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Quantitative determination of morphine in biological samples by gas-liquid chromatography and electron-capture detection

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A highly sensitive method for the determination of morphine in plasma and brain samples down to 500 pg ml^{-1} in plasma and 100 pg in 30 mg of brain tissue is described. This sensitivity is in the range of that described for radioactive and radioimmunological techniques. The samples (50–500 μl) are extracted with toluene–butanol (9:1) at pH 8·9 and then re-extracted into 0·1N sulphuric acid and back extracted to a toluene–butanol phase at pH 8·9. The pentafluoropropionic anhydride derivatives of morphine and the internal standard nalorphine were quantitatively determined by gas–liquid chromatography with a ^3H electron capture detector.

Methods having sufficient sensitivity to determine morphine within the nanogram range are assays based on radioactive, spectrofluorometric and gas–liquid chromatographic techniques (for review see Way & Adler, 1960; Taylor, 1971; Rubin, 1973). Procedures utilizing radioactive labelled morphine are highly sensitive (Yeh & Woods, 1970) although relatively unspecific unless used together with chromatography or isotope dilution (Taylor, 1971). The fluorometric methods have high sensitivity but again lack specificity (Kupferberg, Burkhalter & Way, 1964; Takemori, 1968; Paalzow & Paalzow, 1971). Radioimmunoassay, will allow the measurement of picogram amounts of morphine but here too, the method is not specific for morphine, (Spector & Parker, 1970; Spector, 1971).

Because of the polarity of morphine it is necessary to make derivatives to obtain reliable quantitative determination by gas–liquid chromatography (g.l.c.). Morphine has mostly been converted to its trimethylsilyl derivative and quantified by flame ionization detection (FID) (Brochmann-Hanssen & Baerheim Svendsen, 1963; Schmerzler, Yu & others, 1966; Ikekawa, Takayama & others, 1969; Wilkinson & Way, 1969). A method utilizing g.l.c. mass-fragmentography with a practical lower limit of detection of approximately 500 pg has been reported by Ebbighausen, Mowat & others (1973).

The trifluoroacetic anhydride derivative of morphine was found by Yeh (1973), to be unstable, whereas the pentafluoropropionic derivative (PFP-morphine) shows stability and high electron capturing properties. We now describe a g.l.c. method with electron capture detection of this derivative applied to biological samples.

MATERIAL AND METHODS

Materials

Toluene and ethyl acetate (Merck) of analytical grade were further purified by ractional distillation. Butanol (Merck) was used as received. Pentafluoropropionic

anhydride (PFPA) (Pierce Chemical Co.) was used as purchased and stored under nitrogen at −30°. Morphine and nalorphine (Ph. Nord. 1964) were obtained from the WHO Centre for Chemical Reference Substances (Solna, Sweden). All other chemicals were of analytical grade. All glassware was cleaned in boiling concentrated nitric acid and repeatedly washed with all glass distilled water, then silanized by Silyl-8 (Pierce Chemical Co.). (Siliconization of the glass with Siliclad (Clay-Adams) and extraction with toluene–butanol solvents gave interfering peaks with the electron capture detector.)

Instruments

A *Varian Aerograph* (model 1400) equipped with a tritium foil electron capture detector was employed. The electrometer was connected to an electronic integrator (*Spectra–Physics, Autolab Div.*) and a *Varian* model 20 recorder. The separations were made on a silanized 5 feet × 20 mm glass column packed with 3% OV-17 on 100/120 Gas-Chrom Q. The following conditions were used:

Gas-flow-rate (N_2) 30 ml min^{-1}; injector temperature 245°; column 215°; detector 225°.

Procedure

Male Sprague-Dawley rats, 125–175 g had free access to water, but no food for 16 h before the test. They were then injected with 2·5 mg kg^{-1} morphine (i.v.) and venous blood samples were continuously taken from the plexus orbitalis. In some experiments the animals were decapitated and the brain excised. All tissue samples were stored at −30° until analysed.

(I). To 100 μl of plasma* was added 100 μl of an internal standard (nalorphine 400 ng ml^{-1}) and the subsequent treatment was as outlined under III.

