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Note

Rapid method for the determination of apomorphine in plasma using high-performance liquid chromatography

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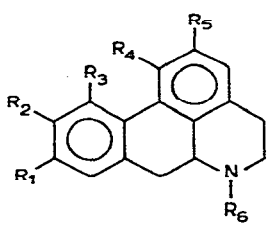
The efficacy of apomorphine (I) and N-*n*-propylnorapomorphine (II) as anti-Parkinsonism agents[1] has stimulated the development of methods for their quantitation in biological media[2–4]. Previously reported high-performance liquid chromatographic (HPLC)[2] and gas chromatographic (GC) analyses[3,4] are limited by their extraction or derivatization procedures, relatively long analysis times, and large sample size requirements for adequate sensitivity. In addition, the HPLC method[2] uses II as an internal standard, which is not readily available commercially and requires careful handling to prevent oxidation similar to that of I. We have developed an HPLC procedure which employs an easily obtainable internal standard, boldine (III). The newly devised method is also more rapidly performed yet possesses similar accuracy and precision.



I: $R_1, R_4, R_5 = H$; $R_2, R_3 = OH$; $R_6 = CH_3$
II: $R_1, R_4, R_5 = H$; $R_2, R_3 = OH$; $R_6 = C_3H_7$
III: $R_1, R_5 = OH$; $R_2, R_4 = OCH_3$; $R_3 = H$; $R_6 = CH_3$
IV: $R_1, R_4, R_5 = H$; $R_2 = OCH_3$; $R_3 = OH$; $R_6 = CH_3$
V: $R_1, R_4, R_5 = H$; $R_2 = OH$; $R_3 = OCH_3$; $R_6 = CH_3$

MATERIALS AND METHODS

All organic solvents were distilled-in-glass quality (Burdick & Jackson, Muskegon, Mich., U.S.A.). Water was deionized and doubly distilled from glass. Apomorphine hydrochloride hemihydrate was obtained from McFarland-Smith (Edinburgh, Great Britain) and determined to have a purity of 99% based on UV spectroscopy[5]. Apocodeine[6] (IV) and isoapocodeine[7] (V) were prepared as previously described. Boldine was obtained from ICN Pharmaceuticals, (Cleveland, Ohio, U.S.A.), and *p*-methoxyphenol from Aldrich (Milwaukee, Wisc., U.S.A.). The HPLC mobile phase consisted of methanol–acetonitrile–buffer (0.02 *M* KH_2PO_4 + 0.03 *M* citric acid, pH = 3.25, containing 0.001 *M* sodium lauryl sulfate) (36:9:55) and was filtered through Whatman GF/F glass fiber paper prior to use. HPLC

columns (μBondapak phenyl, 30 cm × 4 mm I.D., particle size 10 μm) were obtained from Waters Assoc. (Milford, Mass., U.S.A.). Standard solutions of 100, 20, and 10 μg/ml of I and 5 μg/ml of the other aporphines were freshly prepared in 0.015 *M* HCl. Standard solutions of III (10 μg/ml) and *p*-methoxyphenol (34 μg/ml, $t_R = 3.7$ min) in the mobile phase as well as III (2 μg/ml) and *p*-methoxyphenol (1 μg/ml) in acetonitrile (acidified with 1 ml 6 *M* HCl per 200 ml solution) were prepared. The stability of III in the solutions was measured as the 24-h change in the III to *p*-methoxyphenol peak-height ratios. An acetonitrile solution containing 2 μg/ml of III and 1 ml 6 *M* HCl per 200 ml was used in the standard assay procedure. Acetonitrile solutions were stored at 5° and protected from light when not in use.

Equipment

A Tracor Model 995 chromatographic pump attached to a Waters Assoc. U6K 2 ml loop injection valve and a Tracor Model 970A variable wavelength detector were used. Detection was at 273 nm using a sensitivity of 0.03 a.u.f.s. at 1 mV recorder input. A mobile phase flow-rate of 1.2 ml/min at 1700 p.s.i. was employed.

Extraction and recovery studies

Ten μl portions of standard solutions of I in 0.015 *M* HCl were made up to 40 μl with 0.015 *M* HCl and added to 250-μl amounts of mouse plasma fortified with 10 μl of a 150 m*M* ascorbic acid solution in silylated 15-ml centrifuge tubes. Final concentrations of I were in the range of 1–8 μg/ml plasma. A 750-μl portion of acetonitrile containing 3.75 μg III was added to each test sample and the mixture vortexed for 15 sec. The sample was centrifuged at 1230 *g* for 10 min and an 800-μl portion of the clear supernatant was transferred to a 1-ml silylated Reacti-vial-® (Pierce, Rockford, Ill., U.S.A.). The supernatant portion was concentrated for 15 min under a nitrogen stream at 55° to a total volume of approximately 100 μl. This residue was diluted with the mobile phase to a total volume of 200 μl prior to injecting a 40-μl portion into the chromatograph.

Peak heights for I and III were compared to those from injection of solutions prepared by substituting 250 μl of 0.015 *M* HCl for the mouse plasma in the test sample. Recoveries were calculated by comparison of peak heights of I and III in plasma samples *versus* those obtained from standard HCl solutions.

