morphine often make use of the reactivity of the phenol group. Oxidation is used in amperometric detection[1] and formation of dimeric products by pre-column derivatization and fluorescence detection[2].

Derivatization of morphine and related compounds is necessary in order to reduce the polarity and to increase the sensitivity (for electron-capture detection) prior to gas chromatography (GC). Acylation with fluorinated anhydrides and N-heptafluorobutyrylimidazole has been used in combination with electron-capture detection[3–7] or mass fragmentographic detection[8]. Silylation has been used prior to flame-ionization detection[9] and mass fragmentography[10]. Only mass fragmentography and electron-capture detection give detection limits low enough for pharmacokinetic investigations where only a few nanograms per millilitre are to be detected. We have been using a GC method with electron-capture detection for several years[6]. However,

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this method is laborious and we report here the results of our efforts to reduce the time of analysis and to improve the reproducibility at low concentrations with the aid of capillary chromatography.

EXPERIMENTAL

Morphine, codeine and nalorphine were obtained from the WHO Centre for Chemical Reference Substances (Solna, Sweden). Diacetylmorphine was prepared by heating 0.4 g of morphine hydrochloride with 0.2 g of sodium acetate and 2 ml of acetic anhydride for 20 min at 100°C, followed by addition of 30 ml of 1 *M* sodium carbonate and extraction three times with 10 ml of dichloromethane–cyclohexane (1:5). The solvent was distilled off in a rotary evaporator and the product was recrystallized from ethanol–water. 6-Acetylmorphine was synthesized according to Wright[11] and its purity was checked by thin-layer, liquid and gas–liquid chromatography. The product contained 0.5% of morphine but no other impurities were detected by these methods. Pentafluoropropionic anhydride (PFPA) was purchased from Massanalys (Stockholm, Sweden).

Dichloromethane, 1-butanol and ethyl acetate were of LiChrosolv quality (Merck, Darmstadt, G.F.R.). Extractions of samples were performed on columns packed with Extrelut silica (Merck). The silica was packed in a large column and purified with methanol and ethanol until the eluent was free from impurities, followed by drying in an oven at 100°C.

A 1.2-g amount of the pure silica was packed in a glass ion-exchange column (190 × 6 mm I.D.) with silanized glass-wool as outlet filter. Columns and test-tubes were silanized with 2% Drifilm (Pierce, Rockford, IL, U.S.A.) in cyclohexane, followed by three washes with methanol and drying at ambient temperature.

Preparation of buffers

Buffer A. Ammonia solution (1 *M*) was titrated with 2 *M* hydrochloric acid using a pH meter with a glass electrode until pH 9.0 was obtained.

Buffer B. Ammonia solution (5 *M*) was added to buffer A until it gave pH 9.0 when mixed with an equal volume of 0.05 *M* sulphuric acid. This calibration should be checked whenever a new dilution of sulphuric acid is prepared.

Preparation of capillary columns

Borosilicate columns (25 m × 0.36 mm I.D.) were filled with 20% hydrochloric acid and sealed under vacuum. The columns were heated at 160°C for 16 h, followed by washing with ten column volumes of distilled water and drying at 120°C with a flow of nitrogen.

Deposition of sodium chloride according to de Nijs *et al.*[12] was used for the preparation of columns with polar stationary phases. Deactivation of the glass surface was performed with N-cyclohexyl-3-azetidenol (CHAZ) according to Sandra and Verzele[13] or gas-phase deactivation with Carbowax 20M[14,15]. Polar stationary phases were applied by dynamic coating using the mercury plug method. Non-polar stationary phases were applied with static coating by distillation of the solvent under vacuum. The columns were conditioned by temperature programming, followed by further conditioning at 220°C overnight.

Instruments

A Hewlett-Packard gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The injection port was enlarged to take a Packard glass solid injector with a falling needle. Argon containing 5% of methane as make-up gas for the detector was connected with a tee-piece and glass-lined tubing.

The column (25 m × 0.36 mm I.D.) was packed with OV-1. The injection temperature was 250°C, the column temperature 220°C, and the detector temperature 300°C. The flow-rate of the carrier gas (helium) was 35 cm/sec and that of the make-up gas was 40 ml/min.

Extraction procedure

Plasma (1 ml) and 0.1 ml of internal standard solution containing nalorphine and 1 ml of buffer A (pH 9.0) are mixed and poured on to an extraction column. After 10 min, elution is carried out with a 5% solution of 1-butanol in dichloromethane until 8 ml have been collected.

