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SUB-MICROGRAM ESTIMATION OF MORPHINE IN BIOLOGICAL FLUIDS BY GAS–LIQUID CHROMATOGRAPHY*

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Abstract—A specific gas–liquid chromatographic method for the determination of nanogram amounts of morphine in plasma and cerebrospinal fluid is described. The procedure requires conversion of morphine, after extraction and concentration from the biological fluid, to its trimethylsilyl ether derivative. *Bis*(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane is used both as the solvent and silylating agent in the derivatization. Extraction and conversion are linear over the range 25–500 ng/sample with the limit of sensitivity of the method being about 25 ng/sample.

MANY methods have been described for the estimation of morphine in biological fluids, the suitabilities of which have been critically reviewed by Way and Adler.[1] Photometric methods are capable of determining only microgram amounts of drug and, although radioactive techniques allow estimation of nanogram quantities, they are impractical for routine analysis. Since the above review, Kupferberg *et al.*[2] have developed a fluorometric method which enables detection of 0·1 μg morphine in plasma and brain. More recently, Takemori[3] has modified this method and increased its sensitivity 10-fold, but the presence of closely related narcotics, metabolites and tissue constituents reduces the specificity of the procedure. The intrinsic characteristics of gas–liquid chromatography suggested that this technique might be suitable for the specific determination of morphine in the nanogram range.

A number of investigators have reported the gas chromatographic behavior of morphine. Many of these reports have been concerned only with the detection and identification of the drug either as the free base or as "on column" synthesized derivatives.[4–10] It has proved impossible to quantify, with any precision, morphine free base on account of its nonlinear adsorption to the chromatographic system. Consequently, derivatization has been necessary to achieve quantification.[11–13] The *bis*-trimethylsilyl (TMS) ether derivative has been found eminently suitable for this purpose,[12, 13] although the procedures have only been applied to milligram quantities of the drug present in various opium preparations.

In the past, the formation of TMS derivatives has been based on the procedure of Sweeley *et al.*,[14] involving reaction with a mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS), frequently in pyridine. However, a new reagent,

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bis(trimethylsilyl)acetamide (BSA) has been introduced[15] which, because of its increased silylating activity, is supplanting HMDS. More recently, the trifluoro analog of BSA, *bis*(trimethylsilyl)trifluoroacetamide (BSTFA), has become available[16] which, while retaining the potent silylating properties of BSA, has certain advantages.

This communication reports the use of BSTFA in the formation of morphine–TMS for subsequent estimation of nanogram quantities of the drug, extracted from plasma and CSF, by gas–liquid chromatography (GLC) utilizing a flame ionization detector.

MATERIALS AND METHOD

Reagents. Hydrochloric acid, sodium bicarbonate and tetraphenylethylene (TPE) were reagent grade, while ethyl acetate and *iso*propanol were GC-spectrophotometric quality (J. T. Baker Chemical Co.). *Bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA/TMCS) was purchased from Regis Chemical Co. The 10-ml "Oak Ridge" type, screw-cap, polypropylene tubes and the 15-ml polycarbonate conical tubes were obtained from International Equipment Co.

Gas chromatography. A Varian Aerograph model 1200 gas chromatograph equipped with a flame ionization detector and a 7127A Moseley recorder were employed. The chromatographic column was stainless steel, $\frac{1}{8}$ in. o.d. × 5 ft, packed with 3 per cent OV-1 on 100/120 mesh Gas Chrom Q (Applied Science Labs.). Conditioning was carried out at 300° for 4 hr with no carrier gas flow and then at 275° with 15 ml/min nitrogen flow rate for 24 hr. Chromatography was performed under the following conditions: column temperature, 215°; injection block temperature, 250°; detector temperature, 250°; nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; air flow rate, 300 ml/min.

