Journal of Chromatography, 386 (1987) 19–24
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1029

EFFECT OF SAMPLE PREPARATION AND LIQUID CHROMATOGRAPHY COLUMN CHOICE ON SELECTIVITY AND PRECISION OF PLASMA CATECHOLAMINE DETERMINATION

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SUMMARY

Research into the role of the catecholamines has often depended on the reliable determination of plasma catecholamine concentrations, which present a challenge since they are normally in the low pg/ml range. Most methods employ liquid chromatography, with variations in sample preparation, the separation mechanism, and detection. We tested a new approach to sample clean-up using boric acid gel instead of alumina. No advantage was found. We also compared cation-exchange separation with ion-pair chromatography. Several improvements are possible with the former, most notably greater precision, better specificity, and increased throughput.

INTRODUCTION

The three catecholamines dopamine (DA), norepinephrine (NE), and epinephrine (EPI) are all derived from the amino acid tyrosine. Aberrations in the concentrations of the catecholamines have been implicated in depression and other affective disorders[1], psychosomatic complaints and essential hypertension[2], and stress[3]. Plasma NE provides a guide to prognosis in patients with stable, chronic congestive heart failure[4]. Besides a broad research interest, there is a recognized clinical role for catecholamine determination in the diagnosis and management of the neuroendocrine tumor pheochromocytoma[5]. Particularly, regional venous sampling at various sites surrounding the adrenals has proven useful in locating these tumors[6]. In all of this work, most often the best combination of useful data and practical sample collection is obtained by utilizing plasma.

Therefore, numerous attempts have been made to develop acceptable plasma catecholamine methods. Much of the challenge stems from the trace concentration at which catecholamines exist in plasma. Radioenzymatic procedures are sensitive and specific, but they are laborious, require radiolabeled reagents, and do not differentiate between the three individual catecholamines without a thin-layer chromatography step. This specificity is a significant advantage, and is attainable by chromatographic methods. In the last few years, techniques relying on liquid chromatography (LC), especially with electrochemical detection, have proved worthy. Ap-

0021-9673/87/$03.50

proaches most often utilize alumina to isolate the catecholamines from plasma, and reversed-phase LC with ion-pairing reagents[7,8].

Several issues still remain unresolved, however, including the stability of catecholamines in plasma[9]; the proper combination of isolation steps to choose between alumina, boric acid gel, or extraction by ion-pairing with diphenylborate[8,10]; and the optimal chromatographic conditions, whether conventional reversed-phase[7], microbore reversed-phase[10], or cation-exchange chromatography[11,12]. Close examination of the various approaches by a number of investigators should clarify the strengths and weaknesses of each and help a laboratory select the analytical method that is best in their situation. Here we compare sample preparation with boric acid gel and alumina, and report on the first use of a weak cation-exchange silica column in place of reversed-phase chromatography.

EXPERIMENTAL

Reagents and standards

Tris(hydroxymethyl)aminomethane (THAM) buffer (Fisher Scientific, Fair Lawn, NJ, U.S.A.); ethylenediaminetetraacetic acid (EDTA, free acid, Sigma, St. Louis, MO, U.S.A.); perchloric acid, phosphoric acid, sodium citrate and monochloroacetic acid (Mallinckrodt, St. Louis, MO, U.S.A.); hydrochloric acid and acetic acid (J. T. Baker, Phillipsburg, NJ, U.S.A.); sodium hydroxide and sodium metabisulfite (MCB Manufacturing Chemists, Darmstadt, F.R.G.) were all of analytical reagent grade. Sodium octyl sulfate was from Kodak Laboratory Chemicals (Rochester, NY, U.S.A.). Acetonitrile was HPLC-grade from Fisher Scientific. Standards of norepinephrine (bitartrate salt), epinephrine (bitartrate salt), dopamine (hydrochloric acid salt), and 3,4-dihydroxybenzylamine (DHBA, internal standard) were supplied by Bioanalytical Systems (West Lafayette, IN, U.S.A.). These standards were used to make stock solutions of various concentrations in 0.1 *M* perchloric acid, which were stable in the refrigerator for six months.

