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SELECTED ION MONITORING OF APOMORPHINE IN WHOLE RAT BRAIN

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SUMMARY

Mass fragmentography was used for quantitation of apomorphine in whole rat brain. Following extraction with 0.45 *N* perchloric acid, the components were derivatized to the O,O-bis(trimethylsilyl) (TMS) derivatives with N,O-bis(trimethylsilyl)trifluoroacetamide at 30°C. The molecular ions of the TMS derivatives of apomorphine (*m/z* 411) and N-*n*-propylnorapomorphine (*m/z* 439), as an internal standard, were assayed simultaneously by selected ion monitoring. Total chromatography time is 5 min. By this method 50 ng/ml can be determined with a coefficient of variation of 12.0%. Application of the method to apomorphine disposition study in whole rat brain was demonstrated.

INTRODUCTION

Apomorphine induces stereotyped behaviour consisting of sniffing, licking or biting in rodents [1]. This behavioral effect is due to a direct action of apomorphine on central dopaminergic receptors [2, 3]. Since antipsychotic drugs such as phenothiazine and butyrophenone analogues block apomorphine-induced stereotypy, this behaviour has been widely used for screening new antipsychotic drugs.

Recent research indicates that apomorphine-induced stereotypy is affected by non-drug factors such as circadian rhythms [4], aging [5, 6], or food deprivation [7, 8]. Effects of non-drug factors on apomorphine-induced stereotypy may be related to the changes in the sensitivity of dopamine receptors or the changes in pharmacokinetics in the body.

In order to determine brain apomorphine levels, it is necessary to develop a sensitive and selective method for determining the compound. Previously reported techniques for measuring apomorphine in biological materials include

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gas—liquid chromatography [9, 10], high-performance liquid chromatography (HPLC) [11, 12], spectrofluorometric assay [13], enzymatic—radioisotopic assay [14], and more recently selected ion monitoring (SIM) [15]. The spectrofluorometric and enzymatic—radioisotopic assays have been predominantly used for determining brain apomorphine concentrations. However, these methods have a limitation in sensitivity and/or selectivity. Therefore, in this study, we applied SIM for measuring apomorphine in whole rat brain.

EXPERIMENTAL

Chemicals and reagents

Apomorphine hydrochloride hemihydrate (APM · HCl) was a gift from Sandoz (Basel, Switzerland). N-*n*-Propylnorapomorphine hydrochloride hemihydrate (PNAPM · HCl) was kindly donated by Dr. R.V. Smith (Drug Dynamics Institute, College of Pharmacy, The University of Texas at Austin, U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). Benzene and diethyl ether, analytical grade, and tribasic sodium phosphate and 60% perchloric acid (PCA), reagent grade, were purchased from Katayama (Osaka, Japan).

Extraction of samples

One milliliter of a 0.45 *M* PCA solution of PNAPM (10 μg/ml), as an internal standard, and 4 ml of 0.45 *M* PCA solution were added to whole rat brain samples on an ice bath. The mixture was homogenized using a Kinematica Polytron (setting of 7; PT-10 probe) for 1 min and then the homogenate was shaken for 10 min. After centrifugation (4°C, 8000 *g*, 10 min) the supernatant was transferred to a conical glass tube with PTFE-lined screw-cap. To this tube a mixture of diethyl ether and benzene (5:2, v/v) was added and then shaken for 10 min. After the mixture was centrifuged (4°C, 3000 *g*, 10 min) the organic layer was aspirated and discarded. Three milliliters of the remaining aqueous layer were transferred to a conical glass tube with PTFE-lined screw-cap and adjusted to pH 7.0 ± 0.05 with aqueous saturated tribasic sodium phosphate. Then 5 ml of diethyl ether were added to this tube and the whole was mixed for 15 min. At the end of this period, the mixture was centrifuged (4°C, 1200 *g*, 10 min). A 3-ml volume of the organic layer was taken to dryness under a gentle stream of nitrogen at 30°C.

Preparation of standard curves

With each set of rat brain samples a standard curve was prepared adding 1 ml of APM · HCl solution (0.05, 0.1, 0.3, 0.5, 1.0, 3.0, and 5.0 μg in 1 ml of 0.45 *M* PCA solution), 1 ml of PNAPM · HCl solution (10 μg in 1 ml of 0.45 *M* PCA solution) and 3 ml of 0.45 *M* PCA solution to a drug-free whole rat brain. The standard samples were extracted in the same way as described for whole rat brain samples.