The organic phase is extracted with 0.5 ml of 0.05 *M* sulphuric acid and the aqueous phase is separated by centrifugation and transferred into a new tube with a pasteur pipette. The aqueous phase is made alkaline with 0.5 ml of buffer B and extracted with the same solvent as above. The aqueous phase is aspirated off and the organic phase is poured into a 3-ml tube. The organic phase is evaporated with a stream of nitrogen at 50°C in a heating bath and 100 μl of PFPA are added. The tubes are sealed with glass stoppers and heated in a heating block at 65°C for 30 min, then the PFPA is evaporated with a stream of nitrogen at ambient temperature. A 0.25-ml volume of ethyl acetate is added and the contents of the tube are thoroughly mixed on a Whirlmixer. Then 1–3 μl of this mixture is injected to the gas chromatograph. The concentrations of morphine, codeine and 6-acetylmorphine are evaluated by using a calibration graph established by use of six standard samples.

RESULTS AND DISCUSSION

Extraction

Morphine is an ampholytic compound and the optimal pH for extraction has been determined to be 8.96 (ref. 16). We earlier used carbonate buffers for adjustment of pH prior to extraction, but have now changed to ammonium chloride buffers owing to their better pH stability on storage. The extraction method described above is not suitable for the quantitative determination of 6-acetylmorphine in samples containing diacetylmorphine owing to partial hydrolysis of the latter during extraction. For analysis of samples containing diacetylmorphine, addition of enzyme inhibitors and extraction at lower pH must be used[17]. 6-Acetylmorphine is more stable and is not hydrolysed to morphine in the extraction method described above. This was checked by extraction of spiked plasma samples containing 6-acetylmorphine alone. Less than 1% of 6-acetylmorphine was hydrolysed to morphine during extraction.

The hydrolysis of diacetylmorphine in blood or plasma is very rapid. Nakamura and Thornton[18] determined the half-life of diacetylmorphine in blood to be 9 min and concluded that *post mortem* blood specimens should be analysed for the presence of 6-acetylmorphine. Halogenated hydrocarbons mixed with alcohols have been

widely used or the extraction of morphine from biological samples[2,3,8,10,19]. High extraction recoveries are obtained with these polar solvents, but polar solvents also extract more of the sample matrix. Less polar solvents such as toluene–butanol[5,6] and benzene–butanol[20] have been used to achieve clean extracts. If the more polar solvents are used the pH adjustments becomes less critical, and adsorption to glass surfaces will be reduced.

Emulsions are formed when chlorinated hydrocarbons are shaken with buffered plasma samples, which makes phase separation difficult. Column extraction has proved to be useful for solving this problem. Different materials have been used to absorb the aqueous phase, such as cellulose powder[21] silica[22] and gauze sponges[23]. A good absorption matrix to hold the aqueous phase should be inert with respect to adsorption of the compounds of interest and it should not contain extractable impurities. Extrelut silica was found to be suitable after purification with methanol as described above. Extracts with a low background (Fig. 1) and a high recovery were obtained (Table I).

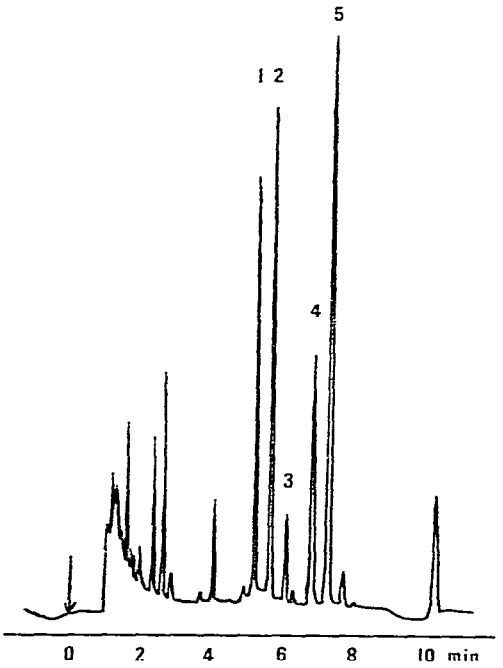

Fig. 1. Chromatogram obtained from a 1-ml serum standard containing 62 ng of morphine (1), 546 ng of codeine (2), 76 ng of normorphine (3), 189 ng of 6-acetylmorphine (4) and 100 ng of nalorphine (5). Injection: 1 μl from 250 μl by falling needle on to a 25 m × 0.36 mm I.D. OV-1 column (column 1, Table II). Carrier gas, helium (35 cm/sec; make-up gas, argon–5% methane (40 ml/min). Injection temperature, 250°C; column temperature, 220°C; detector temperature, 300°C. Hewlett-Packard electron-capture detector (^{63}Ni source).