(II). Each brain was homogenized in 3·0 ml 0·4N ice-cold perchloric acid containing 100 μl internal standard solution. The samples were then centrifuged at 28000 rev min^{-1} for 20 min and 200 μl* of the supernatant taken for further analyses according to III.

(III). The pH of the plasma or brain homogenate was adjusted to 8·9 with a 3M carbonate buffer (pH 8·9). Toluene–butanol (9:1) 3·0 ml was added to the plasma sample and 6·0 ml to the brain sample. They were then shaken for 30 min with a Büchler Evapomixer and centrifuged at 2500 rev min^{-1} for 5 min. The organic layer was transferred to a tube containing 500 μl 0·1M sulphuric acid, shaken for 15 min and again centrifuged. The organic layer was discarded, the aqueous phase adjusted to pH 8·9 with a 3M carbonate buffer (pH 8·9) and 3·0 ml toluene–butanol (9:1) was added. The tubes were then shaken for 15 min and centrifuged as before. The organic phase was transferred to a new tube and evaporated by a gentle stream of dry nitrogen in a heating block at 70°. The residue was dissolved in 50 μl dry ethyl acetate, and 100 μl pentafluoropropionic anhydride was added. The tightly stoppered tubes were then placed in an oven at 60° for 30 min. Excess of the reagent was removed by a gentle stream of dry nitrogen and the residue dissolved in 50 μl dry ethyl acetate. 1–2 μl of this solution was injected into the gas chromatograph.

* Plasma samples from 50–500 μl and brain samples from 200–600 μl have also been examined by keeping the water to organic phase volume ratio constant.

The concentrations of morphine in the samples were obtained from a standard curve prepared by adding known amounts of morphine and nalorphine to blank plasma and brain samples. These samples were analysed according to the procedure described above. The integration of the peak areas was performed with an electronic integrator. The standard curve was computed with the linear least square analysis of the peak area ratio *versus* the weight ratio morphine to nalorphine (Fig. 1).

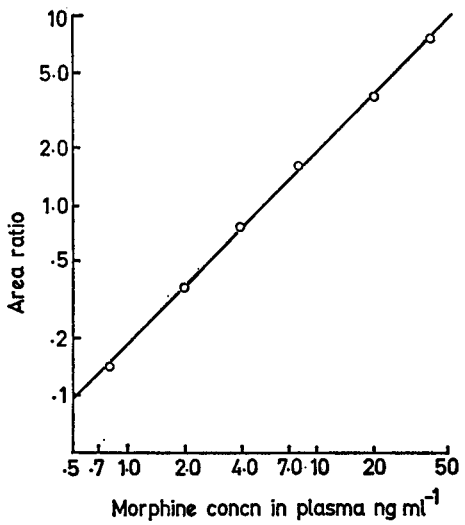

Fig. 1. Peak area ratio illustrated as a function of the concentration of morphine in plasma samples. Note the sensitivity.

RESULTS AND DISCUSSION

The extraction of morphine in the nanogram range with non-polar organic solvents is often a problem owing to the strong adsorption to the glass surfaces (Wilkinson & Way, 1969; Takemori, 1968). Extraction with benzene gives favourable partition of the drugs and also very clean extracts (Way & Adler, 1962), but in the present method toluene was chosen primarily because it has similar extracting properties and because it is less toxic than benzene. Preliminary experiments with toluene as the only extracting solvent resulted in very large losses even though all glassware was carefully silanized. Butanol 10% was therefore added to the toluene since it has been widely used to prevent adsorption (Mulé, 1964; Wilkinson & Way, 1969; Yeh & Woods, 1970). The extraction with toluene–butanol yielded blank extracts showing no interfering peaks and the original baseline was achieved within 1 to 3 min. This allows frequent injection of new samples. At pH 8·9 the morphine exists in its least charged form (Schill & Gustavii, 1964; Schill & Jönsson, 1964) and, consequently, the extraction was made at this pH. Nalorphine was chosen as internal standard because of its close chemical relationship to morphine. Nalorphine was always added to the tissue samples before extraction to correct for recovery and aliquot losses. The absolute recoveries were 70% for morphine and 98% for nalorphine. The stability of the pentafluoropropionic anhydride derivatives was very good and a drop in the peak areas of only some percentage units was noted after 3 days' storage at 4°, but the peak area ratio remained constant.