Preparation of derivatives

BSTFA (100 μl) was added to the dried extract, and the mixture was

incubated at 30°C for 60 min. The residual BSTFA was removed in a stream of nitrogen at 30°C, and the dried residue was dissolved in 1 ml of benzene. Of this solution 3 μl were injected onto the gas chromatograph—mass spectrometer.

Gas chromatography—mass spectrometry

Derivatized samples were chromatographed on a silanized glass column (1 m × 2 mm I.D.) packed with 3% OV-17 on silanized Gas-Chrom Q (80—100 mesh, packed by Shimadzu Co., Kyoto, Japan). The column was maintained at 250°C and the injector port at 300 °C. Helium was used as the carrier gas and its flow-rate was set at 25 ml/min.

A Shimadzu AUTO GCMS-6020 fitted with a Shimadzu PAC-500 data system was used. The jet separator was maintained at 300°C. For recording mass spectra the mass spectrometer was operated in the scan mode at an electron energy of 20 eV and 70 eV with an ionization current of 60 μA, scan speed from *m/z* 0 to 800 in 7 sec, and an accelerating voltage setting of 3.5 kV.

Selected ion monitoring

For SIM a JMS-D300 (JEOL, Tokyo, Japan) was used. The instrument was run in the SIM mode at an ionization energy of 20 eV with 300 μA of emission current and an electron multiplier setting of 1.4 kV. The selected ion detector channels were set at *m/z* 411 and 439. These are the molecular ions and the base peaks in the mass spectra of the TMS derivatives of APM (APM derivative) and PNAPM (PNAPM derivative), respectively, at an ionization energy of 20 eV with 300 μA emission current (Fig. 1). Slits were adjusted to a resolution of approximately 500, and filters were set to a peak width of 3 Hz.

Accuracy, precision and sensitivity

Solutions of APM · HCl at 0.05, 0.5 and 4.0 μg/ml and a solution of PNAPM · HCL at 10 μg/ml in 0.45 *M* PCA were prepared. One milliliter of each solution of APM · HCl, 1 ml of the solution of PNAPM · HCl and 3 ml of 0.45 *M* PCA solution were added to a drug-free whole rat brain. The standard samples were analyzed in the same manner as described above. Each sample was analyzed in quintuplicate.

Application of the method to the APM disposition study

Female Wistar rats, weighing 110—140 g, were sacrificed at 5, 10, 20, 30, 90, and 150 min following subcutaneous administration of 10 mg/kg APM · HCl. Five rats were decapitated at each time interval and brain samples were stored at —20°C until analyzed.

APM concentrations in whole rat brain samples were determined by the method as described.

RESULTS

The TMS derivatives of APM and PNAPM have retention times of 3.12 and 4.15 min, respectively, on the 3% OV-17 column (250°C). The mass spectrum of the APM derivative recorded at 20 eV showed a base peak at *m/z* 411 corre-