Quality control

Discarded fresh and outdated plasma was used to prepare plasma pools. Various amounts of the standard catecholamines were added to reach desired concen-

TABLE I

DAY-TO-DAY PRECISION

Pool	*NE*			*EPI*		
	Mean (pg/ml)	*S.D.*	*C.V. (%)*	*Mean (pg/ml)*	*S.D.*	*C.V. (%)*
(A) Alumina with reversed-phase LC						
1	145	7.8	5.4	45	6	13
2	564	14.4	2.6	104	4	3.8
(B) Alumina with cation-exchange LC						
A	97	5.8	6.0	15	1.2	8.0
B	330	14.7	4.4	32	2.4	7.5

trations for a calibrator, a "low-concentration" quality control (QC) pool near the upper limit of the reference range, and a "high-concentration" QC pool about 2.5 times normal. (For concentrations, see Table I. Normals in our laboratory are 70–750 pg/ml NE, 0–110 pg/ml EPI, and <30 pg/ml DA for supine, healthy individuals.) These pools were stabilized by adding 50 μl of a 10% $Na_2S_2O_5$ solution to every 10 ml, and were then stored frozen at −20°C in 5 ml plastic vials. Catecholamine concentrations in these plasma pools have not varied noticeably after eight months.

Apparatus

A Model 340 liquid chromatograph from Beckman Instruments (Fullerton, CA, U.S.A.), was equipped with an LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A glassy carbon working electrode was used in all experiments. The analytical column was a Supelco (Bellefonte, PA, U.S.A.), 5-μm LC-18, 5 cm × 4.6 mm I.D. column for reversed-phase chromatography, protected by a Brownlee cartridge RP-18 guard column (Brownlee Labs, Santa Clara, CA, U.S.A.). For cation-exchange chromatography, a Bio-Rad clinical cation-exchange 15 cm × 4.6 mm I.D. column with a similar guard column (Bio-Rad, Richmond, CA, U.S.A.) in a Brownlee cartridge was used. The detector output was monitored and plotted by a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3390A integrator and an Omniscribe (Houston Instrument, Austin, TX, U.S.A.) double-pen recorder.

Chromatographic conditions

For reversed-phase chromatography, a mobile phase of 0.067 *M* monochloroacetic acid buffer adjusted to a pH of 4.0 with 3 *M* sodium hydroxide was utilized. The mobile phase contained 0.54 g/l of sodium octyl sulfate and 9% acetonitrile. The flow-rate was 0.55 ml/min, while the electrode potential was +0.40 V *vs.* Ag/AgCl. The mobile phase used for cation-exchange chromatography was 0.07 *M* citrate adjusted to pH 6.4 with concentrated phosphoric acid, and contained 15% acetonitrile. The flow-rate and electrode potential were the same as above. Both chromatographic procedures were performed at ambient temperature.

Procedure

Plasma was collected in 10-ml evacuated tubes, with EDTA as the anticoagulant, to which 50 μl of a 10% $Na_2S_2O_5$ solution had been added. After gentle mixing, the samples were centrifuged at 2000 *g* for 20 min in a refrigerated centrifuge. Plasma not immediately analyzed was stored frozen.

Isolation on alumina occurred as follows: 50 μl of DHBA (internal standard) was added to 2 ml plasma which is placed in a 6-ml conical reaction vial. To this solution, 400 μl of 3 *M* Tris–5% EDTA buffer (pH 8.6) was added, along with 10 mg acid-washed alumina (Bioanalytical Systems). This combination was mixed on a rotor for 10 min. The alumina was allowed to settle and the supernatant was discarded by aspiration. The alumina was washed with four portions of 5 ml deionized water, and then transferred to a microfilter (Bioanalytical Systems) with 0.5 ml water. Water was removed by centrifugation and the catecholamines were eluted from the alumina with 50 μl 0.1 *M* perchloric acid, of which 25 μl were injected into the LC system.

The boric acid gel (Affi-Gel 601, Bio-Rad) procedure was as described by Ma-

ruta *et al.*[13], except that centrifugation occurred at only 2500 *g* for 20 min instead of 10 000 *g* for 2 min.

In each case, samples were quantitated by comparison of peak height ratios (compound/internal standard) to the calibrator sample. Concentrations in the calibrator had been previously determined by a standard addition experiment. Within each group of samples, the two QC pools were assayed to judge the acceptability of the data.

