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GAS CHROMATOGRAPHIC DETERMINATION OF APOMORPHINE IN PLASMA

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SUMMARY

A method is described for the analysis of 1 to 10 μg/ml concentrations of apomorphine in plasma. The procedure is based on ethyl acetate extraction, a back extraction cleanup-step, derivatization with heptafluorobutyric anhydride, and gas chromatography on a 3% OV-17 column using flame ionization detection. N-*n*-Propylnorapomorphine is employed as an internal standard and quantitative relative recoveries of drug are realized with relative standard deviations of 4.6%. The method permits analysis of apomorphine in the presence of its two monomethyl ether metabolites, apocodeine and isoapocodeine. The latter compounds are also chromatographically resolved as their heptafluorobutyrate derivatives.

INTRODUCTION

The discovery that apomorphine (I) and some of its prodrugs possess antiparkinsonism activity has led to a renewed interest in this group of compounds[1–6]. As part of a systematic examination of the metabolism of these alkaloids, it was necessary to develop a method for the analysis of apomorphine in plasma in the μg/ml range. A procedure that would permit assay of I in the presence of its potential metabolites, apocodeine (II), isoapocodeine (III) and apomorphine dimethyl ether (IV)[7–11], was also desired.

Colorimetric assay of I, based on mercuric chloride oxidation to its *o*-quinone, offers adequate sensitivity[12] but lacks the necessary selectivity as II and III can also be converted to the *o*-quinone[13]. Other colorimetric[14,15] and fluorometric[16,17] methods lack either the desired sensitivity and/or selectivity for the analysis of I in plasma.

A gas chromatographic (GC) method using 5% SE-30 and non-derivatized I[18] suffered from decomposition on the column at temperatures needed for elution[19]. GC of I as its O,O-bis(trimethylsilyl) ether derivative permitted satisfactory development but lacked needed selectivity[19]. It was proposed that derivatization of I with

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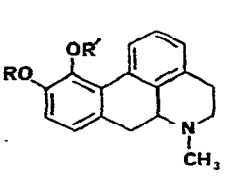
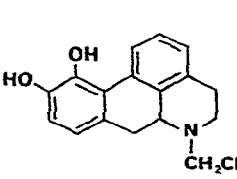
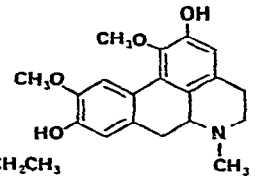






I. R=R'=H
APOMORPHINE

II. R=CH_3, R'=H
APOCODEINE

III. R=H, R'=CH_3
ISOAPOCODEINE

IV. R=R'=CH_3
APOMORPHINE DIMETHYL ETHER

V. N-*n*-PROPYLNOR-APOMORPHINE

VI. BOLDINE

heptafluorobutyric (HFB) anhydride might improve selectivity and provide the potential ability to use electron capture detection with its attendant high sensitivity.

MATERIALS AND METHODS

Gas chromatography

A Hewlett-Packard model 5710 A gas chromatograph equipped with dual flame ionization detectors (FID) was employed throughout. The oven and detector were maintained at 190° and 300°, respectively. Gas flow-rates were: carrier gas (nitrogen), 50 ml/min; hydrogen, 60/ml min; and compressed air, 240 ml/min. Silylated glass columns, 76 cm × 6 mm O.D., 4 mm I.D., were packed with 3% OV-17 on Chromosorb W-HP, 100–120 mesh (Analabs, North Haven, Conn., U.S.A.) and conditioned at 275° for 18 h with normal carrier gas flow. They exhibited an average continuous life of approximately 90 days.

Materials

Apomorphine hydrochloride hemihydrate (Merck, Rahway, N.J.) U.S.A. and boldine (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) were used as purchased. N-*n*-Propylnorapomorphine was a gift of Sterling Winthrop Research Institute (Rensselaer, N.Y., U.S.A.). Apocodeine, isoapocodeine and 10,11-dimethoxyaporphine were prepared in our laboratory as previously described[10]. HFB anhydride and dithiothreitol were used as purchased (Aldrich, Milwaukee, Wisc., U.S.A.). Pesticide grade acetonitrile and spectroquality methanol were employed (Matheson, Coleman & Bell, Norwich, Ohio, U.S.A.); all other solvents and reagents were analytical reagent grade.

All glassware was silanized by rinsing with 2% trimethylchlorosilane in benzene solution and heating at 110° for 30 min.

