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DETERMINATION OF APOMORPHINE IN PLASMA AND BRAIN TISSUE BY ION-PAIR EXTRACTION AND LIQUID CHROMATOGRAPHY

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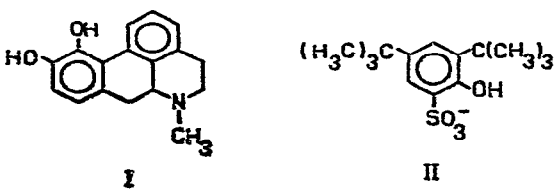
SUMMARY

Apomorphine is extracted from plasma or tissue homogenate with ethyl acetate. After back-extraction into hydrochloric acid, the apomorphine is extracted as an ion pair with 3,5-di-*tert.*-butyl-2-hydroxybenzene sulphonate into a small volume of methylene chloride and the solution is injected into the chromatographic column. Apomorphine is separated on microporous silica with a mixture of aqueous perchloric acid, methanol and methylene chloride as the mobile phase. With absorbance measurement of the eluent at 254 nm the method permits the determination of 15 pmol of apomorphine in 1 ml of plasma or in a rat brain. The coefficient of variation was 4% at the 100 pmol level.

INTRODUCTION

Apomorphine and related aporphines are dopamine receptor agonists and for several years have attracted much interest in the neurophysiological research field. The drug shows pharmacological activity even at low concentrations but so far the possibilities of measuring the apomorphine content in blood, plasma and tissues have been limited, as the available assay methods suffer from poor selectivity and sensitivity.

Apomorphine is a tertiary amine with a catechol function (Fig. 1) and exhibits high absorbance and fluorescence. Like other catecholamines it is sensitive to oxidation, which has to be considered in the sample preparation procedure.



I II

Fig. 1. Structural formulae of apomorphine (I) and BPS (II).

Different techniques have been used for the assay of apomorphine in biological materials. Fluorimetry[1,2] has most often been the method of choice, but in the last few years gas chromatography with flame-ionization[3,4] or electron-capture detection[5] has been suggested. Recently, reversed-phase liquid chromatography has been employed with electrochemical[6] or UV absorption detection[7].

This paper describes a method for the determination of apomorphine in plasma and brain tissue. Apomorphine is isolated from the biological material and brought into a small volume of an organic phase by a solvent extraction procedure. The drug is then separated from co-extractants by liquid chromatography on silica gel and quantified by UV absorbance measurement at 254 nm. Major efforts have been made to develop an optimal sample preparation procedure based on solvent extraction systems. The liquid chromatographic studies have been restricted to systems with mobile phases containing acids as ion-pairing agents.

EXPERIMENTAL

Reagents and materials

All solvents, buffer components and other chemicals were of analytical-reagent grade.

Glutathione (reduced), crystalline (GSH), was obtained from Sigma (St. Louis, Mo., U.S.A.). Apomorphine hydrochloride was of pharmacopoeial grade. 3,5-Di-*tert*.-butyl-2-hydroxybenzene sulphonate (BPS) was supplied as the potassium salt by the Department of Organic Chemistry, AB Haessle[8] (see Fig. 1) and a 0.01 *M* solution was prepared by dissolving 1.6 g of the potassium salt in 500 ml of distilled water. The EDTA solution consisted of 10 g of the disodium salt in 100 ml of 0.25 *M* sodium hydroxide solution (pH 7) (prepared freshly once a month). The ascorbic acid solution consisted of 0.2 g of ascorbic acid in 100 ml of distilled water (pepared freshly every day). A phosphate buffer solution was prepared by mixing 85 ml of 0.5 *M* disodium hydrogen orthophosphate solution with 15 ml of 1 *M* sodium dihydrogen orthophosphate solution.

Collection of samples

Blood samples are collected in cold tubes containing heparin and solid reduced GSH (5 m*M*). The tubes are inverted, cooled in an ice-bath and the plasma is separated as soon as possible by centrifugation at 4° and frozen at −70°.

Rat brains are rapidly removed following decapitation and immediately frozen at −70°.