Assay procedure

A 750-μl portion of acetonitrile solution containing 1.5 μg III was added to a 250-μl plasma sample fortified with 10 μl of 150 m*M* ascorbic acid and 40 μl 0.015 *M* HCl (containing between 0.4 and 8 μg I/ml plasma, the exact concentration being unknown to the operator). The mixture was vortexed and centrifuged at 1230 *g* for 10 min. An 800-μl aliquot of the clear supernatant was transferred to a 1 ml silylated vial and was concentrated to approximately 100 μl under a nitrogen stream at 55°. This concentrate was made up to 200 μl with mobile phase and a 40-μl portion was taken for injection into the chromatograph. Standards containing 0.4, 0.8, 1.6, 2.4, and 4.0 μg of I/ml of plasma were treated in the same manner as the unknowns.

The peak-height ratio I/III was calculated for each injection. The test results were compared to a standard curve generated from the standards for each day.

RESULTS AND DISCUSSION

The separation of apomorphine, its O-methyl ether metabolites, and the internal standard, boldine was accomplished using a solvent system consisting of a methanol–acetonitrile–citrate/phosphate buffer (pH 3.25) incorporating lauryl sulfate as a counter ion[2]. As noted in Fig. 1, chromatographic analysis was completed within 17 min with boldine eluting before the other aporphines. The clean-up procedure, which removed plasma proteins by acetonitrile precipitation followed by centrifugation, produced a final solution which showed no noticeable chromatographic interference by the standard assay method. The previous technique[2] required dithionite-treated ether for extraction of a 1-ml sample and utilized only 60% of the total apomorphine extract in a final volume of 1 ml. The present procedure uses the entire acetonitrile extract in a final volume of only 200 μl, thus allowing a small plasma sample (250 μl) to be taken to achieve the same sensitivity as found in the previous method. Recoveries were consistent when ascorbic acid was added as an antioxidant to the plasma sample. It was also found necessary to evaporate excess acetonitrile from the plasma treated supernatant and to dilute with mobile phase prior to injection. Attempts to chromatograph the acetonitrile supernatant directly produced broad, non-reproducible peaks apparently due to a possible disruption of equilibrium in the column.

The absolute mean recovery of I from plasma was 93.9 $\pm$ 2.2% ($n = 8$) at levels from 1 to 4 μg/ml; recovery of III averaged 98.9 $\pm$ 3.9% ($n = 17$) for samples containing 2 μg/ml. The relatively small loss of I may be due to a constant

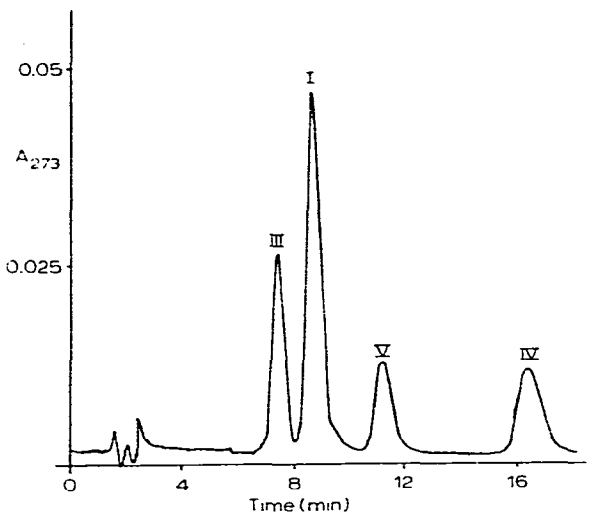


Fig. 1. Separation of aporphines on a μBondapak phenyl column with methanol–acetonitrile–phosphate/citrate buffer (36:9:55, pH 3.25) containing 0.001 *M* sodium lauryl sulfate as the mobile phase; peaks: III = boldine (0.61 μg), I = apomorphine (0.77 μg), V = isoapocodeine (0.25 μg), IV = apocodeine (0.43 μg); a 20-μl injection with detection at 273 nm, and 0.128 a.u.f.s.

fraction of standard co-precipitated with plasma proteins. The internal standard, boldine, was found to be relatively stable over a one week period, with a daily decomposition rate of less than 1% in mobile phase and about 0.8% in acidified acetonitrile (compared to *p*-methoxyphenol). The minimum detectable concentration of I was 0.4 μg/ml plasma at a signal-to-noise ratio of 3. Consistently linear calibration curves were obtained for the I/III ratio in the concentration range of 0.4 to 8 μg I/ml plasma (slope = 0.19, corr. coeff. = 0.999).

The results from blind studies, performed according to the standard assay procedure, are presented in Table I. The precision and accuracy of the method are relatively good.

TABLE I

ACCURACY AND PRECISION OF HPLC ASSAY FOR APOMORPHINE IN BLOOD PLASMA

Apomorphine concentration prepared (μg/ml)	*Mean concentration determined by HPLC (μg/ml)*	*Relative standard deviation (%)*
1.20	1.25 ($n = 6$)	7.0
2.00	2.22 ($n = 4$)	5.2
3.20	3.08 ($n = 4$)	3.8

The HPLC method described herein provides improved sensitivity by a factor of approximately 10 × compared to the previous method.[2] This was due principally to a larger injected sample to total sample ratio. In addition, the use of boldine as an internal standard and the development of a new clean-up procedure improved the utility and shortened the time of analysis.

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