Recovery experiments

Stock solutions of I and V were prepared in 5- or 10-ml volumetric flasks by dissolving 5.0–10.0 mg of the compound in methanol or acetonitrile. Between experi-

ments, samples were stored at −10°. Standard GC solutions were prepared by reducing to dryness aliquots placed in Reacti-vials (Pierce, Rockford, Ill., U.S.A.) with a stream of nitrogen. Acetonitrile (50 μl) and HFB anhydride (25 μl) were added, and the solution was allowed to stand at room temperature for 30 min. Injections were made using a Hamilton 701 N 10-μl syringe. N-*n*-Propylnorapomorphine (V) was used as an internal standard to control the extraction procedure. Standard curves of the peak height ratio of I and V *versus* the concentration (μg/ml) of I in plasma were used analytically. Boldine (VI) was used as an internal standard to control the GC step when absolute recovery data were needed and was dissolved in the acetonitrile used as a derivatization solvent. Peak heights "corrected" to a "standard" boldine value were plotted *versus* μg of I (or V) per ml of plasma to yield a standard curve.

Preparation of standards

To two sets of five 2-ml human blank plasma samples was added 2, 5, 10, 15, and 20 μg of I and 10 μg of II (in 100 μl or less of methanol). The standards were extracted and analyzed in the same manner as described for plasma samples below.

Determination of I in plasma

Each 2-ml human plasma sample to be analyzed had 10 μg N-*n*-propylnorapomorphine and 3 ml of 0.25 *M* phosphate buffer (pH 7.0) added[19]. Ethyl acetate (10 ml) was added, and the two phases were mixed for 10 min. The ethyl acetate layer was removed and reduced in volume to approximately 1 ml with a stream of nitrogen at room temperature. The ethyl acetate was extracted with 2 ml of 0.012 *N* HCl. The acid layer was neutralized with 250 μl of 8% Na_2CO_3. Three milliliters of 0.25 *M* phosphate buffer (pH 7.0) were added, and the aqueous layer was again extracted with 10 ml of ethyl acetate. The organic layer was reduced to dryness at room temperature by a nitrogen stream. The residue was dissolved in 50 μl of acetonitrile. HFB anhydride (25 μl) was added. Samples were injected directly into the GC after 30 min at room tempera-

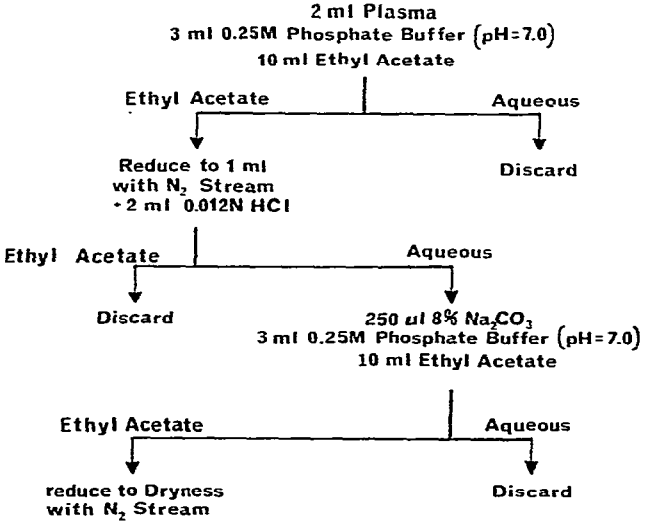


Fig. 1. The extraction of compounds I and V from plasma

ture (Fig. 1). This extraction procedure resulted in a blank plasma extract which contained no detectable peaks when chromatographed in the usual manner.

Calculations

The peak heights of I and II were measured. Peak height ratios were obtained by dividing the peak height of I by the peak height of II. Calibration curves were prepared by plotting peak height ratios of the standards *versus* the concentration of I in plasma expressed as μg/ml. Least squares regression was used to determine the best fit line for the data obtained from the standards. Values for the unknown concentrations were determined by calculation using the peak height ratio of I and II and the parameters determined by the regression analysis.

RESULTS AND DISCUSSION

Reaction of apomorphine (I) with acetic anhydride has been reported to yield the ring-opened product[20]. By analogy, it was anticipated that products of the reaction of I–III and V with HFB anhydride would also be ring-opened products. Mass spectra of these derivatives, however, showed that I and V contained only two equivalents of HFB acid, while the derivatives of II and III showed only one equivalent of HFB acid (Table I). It is, therefore, proposed that the derivatives formed are the phenolic esters of the non-ring-opened compounds (Table II). It is assumed that VI acts in a similar manner and that IV is unaffected by HFB anhydride. Since no extraordinary efforts

