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ELECTRON CAPTURE DETECTION OF AN APOMORPHINE HEPTAFLUOROBUTYRATE DERIVATIVE AT LOW PICOGRAM LEVELS.

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SUMMARY

An electron capturing derivative of apomorphine was prepared by incubating the drug with heptafluorobutyric anhydride (HFBA), triethylamine and heat. Mass spectral analysis suggests that HFBA reacts with both phenolic hydroxyl groups on apomorphine to give a derivative detectable at low picogram levels. This method is sufficiently sensitive for pharmacokinetic studies in the horse and is likely applicable to other dopaminergic analogues of apomorphine.

INTRODUCTION

Relatively small (0.02 mg/kg or about 12 mg) doses of apomorphine intravenously produce marked signs of central excitation in the horse. Apomorphine is thus not infrequently used as a stimulant "dope" in horse racing. However, because of the small amounts of drug which are effective, pharmacokinetic studies and correlations between plasma levels of apomorphine and behavioral and performance effects are difficult to perform. Thus in our hands, the fluorimetric methods of Butterworth & Barbeau (1975) and VanTyle & Burkham (1971) were not sufficiently sensitive for pharmacokinetic work in horses. Further, recent studies showing that apomorphine is a direct dopamine receptor agonist (Seeman et al, 1975; Moore, 1974) and studies on the treatment of Parkinson's disease with apomorphine and its congeners (Cotzias et al, 1976) suggest a need for a highly sensitive and specific assay for apomorphine analogues. In this communication we report a heptafluorobutyric anhydride (HFBA) derivatization method for determining apomorphine in equine plasma

which is sensitive at low picogram levels. This method is more than adequate for pharmacokinetic studies in the horse and thus presumably in other species.

METHODS

To 2 ml of spiked aqueous or equine plasma samples, 0.2 ml of 0.5 M phosphate-citrate buffer, pH 7.0 and 4 ml of 4:1 benzene-ethylacetate was added and the whole rotoracked for 15 minutes. At the end of this period the tubes were centrifuged at 2000 x g for 10 minutes and the organic layer removed. The samples were then re-extracted with 2 ml of benzene-ethylacetate (4:1) and the organic phases pooled. The organic phase was evaporated to dryness and the residue taken up in 0.5 ml of benzene. The benzene layer was transferred to a 16 x 125 mm culture tube and 40 µl of heptafluorobutyric anhydride (HFBA) and 10 µl of a 5% solution of triethylamine (TEA) in benzene added. These were then incubated at 60° for 40 minutes. At the end of this period, the reaction mixture was washed with 10 ml of saturated sodium tetraborate, pH 9.0. After washing, the benzene layer was placed in a 6 x 50 mm glass culture tube with care taken to transfer as little of the aqueous phase as possible. These tubes were then centrifuged at 2,000 x g for 5 minutes and aliquots of the supernatant taken for gas chromatography.

Chromatography was on a Varian Aerograph (Model 2740) equipped with tritium foil electron capture detectors. Separation was on a 3 foot glass column packed with 3% OV 101 on 100/120 Gas Chrom Q., with nitrogen at 30 ml/min as the carrier gas. The injector temperature was 280° C, the column temperature 235° C, and the detector foil temperature 255° C.

The heptafluorobutyric anhydride was from Pierce Chemicals, Ltd., Rockford, Illinois. All glassware used in these experiments was silanized in a 1% solution of dichlorodimethylsilane in toluene. Authentic apomorphine HCl

used in the preparation of standards was a kind gift of Professor J. G. Cannon of the College of Pharmacy, University of Iowa. For equine administration experiments, commercially available 6 mg injectable tablets of apomorphine from Eli Lilly, Ltd., Indianapolis, Indiana, were used.

RESULTS

Figure 1 shows that best recovery of an electron capturing derivative from spiked apomorphine standards was obtained with an 8:2 benzene-ethylacetate mixture. If the proportion of ethylacetate in this system was high, the yield of the electron capturing derivative was reduced and the non-specific background tended to increase. Thus, in all subsequent experiments reported in this paper the initial extraction of apomorphine was made into an 8:2 mixture of benzene-ethylacetate and the derivatization reaction performed in benzene as outlined in METHODS.