General procedure. About 250 mg sodium bicarbonate was placed in a 10-ml "Oak Ridge" type polypropylene tube and 0·1–1·0 ml of plasma or cerebrospinal fluid was added. The aqueous phase was adjusted to a volume of 2·0 ml with distilled water and then extracted with 5·5 ml of a mixture of ethyl acetate containing 10% (v/v) *iso*propanol (10 min on a tilt-action shaker, set at 30 rpm). After centrifugation, exactly 5·0 ml of the upper organic phase was transferred to another polypropylene tube containing 1·2 ml of 1 N HCl and the morphine was back-extracted by shaking for 5 min. The organic layer was discarded and 1·0 ml of the acid was transferred to a 15-ml conical polycarbonate tube, care being taken to add the extract to the bottom of the tube and not down the side. The acid was then evaporated to dryness under vacuum and gentle shaking at 35°, using a Buchler Evapomix. When dry, 50 μl of a methanolic solution of TPE (10 μg/ml) was added to the bottom of the tube and the methanol was evaporated on the Evapomix. About 25 μl of BSTFA/TMCS reagent was added to the residue and the lower 4 cm of the tube was rinsed with the mixture. The silanizing reaction was allowed to proceed for 20–30 min at room temperature. After a further rinsing of the tube, 1–2 μl of the reaction mixture was injected into the chromatograph. The amount of morphine present in the sample was calculated from the peak height ratio of morphine–TMS to TPE and relating this to a previously constructed calibration curve.

RESULTS AND DISCUSSION

Under the described chromatographic conditions, TPE had a retention time of 5·0

min and was well separated from the morphine–TMS peak, retention time 6·5 min. Both peaks were sharp and symmetrical (Fig. 1). Despite the rapid drifting of the baseline at the low electrometer attenuations required for the determination of the smallest amounts of morphine, these latter two characteristics and the absence of any interfering peaks allowed easy and accurate interpolation of the baseline of the morphine–TMS peak, an essential operation in determining its height.

Preliminary studies, using morphine solutions directly, indicated that 100 per cent conversion of morphine to its TMS ether by BSTFA/TMCS was achieved at room temperature within 20 min. In the absence of TMCS, it was necessary to heat the

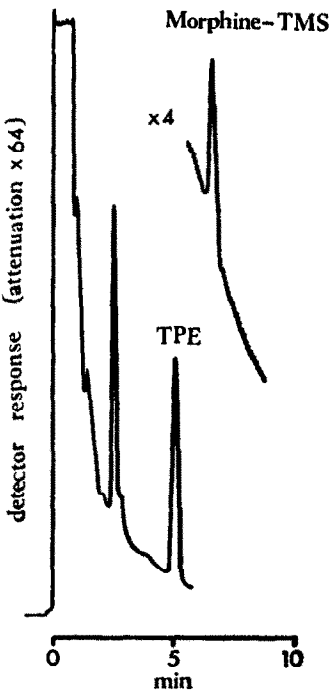

FIG. 1. Chromatogram of morphine–TMS and internal marker (TPE) after extraction of 0·5 ml plasma, containing 150 ng morphine/ml, obtained from an anesthesized dog 2 hr after i.v. injection of 5 mg morphine/kg (electrometer attenuation was changed from ×64 to ×4 between the elution of TPE and morphine–TMS).

reaction at 55° for 30 min to obtain the same degree of conversion. The reaction was found to be quantitative over the absolute range of 0·005 to 2·5 μg morphine, with 2–5 ng being the sensitivity limit of the detector.

Prior to the commercial availability of BSTFA, derivatization was achieved by using BSA/TMCS. However, it was found that the response of the flame ionization detector to BSA was quite large so that at drug levels below 0·1 μg the morphine–TMS peak was eluting off-scale. Furthermore, the BSA/morphine solution was prone to rapid hydrolysis which gave rise to spontaneous crystallization. After such crystallization it was difficult, possibly because of occlusion, to obtain 100 per cent conversion

and recovery by further addition of BSA. BSTFA, however, gave a much reduced FID response and even at the lowest practical attenuation (×4, FSD 12·5 × 10^{-12} amp) the morphine–TMS peak eluted from a not too steep baseline (Fig. 1). Also, spontaneous crystallization of BSTFA occurred very infrequently and the deposition of silicon dioxide on the detector was reduced compared to BSA. Deposition did occur, but daily cleaning of the anode and flame-tip prevented buildup to a level which affected sensitivity and noise levels.