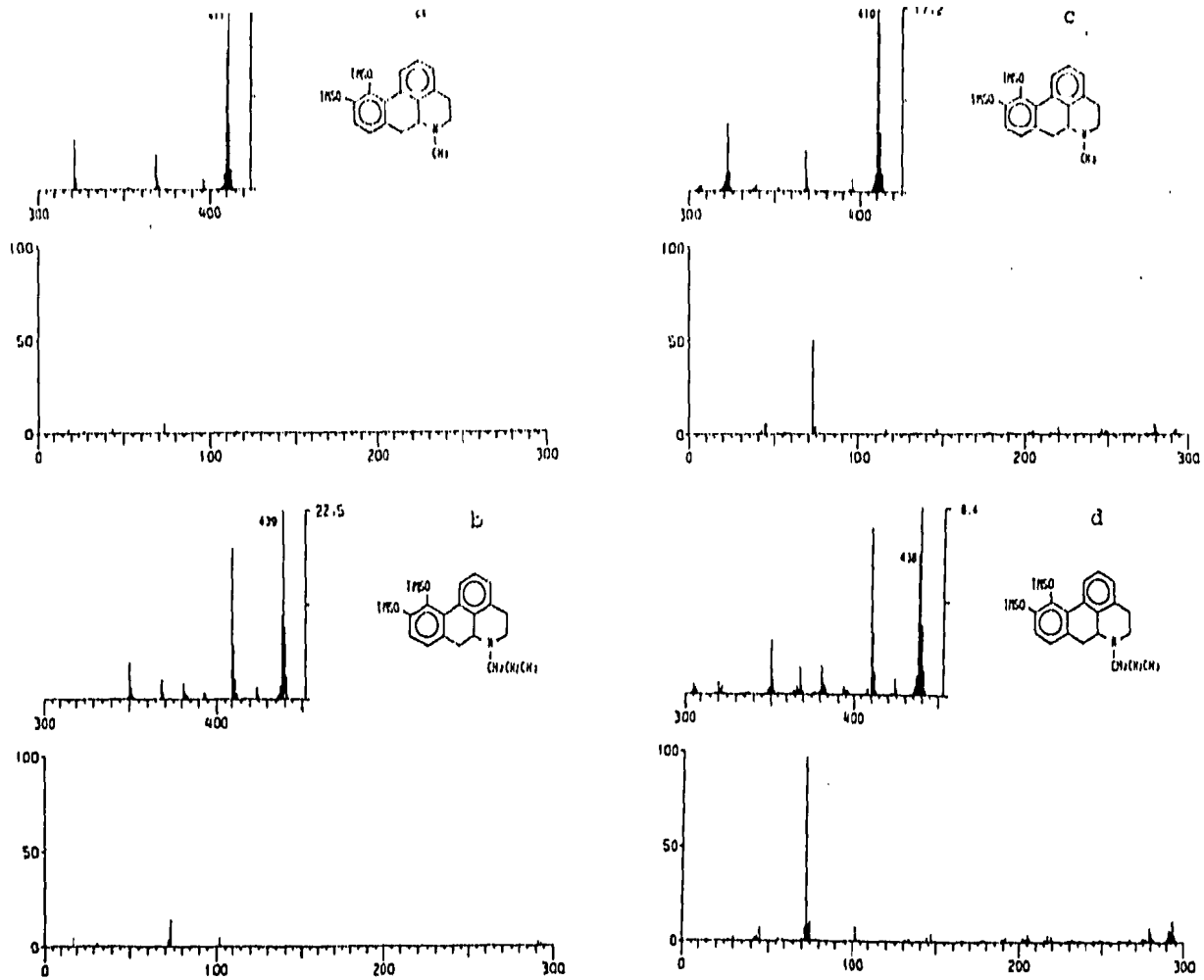

Fig. 1. Mass spectra of the TMS derivatives of APM and PNAPM (a and b recorded at 20 eV, c and d at 70 eV). The number at the right-hand side of each spectrum gives the ratio of the peak height of the ion shown in each spectrum to that of the total amount of ions.

sponding to the molecular ion and strong peaks occurring at *m/z* 410 (75.5%), 368 (18.7%) and 322 (27.0%) (Fig. 1a). The fragment ions at *m/z* 410 and *m/z* 368 presumably arise from losses of $H^{\cdot}$ and $CH_2 = N{-}CH_3$ groups, respectively, from the molecular ion. The fragment ion at *m/z* 322 results from the elimination of $Si(CH_3)_4$ from the *m/z* 410 fragment ion. As recorded at 70 eV, the mass spectrum of the APM derivative showed a base peak at *m/z* 410 with a strong molecular ion (*m/z* 411, 91.9%) (Fig. 1c). The fragment ions at *m/z* 368 (22.7%) and *m/z* 322 (36.8%) also appeared (Fig. 1c). The mass spectrum of the PNAPM derivative is characterized by a base peak at *m/z* 439, corresponding to the molecular ion, and strong fragment ions at *m/z* 438 and *m/z* 410 (Fig. 1b and d).