Injection and chromatography

A solid injector with a falling glass needle was used due to its inertness and the possibility of injecting large fractions of the total sample. It is possible to evaporate

TABLE I

RECOVERY AND PRECISION

Compound	*Elution time (sec)*	*Amount added (ng)*	*Recovery ± S.D. (%)*	*Coefficient of variation* (%)*	*No. of determinations*	*Detection limit (ng/ml)*
Morphine	314	62	98 ± 6	4	7	1
		0.8		10	8	
Codeine	341	546	98 ± 4	3	7	5
		4		7	8	
Normorphine	368	76	24 ± 2	7	7	
6-Acetylmorphine	389	409	98 ± 2	2	7	2

* Relative peak height *versus* nalorphine.

the PFPA directly on the falling needle, but this is not recommended for routine work. The density of PFPA is high, which makes it difficult to inject without releasing a drop down to the column.

Our first attempts to use capillary columns for morphine and codeine analysis gave contradictory results. No peaks appeared after injection of nanogram amounts of the derivatives on some columns, while others appeared to be inert. Surface pretreatment and deactivation are usually not carried out with commercially available columns. Therefore, we decided to test some different methods described in the literature to find one suitable for opiate analysis. After some preliminary tests it became clear that some kind of standard chromatographic test for opiates was needed in order to be able to compare different columns and to control a column in use. A methanolic solution of morphine, codeine and pentazocine was evaporated and treated with PFPA. The PFPA was evaporated and the test sample was dissolved in ethyl acetate containing aldrin. The test sample was chromatographed and the peak area per mole relative to aldrin was calculated. The results are presented in Table II.

TABLE II

RESULTS FOR DETERMINATION OF OPIATES USING DIFFERENT COLUMNS

Columns 3 and 6 were supplied by Hewlett-Packard; the others were prepared as described under Experimental. Carbowax 20M deactivation, columns 1, 2 and 3; CHAZ deactivation columns 4 and 5.

Column	*No.*	*Column temperature (°C)*	*Relative peak area/mole versus aldrin*		
			Pentazocine	*Morphine*	*Codeine*
OV-1	1	220	0.53	1.34	0.25
OV-1	2	220	0.41	1.13	0.15
Methylsilicone fused silica	3	220	0.38	0.63	0.24
OV-17	4	220	0.31	0.74	0.09
OV-225	5	180	0.59	1.82	0.27
		200	0.47	1.56	0.19
		220	0.52	1.62	0.103
		240	0.48	0.58	0.013
Carbowax 20M fused silica	6	220	0.19	0.13	0.074

Table II indicates that degradation of morphine and codeine occurs on all columns to some extent, and the sensitivity to degradation decreases in the order codeine ≈ morphine ≫ pentazocine.

It is difficult to estimate how much of the derivatives have survived chromatography from chromatographic data alone, as no absolutely inert column is available, and the degradation might proceed without catalysis. It seems probable that the degradation follows a first-order reaction with catalysis (pyrolysis of esters on glass beads has been used for the synthesis of alkenes; Tschugaev elimination). De Nijs *et al.*[15] measured the first-order rate constant for the degradation of endrin in order to compare the activity of different columns. It should be possible to make similar measurements for opiate derivatives, but it is very time consuming. To be able to obtain a figure for the peak area ratio with small or negligible degradation and for kinetic investigations, a 10 m × 0.5 mm I.D. capillary coated with OV-225 was prepared. Test mixtures of aldrin, morphine and codeine derivatives were chromatographed at almost constant elution time at different temperatures using extreme flow-rates (Fig. 2). One experiment with variation of the flow-rate at constant temperature was also carried out (Fig. 3).

