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Note

Determination of apomorphine in rat plasma and brain by high-performance liquid chromatography with electrochemical detection

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Several analytical methods for the determination of apomorphine in biological samples, including gas chromatographic [1, 2], spectrophotometric [3], fluorimetric [4], radioenzymatic [5] and radiochemical [6] assay, have been described.

In recent years high-performance liquid chromatographic (HPLC) methods for apomorphine have been employed using ultraviolet [7—9] or fluorimetric detection [10]. HPLC with electrochemical detection (ED) has also been employed for measuring brain levels of apomorphine [11]. This procedure shows greater sensitivity but has not been extended to biological fluids.

This paper describes a simple and sensitive HPLC—ED method for the detection of apomorphine in plasma and brain (striatum) with detection limits of 0.3 ng/ml and 3 ng/g, respectively. The method has been used to investigate apomorphine pharmacokinetics in rats.

MATERIALS AND METHODS

Reagents and materials

Apomorphine hydrochloride was kindly supplied by Sandoz (Basel, Switzerland). All solvents, buffer components and other chemicals were analytical-reagent grade or better and used as received. Aqueous standard solutions of apomorphine (1 μg/ml) and ascorbic acid (2 mg/ml) were freshly prepared every day. The EDTA solution was 100 g/l in 0.25 *M* sodium hydroxide (final pH 7).

High-performance liquid chromatography

The HPLC system, composed of a Hewlett-Packard 1084 B liquid

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chromatograph and controlled by a computer terminal (HP 7985 B LC model), was used in conjunction with an electrochemical detector. The electrochemical cell (TL-5, Bioanalytical System, Lafayette, IN, U.S.A.) contained a glassy carbon working electrode, a stainless-steel auxiliary electrode and an Ag/AgCl reference electrode. The working electrode was maintained at an applied potential of +0.77 V versus Ag/AgCl. Separation was achieved with a μBondapak CN reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc.) and the mobile phase was acetonitrile—0.02 *M* potassium dihydrogen phosphate (11:89) containing 0.5 m*M* EDTA adjusted to pH 3 with phosphoric acid. The mobile phase was filtered through a Millipore filter (0.4 μm) and degassed before use. The flow-rate was set at 1 ml/min.

Animals

Male CD-COBS rats (Charles River, Como, Italy), average weight 200 g, were used.

Collection of samples

Blood samples were collected in heparinized tubes; plasma was obtained by centrifugation. Ascorbic acid solution was added (0.05 ml/ml of plasma) and the samples were stored at −25° C until assayed. Rat brains were dissected by the method of Glowinski and Iversen [12]; striatum was removed, immediately frozen on solid carbon dioxide and stored as for plasma.

Extraction procedure

Plasma. To 1 ml of plasma in a plastic centrifuge tube 0.1 ml of EDTA solution was added; the pH was controlled or adjusted to between 7 and 7.5 using a pH meter. Apomorphine was successively extracted with 2 ml of ethyl acetate.

After centrifugation (1500 *g*, 5 min) 1.6 ml of the organic layer were transferred into another tube and apomorphine was back-extracted with 0.4 ml of 0.1 *M* hydrochloric acid. After shaking and centrifugation, 0.37 ml of the aqueous phase was transferred into an eppendorf vial and 10—200 μl were injected into the HPLC system.

Brain. After addition of 0.15 ml of EDTA and ascorbic acid solution, tissue (about 100 mg) was homogenized in 0.7 ml of 0.1 *M* hydrochloric acid using an Ultra Turrax. The Ultra Turrax was then washed with 0.7 ml of 0.1 *M* hydrochloric acid which was combined with the homogenate. After adjustment of the pH to 7.0—7.5 with 1 *M* tripotassium phosphate, the sample was processed as indicated for plasma. All operations described were performed at about 5° C.

Calibration and quantification

Calibration was effected by adding known amounts of apomorphine to plasma and brain tissue, and processing the samples as described above.

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 unknown samples.

RESULTS AND DISCUSSION

Detector response

Fig. 1 shows the change in response current against applied potential (current—potential curve) for apomorphine. The onset potential of apomorphine oxidation was about 0.4 V and the detector response increased up to (at least) 0.85 V.

The applied potential selected in this study was 0.77 V because a higher detector potential resulted in a greater noise level and also in lower specificity of the detector [13].

The glassy carbon electrode produces a linear response using a range of 0.1—100 ng of apomorphine injected.

The electrochemical detector shows a slight loss of sensitivity over a day's chromatographic runs ($\leqslant$ 10%), probably due to build-up of oxidation products of apomorphine on the working electrode surface [14]. The performance of the detector was partially restored by overnight delivery of the mobile phase through the chromatographic system. However, in our conditions the electrode was used for about 200 determinations of apomorphine in tissue extracts or body fluids, after which it was polished with alumina.