For the purpose of an ion selection employed in the mass fragmentography, a comparison of sensitivity was made by monitoring both the *m/z* 411 ion at 20 eV and the *m/z* 410 ion at 70 eV. These were the base peaks of the APM derivative as recorded at 20 eV and 70 eV, respectively. It was found that the recording of the *m/z* 411 ion at 20 eV was approximately two times more sensitive than the recording of the *m/z* 410 ion at 70 eV. The effect of ionization energy on the peak intensity at *m/z* 411 was also studied. The most intense peak was obtained by monitoring at 20 eV. Therefore, the quantitative assay was performed to record, in the SIM mode, the molecular ions at *m/z*

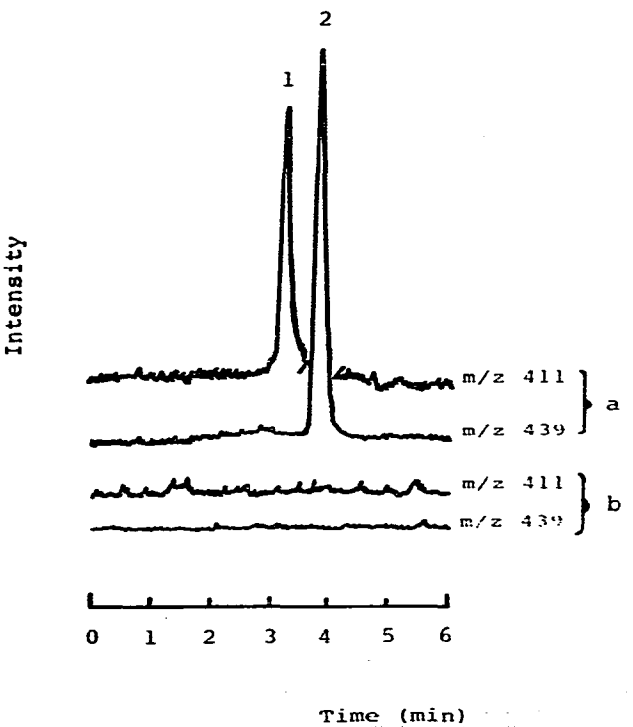

Fig. 2. Selected ion recordings of the ions at *m/z* 411 and *m/z* 439 for (a) the TMS derivatives of extracts of whole rat brain to which were added 0.2 μg/ml APM · HCl and 10 μg/ml PNAPM · HCl, and (b) materials extracted from drug-free whole rat brain. Peaks 1 and 2 correspond to the TMS derivatives of APM and PNAPM, respectively.

411 (APM derivative) and *m/z* 439 (PNAPM derivative, as an internal standard) with an ionization energy of 20 eV. The SIM profile of the TMS derivatives of APM and PNAPM in whole rat brain are shown in Fig. 2 together with the profile of the drug-free whole rat brain extracts. As shown in Fig. 2, no peaks interfering with the monitoring of the ions at *m/z* 411 and *m/z* 439 were present.

TABLE I

ANALYSIS OF REPLICATE SAMPLES, TO WHICH KNOWN AMOUNTS OF APM - HCl WERE ADDED

Added (μg/ml)	Found (μg/ml)	
	Mean ± S.D. ($n = 5$)	C.V. (%)
0.05	0.050 ± 0.006	12.0
0.50	0.504 ± 0.020	3.97
4.00	4.045 ± 0.102	2.52