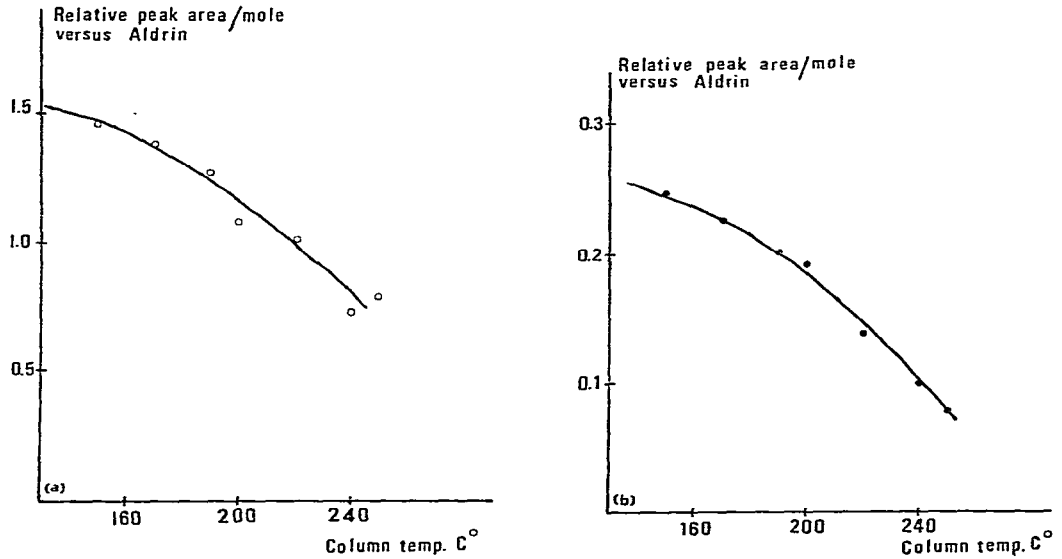

Fig. 2. Variation with column temperature of relative peak area for PFPA-acylated morphine (a) and codeine (b). The elution time was kept constant by variation of the flow-rates. Column: 10 m × 0.5 mm I.D. with sodium chloride deposition and CHAZ deactivation followed by coating with OV-225.

From Figs. 2 and 3 it is clear that degradation will always occur on columns used for analysis as excessive flow-rates cannot be used and a column temperature of at least 200°C is needed in order to achieve a reasonable time of analysis. However, the degradation is very reproducible so that analyses can be carried out in spite of degradation, but regular column control and calibration are advisable. If there is interest in analysing codeine alone, 3-O-ethylmorphine might be used rather than nalorphine as a more suitable internal standard.

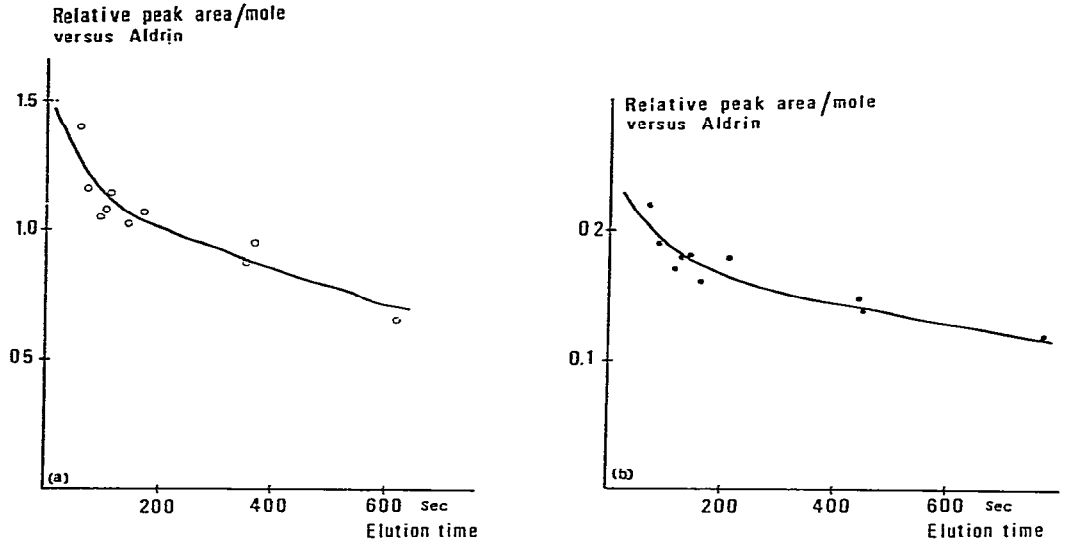

Fig. 3. Variation of relative peak area peak area for PFPA-acylated morphine (a) and codeine (b) at different elution times by variation of flow-rate. Column as in Fig. 2, operated at 200°C.

Both the CHAZ and Carbowax 20M deactivation methods gave useful columns. The advantage of using gas-phase deactivation with Carbowax 20M is that it can be used to deactivate old columns. Good results were most easily obtained with Carbowax 20M deactivation and coating with OV-1. One of these OV-1 columns has been used for the analysis of at least 500 biological samples so far without any sign of deterioration.

The method described is suitable for the simultaneous determination of morphine, codeine and 6-acetylmorphine in biological samples. Normorphine can also be determined, but the extraction recovery is only 24%. The method has been used for the investigation of the specificity of radioimmunoassay of morphine in plasma, the determination of morphine and codeine after codeine administration and the determination of 6-acetylmorphine and morphine in different tissues after acute heroin poisoning to determine the cause of death.

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