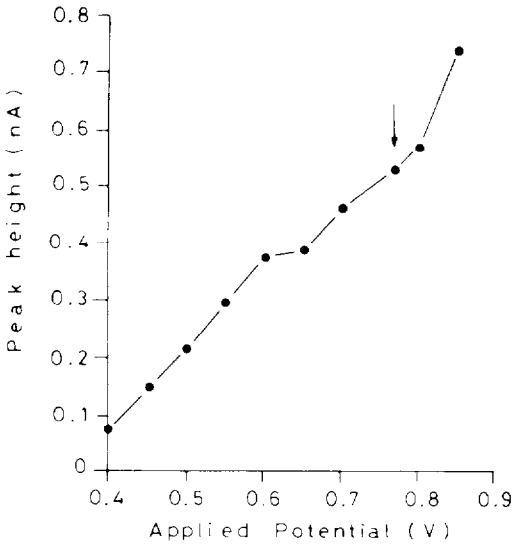

Fig. 1. Current—potential curve for apomorphine (3.5 ng injection). The arrow shows the applied potential used in the present study (0.77 V).

Chromatography

Apomorphine is usually analysed using mobile phases containing a high percentage of organic solvents [7, 10]. However, a high concentration of organic solvent is not, in general, suitable for electrochemical reactions [13]. The use of a relatively polar column, such as a CN column, permits reduction of the organic component in the mobile phase without increasing retention time. Apomorphine separation on a CN reversed-phase column with 11% acetonitrile in pH 3 phosphate buffer made for rapid analysis with symmetrical peaks.

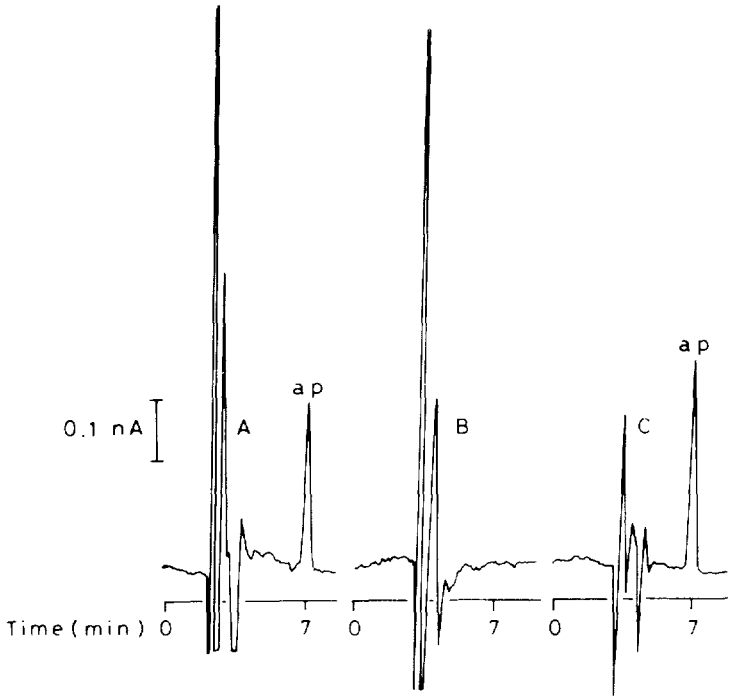

Fig. 2. Chromatograms from a striatum sample containing 10 ng of apomorphine (ap) (A), from drug-free striatum (B) and from striatum of rats treated with apomorphine (C).

Fig. 2 shows typical chromatograms of extracts from (A) striatum to which 10 ng of apomorphine were added, (B) drug-free striatum, and (C) striatum of a rat treated with apomorphine. The extract from untreated striatum shows no peaks that could interfere with drug analysis.

Under the chromatographic conditions specified, apomorphine eluted at 7 min; thus, a sample can be injected every 9 min.

TABLE I

RECOVERY AND LINEARITY OF APOMORPHINE FROM PLASMA (1 ml) AND BRAIN TISSUE (100 mg WEIGHT)

Each value is the mean of four determinations.

Sample	Amount added	Recovery (%) ± S.D.	Apomorphine peak height (nA per 100-μl injection) ± S.E.M.
Plasma	1	74.8 ± 7.2	0.025 ± 0.003
	3	77.8 ± 3.4	0.076 ± 0.003
	10	80.4 ± 1.5	0.261 ± 0.006
	30	84.8 ± 3.9	0.815 ± 0.028
	100	81.8 ± 2.8	2.654 ± 0.089
Brain	1	79.8 ± 2.5	0.026 ± 0.001
	3	85.6 ± 6.3	0.077 ± 0.005
	10	78.2 ± 2.9	0.249 ± 0.010
	30	84.2 ± 1.6	0.806 ± 0.004
	100	82.4 ± 2.0	2.680 ± 0.050

Recovery, linearity and sensitivity of the method

Apomorphine was well extracted from plasma or brain tissue at pH 7—7.5 with ethyl acetate [9], and the back-extraction into a small volume of hydrochloric acid gave a clean chromatogram with good recovery.