Sample preparation

Plasma. A 1-ml volume of plasma in a 4-ml centrifuge tube on an ice-bath is stabilized with 0.1 ml of EDTA solution and 0.05 ml of ascorbic acid solution. After addition of 0.5 ml of phosphate buffer solution, the aqueous phase is extracted with 2.00 ml of ethyl acetate. All extractions are made by mechanical shaking for 10 min followed by centrifugation for 5–10 min. After centrifugation, 1.50 ml of the ethyl acetate phase is transferred into another centrifuge tube and apomorphine is back-extracted into 1.00 ml of 0.1 *M* hydrochloric acid. After centrifugation, 0.75 ml of the aqueous phase is mixed with 0.75 ml of 0.01 *M* BPS solution in a conical tube and

apomorphine is re-extracted in 250 μl of methylene chloride. A 250-μl volume of the organic phase is injected into the chromatographic column.

Rat brain. The rat brain is homogenized in 2.5 ml of 0.1 *M* hydrochloric acid after addition of 0.4 ml of EDTA solution and 0.4 ml of ascorbic acid solution. The homogenization is performed in a centrifuge tube on an ice-bath by an Ultra Turrax, which is washed with 1 ml of water, which finally is combined with the homogenate. After adjusting the pH to about 7 by addition of 3 ml of phosphate buffer, apomorphine is extracted with 8.00 ml of ethyl acetate. After centrifugation, 6 ml of the organic phase is back-extracted with 2.00 ml of 0.1 *M* hydrochloric acid. After centrifugation, 1.5 ml of the aqueous phase is mixed with 1.5 ml of 0.01 *M* BPS solution and apomorphine is extracted with 250 μl of methylene chloride. A 250-μl volume of the organic phase is injected into the chromatographic column.

Chromatography

The liquid chromatograph consisted of an LDC Model 71147 pump, a Rheodyne microsampling valve with a 250-μl loop and a Waters 440 UV detector operated at 254 nm. The separation column was a 20 cm $\times$ 3.0 mm I.D. stainless-steel tube packed with 10-μm particles of LiChrosorb SI 100 silica gel (E. Merck, Darmstadt, G.F.R.). The chromatograph was placed in a room thermostated at 22 $\pm$ 1°.

The mobile phase was a mixture of 1 *M* perchloric acid, methanol and methylene chloride (0.4:4:95.6). A flow-rate of 1 ml/min was used, giving a retention time of about 4 min for apomorphine. The samples were injected with an interval of about 10 min.

Calibration and accuracy

Calibration was effected by adding known amounts of apomorphine to plasma and brain tissue homogenate and taking these standards through the assay procedure. The peak heights for apomorphine in the chromatograms were measured and a calibration graph was plotted which was used to calculate the drug concentration in authentic samples. The reproducibility of the method was evaluated by analysing replicate samples of plasma and tissue homogenate spiked with 100 pmol of apomorphine. A standard deviation of 4% was obtained with a recovery of 95% compared with an analysed pure aqueous solution of apomorphine. The theoretical recovery of the method as calculated from distribution constants and phase volume ratios was 95% and 93% for plasma and brain tissue samples, respectively.

The stability of apomorphine in plasma samples stored in a freezer at —20° was monitored. After 2 months no decrease in concentration could be seen, but 6 months after the samples had been collected there was a decline of about 10%.

The stability was also checked at the different stages of the sample preparation. It is obvious that the presence of EDTA and ascorbic acid is necessary and the samples have to be kept in an ice-bath before extraction. In the ethyl acetate extract there was no decrease in apomorphine concentration after 30 min. In the final extract, where apomorphine is present as a BPS ion pair in methylene chloride, no losses were seen even after 24 h, provided that the samples were stored in the dark prior to injection. Without this precaution there was a decrease of more than 10% in a day.

RESULTS AND DISCUSSION

Extraction

Protein precipitation often precedes the extraction of catecholamines from plasma or tissue samples. This procedure works well for adrenaline, noradrenaline and other hydrophilic compounds, but for apomorphine the recovery was lowered significantly owing to losses in the precipitate[2]. Therefore the extraction had to be made from an intact sample; similar observations were reported earlier for other moderately hydrophobic ammonium compounds.[9].

Different solvent extraction systems were evaluated in order to accomplish the isolation of apomorphine from the biological material. The distribution constants are summarized in Table I. BPS has earlier been used as an efficient ion-pairing agent for the extraction of acetylcholine from deproteinizated rat-brain samples into methylene chloride[10]. The same ion-pair extraction system gave low recoveries of apomorphine, probably owing to interactions between the hydrophobic BPS anion and the proteins still present in the samples.