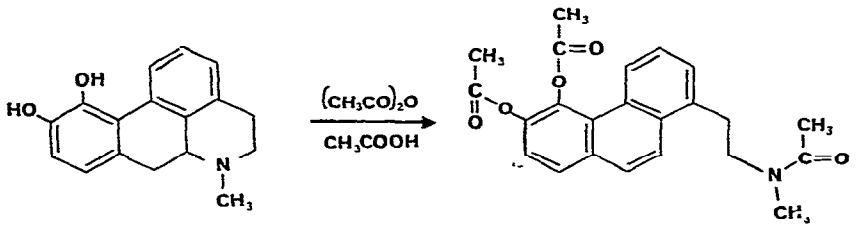


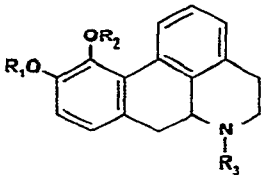
TABLE I

MASS SPECTRAL DATA FOR HFB DERIVATIVES OF COMPOUNDS I, II, III and V

Compound	*Molecular weight*	*Electron impact*			*Chemical ionization*				
		Base peak	*M*+		*Base peak (M + 1)*	*M + 29*		*M + 41*	
			m/e	*% Base*		*m/e*	*% Base*	*m/e*	*% Base*
Apomorphine diheptafluorobutyrate	659	659	659	100	660	688	17.3	700	6.2
N-*n*-Propylnorapomorphine diheptafluorobutyrate	687	658	687	77.1	688	716	22.2	728	9.8
Isoapocodeine heptafluorobutyrate	477	69	477	19.8	478	506	22.9	518	7.0
Apocodeine heptafluorobutyrate	477	69	477	32.2	478	506	30.7	518	6.3

TABLE II

STRUCTURES OF HFB DERIVATIVES OF I, II, III AND V



No.	*Compound*	R_1	R_2	R_3
I	Apomorphine	$CF_3(CF_2)_2CO$	$CF_3(CF_2)CO$	CH_3
II	Apocodeine	CH_3	$CF_3(CF_2)_2CO$	CH_3
III	Isoapocodeine	$CF_3(CF_2)_2CO$	CH_3	CH_3
V	N-*n*-Propylnorapomorphine	$CF_3(CF_2)_2CO$	$CF_3(CF_2)_2CO$	$CH_3CH_2CH_2$

were made to purify the HFB acid, it must be assumed that some HFB acid is present and that more is produced by the reaction of the anhydride with any residual water present in the sample. It is suggested that the weakly basic heptafluorobutyryl anion is unable to remove the proton from position 7 of aporphines, thereby preventing the rearrangement observed with acetic anhydride[20].

The time needed for derivatization was studied. I and V were found to be completely derivatized almost immediately as indicated in Fig. 2. Boldine was completely derivatized after 10 min. The derivatives were stable for at least 2 h. In the studies described our samples were developed 30 min after derivatization.

Although the developed procedure provides standard curves that are linear over three orders of magnitude (1 μg to 1 mg) only the range 1–10 μg/ml was extensively studied because other methods already exist for analyses at higher concentrations. A sample chromatogram is depicted in Fig. 3 along with a similarly processed blank plasma sample which shows no interferences in the region of interest.

Back extractions of samples were found to be necessary to eliminate two large, late chromatographing substances which did not interfere with the analytical region

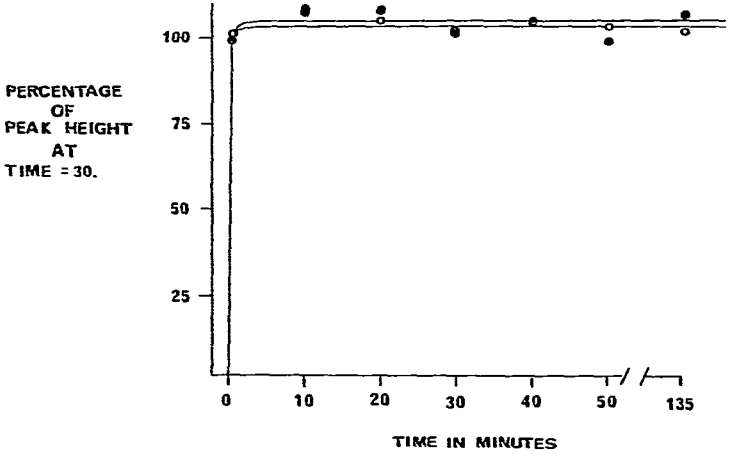


Fig. 2. Rate of derivatization of I (○) and V (●) with HFB anhydride.