The linearity and recoveries (corrected for solvent losses) of apomorphine obtained by this method are summarized in Table I.

The calibration curves of peak height versus amount of apomorphine added to the samples were linear over the concentration range 1—100 ng of apomorphine base ($y = 0.0266x + 0.0008$, $r = 0.99996$ for plasma; $y = 0.0269x - 0.0062$, $r = 0.99997$ for brain tissue). The mean recovery was 80.3% with a coefficient of variation (C.V.) between 1.9% and 9.6% for plasma, and 82% with a C.V. between 1.9% and 7.4% for brain tissue.

The detection limit (signal-to-noise ratio ca. 4) was 0.3 ng/ml for plasma and 3 ng/g for striatum (about 100 mg weight).

Animal studies

In order to obtain information on the kinetic profile of apomorphine, male rats were given an intraperitoneal injection of apomorphine hydrochloride (5 mg/kg), and plasma and striatum were analysed as described.

Fig. 3 shows the time course of plasma and striatum concentrations for the drug. Apomorphine in plasma rose rapidly to a peak (0.31 μg/ml) within 10 min, after which the peak disappeared with an apparent half-life ($t_{½}$) of 9.65 min. Apomorphine levels in striatum were higher than in plasma, the maximum concentration being reached after 10 min (1.92 μg/g) declining thereafter with a $t_{½}$ comparable to that of plasma. The area under the curve (AUC) for apomorphine in striatum was approximately eight times that of the plasma AUC (see Table II), confirming that apomorphine concentrates in this tissue [5, 11, 15].

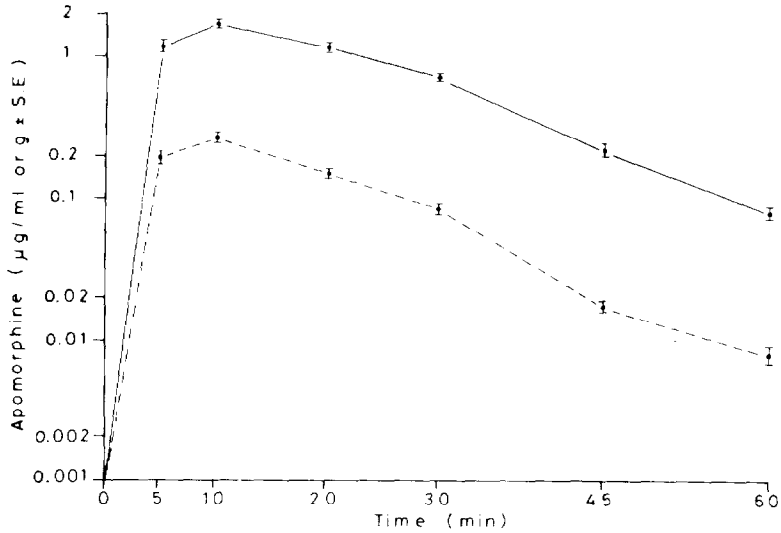

Fig. 3. Plasma (- - -) and striatum (——) concentration—time curves for apomorphine after administration of apomorphine hydrochloride (5 mg/kg, intraperitoneally) to rats. Each point is the mean ± S.E. of four animals.

TABLE II

PEAK CONCENTRATIONS (C_{max}), HALF-LIVES ($t_{1/2}$) AND AREA UNDER THE CURVE (AUC) FOR APOMORPHINE IN PLASMA AND STRIATUM OF RATS TREATED WITH APOMORPHINE HYDROCHLORIDE (5 mg/kg INTRAPERITONEALLY)

Compartment	C_{max} (μg/ml or μg/g)* ± S.E.M.	$t_{1/2}$** (min)	AUC*** (μg/ml or μg/g × min)
Plasma	0.31 ± 0.02	9.65	6.15
Striatum	1.92 ± 0.09	11.07	51.51

*Observed values.
**Plasma and striatal $t_{1/2}$ values were calculated assuming a one-compartment open model.
***AUC was calculated by the trapezoidal rule and extrapolated to infinity.

The $t_{1/2}$ values were obtained assuming a one-compartment model, but our data do not exclude the presence of a β-phase after the time considered, as reported in mice by Burkman et al. [16].

CONCLUSION

The present HPLC—ED method appears specific and sensitive; it shows good recovery and precision and thus does not necessarily require the use of an internal standard. Chromatographic analysis is relatively rapid (9 min) and the extraction procedure does not involve a time-consuming evaporation step (too risky because apomorphine has limited stability).

The procedure has proved particularly useful for pharmacokinetic studies of apomorphine in laboratory animals. A trained technician can analyse 40 samples of biological material per day.

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