TABLE I

DISTRIBUTION AND ION-PAIR EXTRACTION CONSTANTS OF APOMORPHINE

Aqueous phase	*Organic phase*	*Log K_D (K_{ex})*
Buffer, pH 7.4	Ethyl acetate	1.7
Perchlorate	Ethyl acetate	1.6
BPS	Methylene chloride	4.6

The second ion-pair system that was tested, with perchlorate as the counter ion and ethyl acetate as the organic solvent, was useful. The extractions were made at pH 5–6 and the same conditions could be used for plasma samples from different species. A recovery of about 85% was obtained compared with a theoretical recovery of 95% for this extraction. Extraction of apomorphine in an uncharged form is chosen in the method, as a 10% higher recovery was obtained compared with the perchlorate system. Maximal and constant extraction is obtained between pH 7.0 and 7.6 and the pH should be checked when starting with samples from a new animal species.

The volume of the extract has to be reduced before injection. Owing to the limited stability of apomorphine, evaporation to dryness is too risky and an extraction procedure is to be preferred. Apomorphine is back-extracted into hydrochloric acid, and is finally extracted into a small volume of methylene chloride as a BPS ion pair. This extraction is efficient despite the unfavourable phase volume ratio. Less than 3% of apomorphine is not extracted. The final volume of the methylene chloride phase is increased to some extent by extraction of ethyl acetate, which is compensated for by the standard samples.

Liquid chromatography

Liquid chromatography on regular microporous silica was found to give highly efficient and extremely stable separation systems for the analysis of antiarrhythmic drugs in plasma samples[11]. Ammonia solution or perchloric acid was added to the organic mobile phase in order to produce different separation characteristics. The

method could then be restricted to a single extraction and direct injection of an aliquot of the methylene chloride extract. In this study six different acids were compared with respect to the retention behaviour of apomorphine, the capacity factors being listed in Table II. The three inorganic acids gave the lowest capacity factors, perchloric acid showing the shortest retention. The order between the inorganic acids is the same as would be expected for a true liquid–liquid system. The column efficiency was also measured and was of the same magnitude for all six acids. So far the retention mechanism for this kind of chromatographic system has not been studied very much and is not well understood. The solute is probably both retained and migrating as an ion-pair.

TABLE II

CAPACITY FACTORS (k') FOR APOMORPHINE

Packing material: LiChrosorb SI 100, 10 μm. Mobile phase: 1 *M* acid–methanol–methylene chloride (0.4:8:91.6).

Acid	k'
Perchloric	1.19
Nitric	3.19
Hydrochloric	3.95
Trichloroacetic	6.24
Methanesulphonic	7.81
Monochloroacetic	9.57

In addition to apomorphine and other extracted components, the injected sample contains a large excess of BPS which has to be well separated from apomorphine. While keeping the perchloric acid concentration constant, the methanol content of the mobile phase was varied, the capacity and separation factors being measured (Table III). With 12% methanol, apomorphine and BPS are eluted almost simultaneously but are well separated when the methanol content is decreased to 4%, the separation factor being 1.90. With this mobile phase, which is used in the method, the retention of apocodeine, the O-methylated derivative and a major metabolite of apomorphine, was determined. Apocodeine is eluted with a k' value of 0.85 and is thus easily separated.

TABLE III

CAPACITY FACTORS (k') AND SEPARATION FACTORS (α) FOR APOMORPHINE AND BPS

Packing material: LiChrosorb SI 100, 10 μm. Mobile phase: 1 *M* perchloric acid (0.4 parts) + methanol + methylene chloride (100 parts).

Methanol	$k'_{apomorphine}$	k'_{BPS}	α
4	3.40	6.45	1.90
6	1.88	2.90	1.54
8	1.30	1.58	1.22
12	0.90	0.90	1.00

Detectors

In this method the eluent was monitored with a fixed-wavelength UV detector at 254 nm. Later studies showed that measurement at the absorbance maximum, 270 nm, gave about a 50% higher detector response. The size of the BPS peak also increased and the signal-to-noise ratio was not much improved. A fluorescence detector gave about double the signal-to-noise ratio and the BPS peak was as large as in UV detection at 254 nm. The electrochemical detector should give a response superior to that obtained by spectrometric detectors.