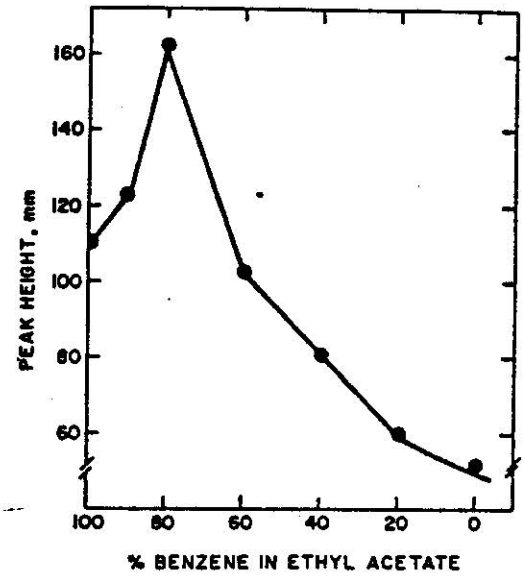

Fig. 1 Recovery of apomorphine from aqueous standards.

Aqueous samples of 80 ng/ml of apomorphine, buffered to pH 7.0 with 0.5 M phosphate buffer were extracted into benzene-ethylacetate mixtures of the indicated proportions. Subsequent treatment was as indicated in METHODS. The symbols show the relative amounts of apomorphine-HFB derivative recovered with the differing proportions of benzene:ethylacetate. All data points are the means of three separate determinations.

Figure 2 shows typical chromatograms of the HFBA derivative of apomorphine. At apomorphine levels of 80 ng/ml in plasma, a clear sharp peak with a retention time of about 1.2 minutes was observed. This peak was not observed in blank samples and was appropriately reduced in a 20 ng/ml sample.

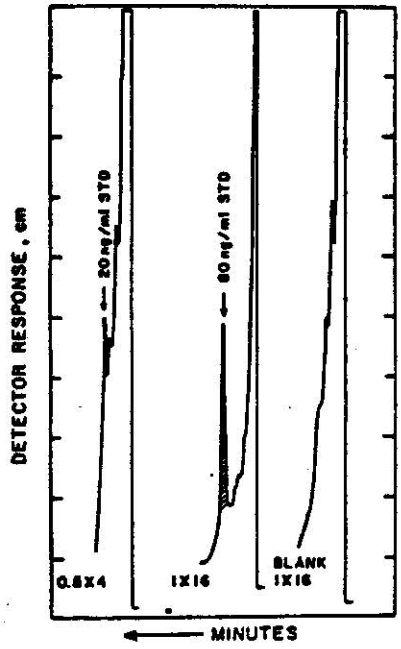

Fig. 2 Gas chromatographic analysis of apomorphine-HFB derivatives.

The right-hand chromatogram is that of a blank sample of equine plasma treated as outlined in METHODS. The center chromatogram is from a plasma sample spiked with 80 ng/ml of apomorphine. The left-hand chromatogram is that of a plasma sample spiked with 20 ng/ml apomorphine. The figures at the bottom of each chromatogram represent microliters injected by electrometer attenuator setting.

Figure 3 shows a standard curve for the recovery of apomorphine from plasma, showing that the method is linear and sensitive down to low nanogram/ml levels. Other experiments, not presented, shwed that this method readily picked up the low (40 ng/ml) levels of apomorphine associated with signs of central nervous system excitation by apomorphine in the horse.

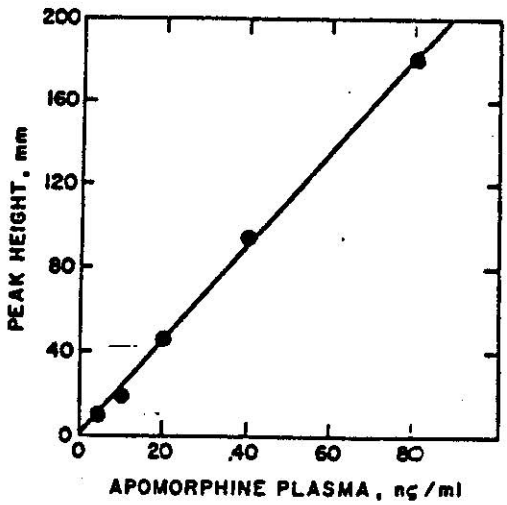

Fig. 3 Standard curve of apomorphine-HFB derivative from plasma.

To samples of equine plasma the indicated concentrations of apomorphine were added and extracted and derivatized as outlined in METHODS. The solid symbols show the relative amounts of apomorphine-HFB recovered at each concentration of apomorphine. Data points are the means of three separate determinations.