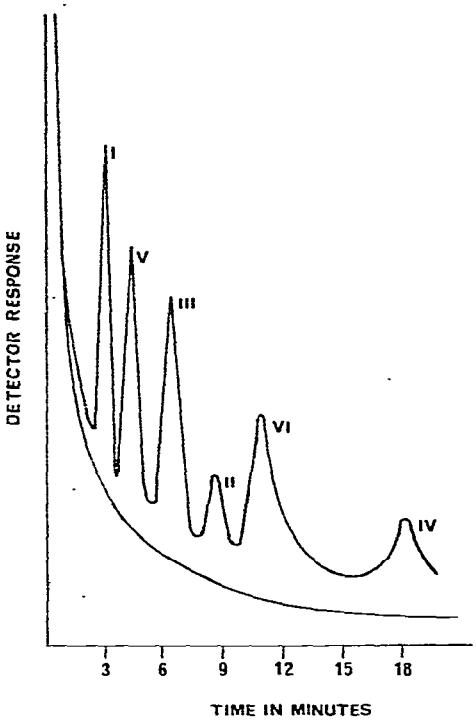


Fig. 3. GC (FID) of a mixture of aporphines using a 64 cm × 4 mm I.D. silylated glass column packed with 3% OV-17 on Chromosorb W-HP. A Hewlett-Packard 5710A gas chromatograph was operated isothermally at 190° and a nitrogen flow-rate of 50 ml/min.

but greatly added to the time needed for repetitive samples. Boldine was used as an internal standard to control the GC step in absolute recovery experiments. The absolute recovery of apomorphine from plasma in the 1–10-μg/ml range averaged 87.9% (n=6), while average absolute recovery of N-n-propylnorapomorphine (the internal standard) was 84.6% (n=6).

In the range 1–10 μg of appmorphine per ml of plasma, correlation coefficients of 0.99 or greater were consistently observed for standard curves (Fig. 4). Over this range, an average relative standard deviation of 4.6% was found.

When dealing with biological specimens, it is often necessary to store them for extended periods, usually by freezing. It has been found that these samples lose apomorphine as time passes. It was assumed that this was due to oxidation of the apomorphine, probably to quinone-type substances. Acid was found to slow or prevent this process[21], but the addition of sufficient acid to retard degradation tended to precipitate plasma proteins which resulted in losses of apomorphine. The acid treatment also made the adjustment of the pH prior to extraction a more tedious task. Dithiothreitol (DTT) (1 mg/ml) was found to be an effective stabilizing agent when added to the plasma prior to freezing. Analysis of DTT-treated samples that were stored for 6 weeks at −10° showed no significant loss. The DTT also did not interfere with the extraction or the actual determinations.

Compounds II, III and IV were found to separate from I and V when added to

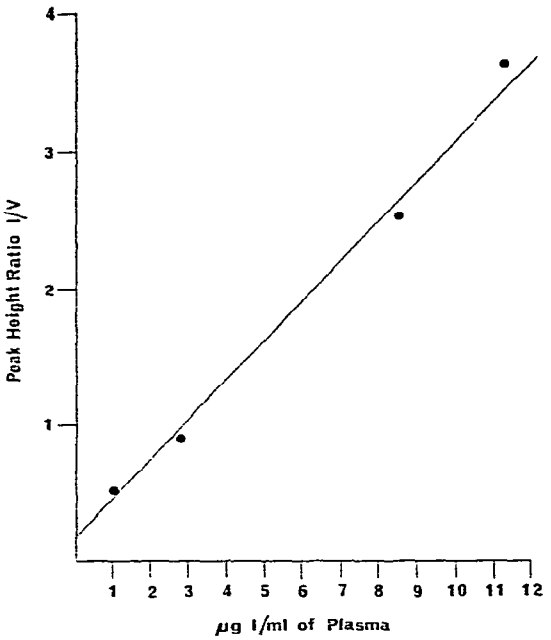


Fig. 4. Typical standard curve for the determination of I in plasma. Least squares data: slope, 0.30; y-intercept, 0.15; r, 0.996.

plasma and analyzed with the developed procedure. The analytical procedure should be applicable to all of the above named compounds, though recoveries were not specifically examined. However, from previous works[22,23], it is expected that recoveries should be excellent.

HFB derivatives offer the potential of analysis using an electron capture detector (ECD), thus, achieving significantly greater analytical sensitivity. The HFB derivative of I was reportedly used to detect apomorphine in ng/ml concentrations in horse blood plasma[24]. However, the authors of this study provided little information on recoveries or the precision of their procedure. Studies are currently being performed in our laboratories to evaluate the utility of ECD for the ppb analysis of apomorphine and its analogs as their HFB derivatives.

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