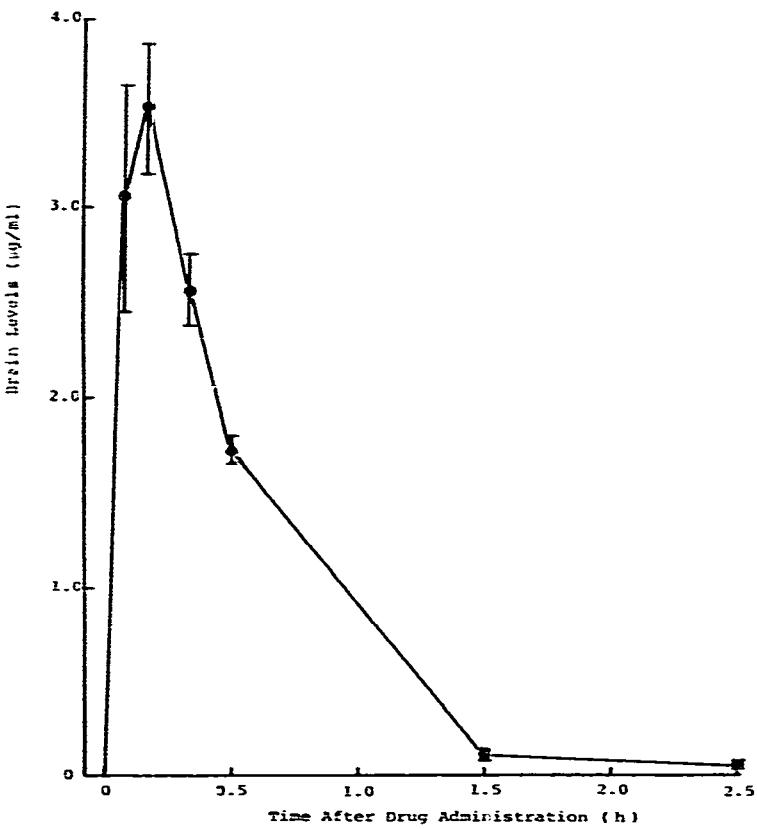

Fig. 3. Concentrations of APM in whole rat brain during 2.5 h after subcutaneous administration of 10 mg/kg APM - HCl. Each point represents the mean ± S.E.M. of five samples.

The standard curve obtained was $y = 2.065x + 0.005$ ($r = 0.999$; $p < 0.01$) and was linear over the concentration range 0.05—5.0 μg/ml APM · HCl.

The accuracy, precision and sensitivity of the method were determined by analysing samples of drug-free whole rat brain to which APM · HCl had been added over the range 0.05—4.0 μg/ml. The results are shown in Table I. The lower limit of detection of the assay was judged to be 0.05 μg/ml, as added APM · HCl concentration to whole rat brain, in which signal intensity was twice as high as the noise value.

As an application of the method to an APM disposition study, a time course of whole rat brain APM levels following subcutaneous administration of 10 mg/kg APM · HCl was examined. The results are shown in Fig. 3. The peak brain APM concentration occurred at 10 min (3.562 ± 0.338 μg/g tissue, mean ± S.E.M.) and then the level of APM declined exponentially. Only a trace amount was detectable at 150 min (0.058 ± 0.016 μg/g).

DISCUSSION

When we re-examined the extraction procedure suggested by Kaul et al. [16] for the estimation of APM in brain tissue, formation of an emulsion and a reduced recovery of APM due to adsorption of the compound on the emulsion were occasionally observed. To remove these defects, we attempted to employ an extraction procedure with 0.45 *M* PCA solution as an extraction medium for APM in the rat brain. No formation of emulsion was observed and satisfactory results were obtained using the extraction procedure indicated above. The acidic extracts obtained were clean enough to permit the estimation of brain APM levels by SIM. No compound interfering with the monitoring of the ions at *m/z* 411 and *m/z* 439 was present in the acidic extracts of drug-free whole rat brain.

PNAPM, a chemical analogue of APM, proved to be satisfactory for use as an internal standard. The similar chemical properties of APM and PNAPM permit their co-extraction and derivatization in adequate yield.

Since the silylation reaction of APM with BSTFA was simple and proceeded at a relatively low temperature (30°C), no decomposition products were found. The silylation reaction time seems to affect the peak intensity of the ion at *m/z* 411. Therefore, the effect of reaction time on the peak intensity of the ion at *m/z* 411 monitored at 20 eV was examined. APM was reacted with BSTFA for 0.33, 0.5, 1, 1.5, 2, and 21 h at 30°C. A constant peak intensity was obtained after 0.5 h.

Since the peak intensity of the ion at *m/z* 411 was also influenced by the ionization energy, the effect of the ionization energy was examined. The most intense peak was obtained when monitoring at 20 eV. Thus, the ionization energy of 20 eV was employed throughout the experiments described in this paper.

Spectrofluorometric methods, which have been used for determining APM concentrations in biological materials [16—18], lack adequate specificity and sensitivity for APM disposition studies in whole rat brain. The selected ion monitoring method is much more sensitive and specific to APM than spectrofluorometric methods.

The range of values obtained for whole rat brain APM concentrations following subcutaneous administration of 10 mg/kg is reasonable when compared with other published data [14] (Fig. 3). As shown in Fig. 3, it is suggested that this selected ion monitoring method is sensitive enough to allow determination of APM concentrations in whole rat brain following an APM · HCl injection at a dosage of less than 10 mg/kg.

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