Figure 4 shows the mass spectrum of the apomorphine-HFB derivative. The spectrum shows numerous peaks of molecular weight greater than 464, suggesting derivatization of both phenolic OH groups on apomorphine by HFBA. Consistent with this hypothesis, the largest peak observed was an ion of molecular weight 645. This mass is consistent with double derivatization of the apomorphine molecule and exchange of the N methyl group for a proton during fragmentation.

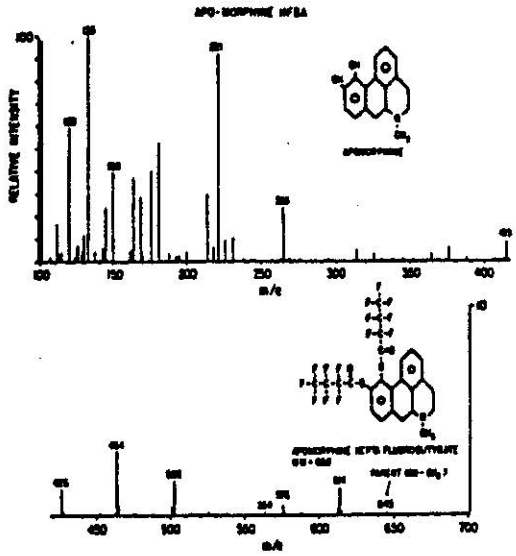

Fig. 4 Mass spectrum of apomorphine-HFBA prepared as outlined in METHODS.

DISCUSSION

Previously published methods for the detection of apomorphine in biological fluids have depended on either UV or fluorescence spectrometry (VanTyle & Burkman, 1971) or flame ionization detection (Smith & Stocklinski, 1975). Both the sensitivity and specificity (Cava et al, 1972) of these methods is limited and in our hands the fluorimetric method was not sufficiently sensitive for pharmacokinetic studies in the horse. By derivatization with HFBA and utilizing electron capture detection a specific method for apomorphine has been developed with sensitivity in the low nanogram/ml range (Blake et al, 1973).

While ethylacetate has been a widely used solvent for the extraction of apomorphine from aqueous samples, a number or problems were encountered with the use of ethylacetate in these experiments. In the first place, ethylacetate was not satisfactory for injection onto the column since it tails badly and masked the drug peak. Secondly, ethylacetate tended to extract considerable amounts of extraneous materials, so that even if the ethylacetate was evaporated and the derivative formed in benzene, high backgrounds which masked low drug levels were still obtained. In our hands, best results were obtained when the biological fluids were extracted into benzene-ethylacetate 8:2, this solution evaporated to dryness and the derivatization reaction performed in benzene. Triethylamine was used as the catalyst because backgrounds obtained with this were less than those obtained with pyridine. Similarly, the concentration of HFBA in the reaction system was reduced below that used by other workers (Blake et al, 1973) in an effort to reduce the derivatization of extraneous material.

When these precautions were taken, the method turned out to be very sensitive indeed. Concentrations of 20 ng/ml of apomorphine in 2 ml of equine plasma were readily detected and the method was sensitive down to about 5 ng/ml. With suitable manipulation of sample size and the extraction procedure (Tobin et al, 1976), it should be readily possible to detect levels as low as 1 ng/ml or 1 picogram of apomorphine injected on the column. This level of sensitivity is more than adequate for pharmacokinetic work in the horse.

The sensitivity of the method appears to be dependent on the fact that both hydroxyl groups on the phenolic ring of apomorphine are derivatized. Thus, a mono-HFB derivative would have a molecular weight of 464 and should not show molecular ions of greater mass. Figure 4, however, shows that the mass spectrum of the apomorphine-HFB derivative contains numerous mass fragments

greater than 464 and one peak with an apparent mass of 645. This mass suggests a bis-heptafluorobutyric derivative of apomorphine in which the N-methyl group has been replaced by a proton. Therefore, both the mass spectrum presented in Fig 4 and the sensitivity of the derivative to electron capture detection are consistent with double derivatization of the apomorphine molecule by HFBA.

Recently, there has been considerable interest in apomorphine as a direct dopaminergic agonist and studies on the treatment of Parkinson's disease with apomorphine derivatives are in progress (Cotzias et al, 1976). Since this derivatization method utilizes the phenolic hydroxyl groups of apomorphine, which OH groups are likely to be present in all dopaminergic analogues of apomorphine, it appears likely that this method will be effective for analogues of apomorphine such as aporphine. The method should thus be useful in the study of the pharmacokinetics of this group of drugs in animals other than the horse. A further interesting possibility developing from this work is that because of the sensitivity of this method it should enable accurate estimation of the concentrations of apomorphine interacting with dopaminergic receptors in the CNS to be made.

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