The structures of the derivatives were verified by mass spectrometry (LKB 9000) which showed a molecular ion at *m/e* 577 for PFP-morphine and at *m/e* 603 for PFP-nalorphine.

The retention times relative to the pentafluoropropionic amide of protriptyline were: morphine: 0·409 and nalorphine: 0·591. All peaks were well separated and symmetrical.

The sensitivity limit of the detector was about 18×10^{-15} mol of morphine (approx. 5 pg) and the corresponding limit when extracted from plasma samples was about 500 pg ml^{-1}. In the brain, 100 pg of morphine could be determined in a 30 mg brain tissue sample. Analyses of 9 different plasma samples containing 0·750 ng ml^{-1} morphine showed a variation coefficient of $5{\cdot}3 \pm 1{\cdot}2\%$. The sensitivity of the described method is thus in the same range as that reported for the radioimmunological technique (Spector & Parker, 1970). Furthermore, g.l.c. methods have the advantage of combining rapidity and specificity with the high sensitivity and selectivity of the electron capture detector.

The suitability and applicability of the method to pharmacokinetic studies *in vivo* are illustrated in Fig. 2 which shows the chromatogram from 100 μl rat plasma sample obtained 25 min after intravenous administration of 2·5 mg kg^{-1} of morphine. The plasma concentration corresponds to 395 ng ml^{-1}.

The time course of morphine concentrations in rat plasma after intravenous injection of 2·5 mg kg^{-1} morphine is shown in Fig. 3. The whole brain concentration of morphine 40 min after i.v. administration of the same dose was 44 ± 4 ng g^{-1} in 4 rats.

Morphine can to a small extent be metabolized to codeine *in vivo* (Elison & Elliott, 1964). This metabolite gives a peak which is not completely resolved from the nalorphine peak under the present conditions. However, the amount of codeine that could not be resolved and detected was too small to significantly influence the

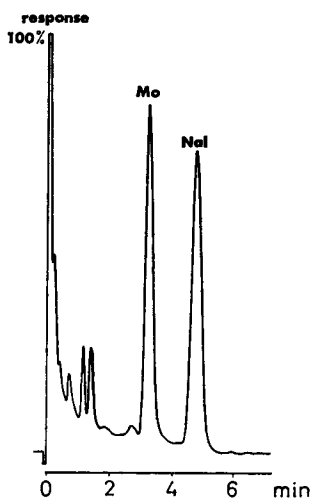

FIG. 2. Gas chromatogram of 100 μl rat plasma taken 25 min after an i.v. injection of 2·5 mg kg^{-1} morphine. The plasma concentration of morphine is 395 ng ml^{-1}. Mo and Nal indicate morphine and nalorphine, respectively.

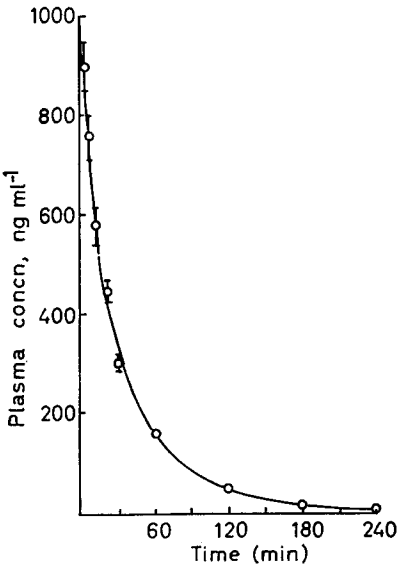

FIG. 3. Mean plasma concentrations of morphine in 4 rats after i.v. injections of 2·5 mg kg^{-1}. Vertical bars represent standard error of mean.

area of the nalorphine peak. Furthermore, in duplicates of the plasma samples without nalorphine, we were not able to detect any codeine with the low dose of morphine used.

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