Analysis of biological samples

Apomorphine was analysed in plasma and brain tissue after administration to rats of 50 mg/kg orally and 0.5 mg/kg subcutaneously (Figs. 2–4). Samples were collected 20 min after administration; blood samples were taken both from an artery and from the portal vein. The results being presented in Table IV.

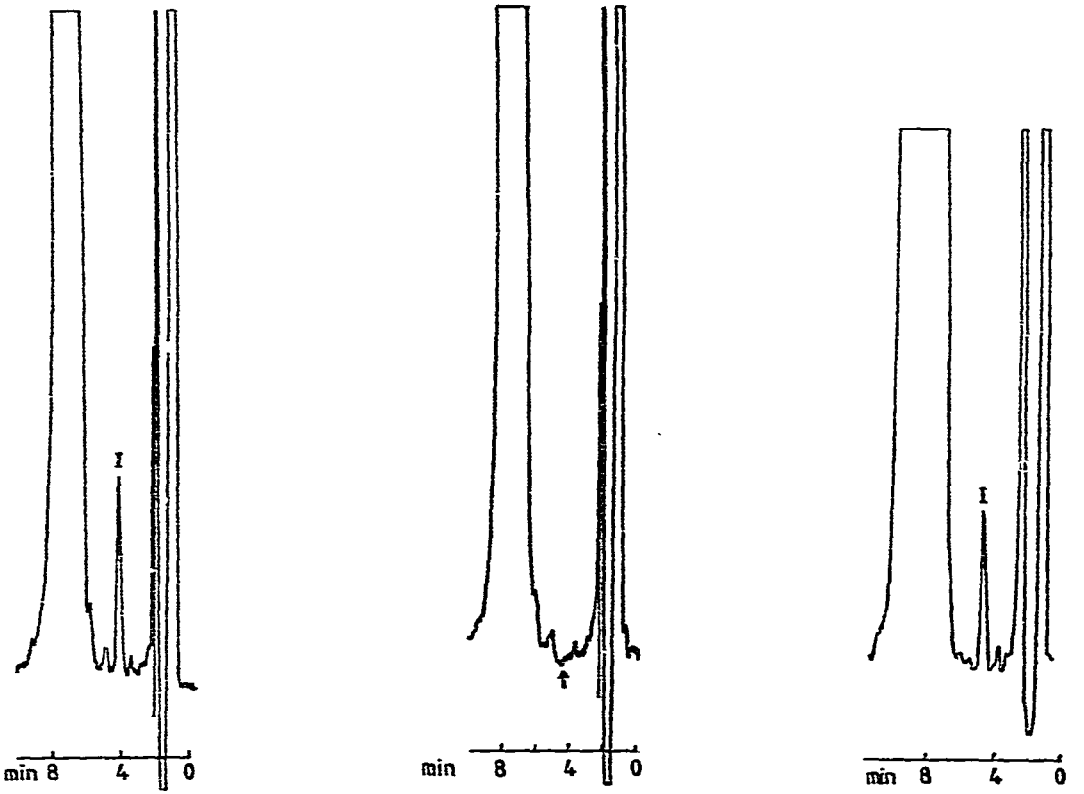


Fig. 2. Chromatogram of extract from 1 ml of plasma containing 100 pmol of apomorphine (I). 0.005 a.u.f.s.

Fig. 3. Chromatogram of extract from blank plasma. 0.005 a.u.f.s.

Fig. 4. Chromatogram of extract from a rat brain containing 200 pmol of apomorphine (I). 0.01 a.u.f.s.

TABLE IV

APOMORPHINE IN RAT BRAIN AND PLASMA

Samples collected 20 min after administration. Results are mean values ± standard deviation ($n = 4$).

Administration	*Dose (mg/kg)*	*Plasma (pmol/ml)*		*Brain (pmol/g)*
		Artery	*Portal vein*	
Oral	50	~4	1050 ± 640	96 ± 36
Subcutaneous	0.5	95 ± 30	43 ± 19	1100 ± 23

After oral administration, much higher plasma concentrations of apomorphine were found in the portal vein than in the artery, indicating a high first pass effect of the drug. This effect is avoided by subcutaneous injection and, as shown by the data presented, apomorphine is strongly accumulated in the brain.

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