Many problems were encountered in the extraction and concentration of nanogram quantities of morphine from water and biological fluids. The major problem was the adsorption of such small quantities of drug on to the glassware. This was particularly troublesome during the evaporation to dryness of the 1 ml of 1 N HCl extract. By using detergent-clean glassware for this step, the recovery of the morphine was found to be nonlinear. Attempts to circumvent this problem by silanizing, siliconizing or scrupulously cleaning the glassware[3] were all unsuccessful. Certain procedures did

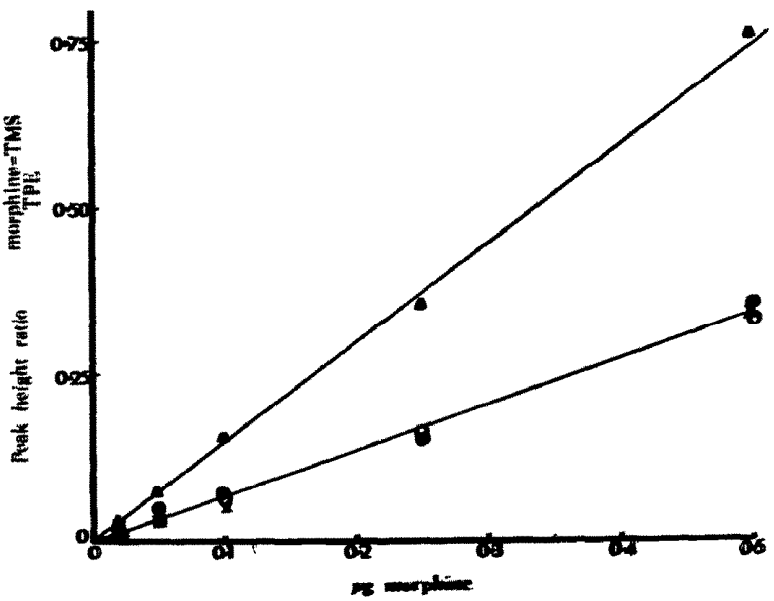

Fig. 2. Absolute (△) and extraction calibration curves for water (○), plasma (●) and CSF (×).

increase individual drug recoveries but the total curve was still nonlinear. The use of polystyrene and polypropylene tubes was investigated, but it was found that the BSTFA extracted various components of the plastic which caused interference during chromatography. Finally, polycarbonate tubes were chosen for the concentration step as they gave 100 per cent relative recovery and no interfering peaks. It was not necessary to treat these tubes in any fashion and they were used as received, then discarded. Vacuum-tight adaptors for fitting the polycarbonate tubes to the Evaporvix were constructed from size B, polypropylene female connectors.

Caution was required in evaporating the 1 ml of 1 N HCl extract to prevent "bumping" and loss of the extract. By freezing the extract in an ethanol-dry ice mixture and then allowing it to thaw slowly with the gradual application of vacuum, this problem was overcome.

The use of polypropylene tubes and inclusion of *iso*propanol in the organic extraction phase were designed to circumvent adsorption of morphine on the vessel during this step of the procedure.

Calibration curves from water, plasma and CSF were found to be linear over the range 25–500 ng morphine per ml for a 1-ml sample (Fig. 2). Recoveries, corrected for aliquot losses, were not significantly different for any of the three solutions (mean recovery, 59 per cent) and the limit of sensitivity of the method was found to be about 25 ng per sample. Interference from normal plasma and CSF constituents, at the retention times of TPE and morphine–TMS, was not found in any of the samples examined, even at the lowest practical attenuation of the chromatograph.

The suitability and applicability of the described method to studies *in vivo* are illustrated in Fig. 1, which shows the chromatogram from 0·5 ml plasma obtained 2 hr after the intravenous administration of 5 mg morphine per kg to a 10 kg anesthesized dog, the plasma concentration corresponding to 150 ng per ml.

The described method is not only sensitive and specific, but may be applied to other narcotics and to related drugs which possess a replaceable labile hydrogen, with possibly the only change being the use of a different chromatographic system. The use of larger samples and more efficient extraction solvents may increase the sensitivity of the method beyond its present limit of 25 ng per sample.

The derivatization procedure, utilizing BSTFA/TMCS as both the solvent and silylating reagent, appears to have a number of advantages over more traditional methods that use a solution of the silanizing reagent in a nucleophilic solvent. By using only 25 μl of reagent considerable concentration of the drug is achieved, compared to its original dilution in the biological fluid. Excess reagent, however, is always present and the solvent peak due to the BSTFA/TMCS is often much smaller than that of the usual solvents (pyridine, dimethylformamide, etc.). Moreover, the constant injection of silanizing reagent maintains the chromatographic system in a deactivated condition. Clearly, such a procedure may be widely applied to the determination of sub-microgram quantities of any compound, drug or otherwise, where TMS derivatization is necessary.

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