RESULTS

The alumina isolation procedure and the reversed-phase chromatographic system are the most extensively utilized. Initial work in our laboratory evaluated a variety of reversed-phase columns and mobile phases, with performance no better than that shown for a plasma sample in Fig. 1. Day-to-day precision for both NE and EPI is indicated in Table IA for two QC pools. Linearity was excellent for NE (extending from 100 to 15 000 pg/ml), but was suspect at low concentrations for EPI (only good from 40 to 3000 pg/ml, with healthy individuals, controls groups, etc., generally well below 100). The total effective recovery of each catecholamine was assessed by supplementing the "low" QC pool with standard. These recoveries averaged 63% for NE, 40% for EPI, 74% for DA, and 47% for DHBA, generally similar to those recently reviewed[7]. Further attempts to determine accuracy involved sending samples to a reputable reference laboratory. NE agreement was satisfactory, while substantial differences from our EPI values were apparent in several samples. Upon investigation, it was discovered that all these samples came from subjects who

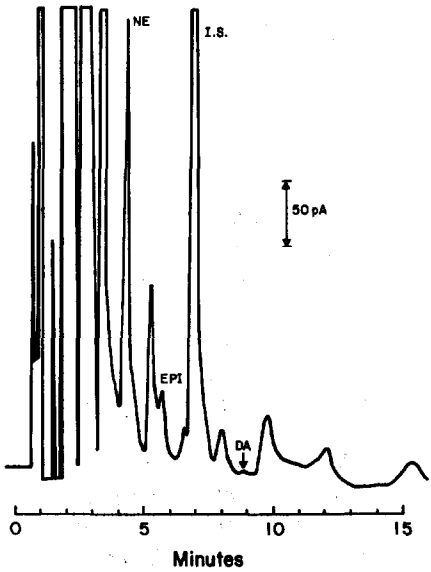

Fig. 1. A plasma catecholamine sample, purified by the alumina procedure, and injected into the reversed-phase column. Chromatographic conditions as described in the text. Concentrations: NE = 424 pg/ml, EPI = 63 pg/ml.

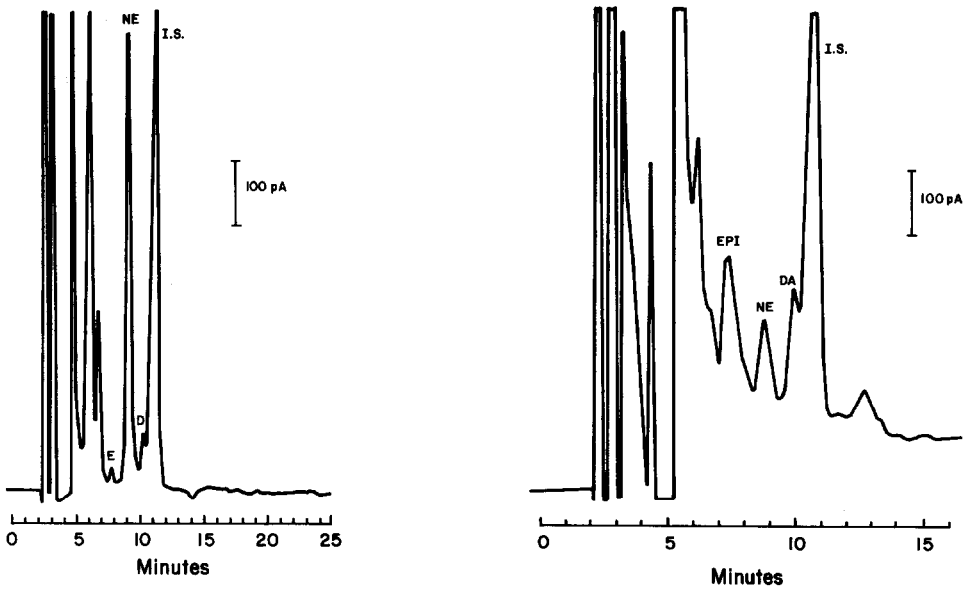

Fig. 2. Plasma catecholamines, purified by the alumina procedure, and injected into the cation-exchange column. Chromatographic conditions as described in the text. Concentrations: NE = 423 pg/ml, EPI = 9 pg/ml, and DA = 28 pg/ml.

Fig. 3. Plasma catecholamines, purified by the boric acid gel procedure and subjected to cation-exchange chromatography. Conditions as described in the text.

had drunk coffee. When these people were retested after abstaining from coffee for at least 6 h, good agreement was obtained. The EPI interference from coffee consumption has been described[14].

Performance of the cation-exchange LC is illustrated by the chromatogram presented in Fig. 2, obtained after alumina isolation. The order of elution is significantly different from that with the reversed-phase system. Precision and linearity of EPI determination in the common range 0–50 pg/ml are greatly enhanced compared to reversed-phase LC. The reversed-phase approach could give no estimates of EPI at concentrations below 30 pg/ml, although samples like those shown in Fig. 2 (9 pg/ml) are typical. Table IB further illustrates the improved precision data at two relatively low concentrations.

An attempt was made to confirm the results of Maruta *et al.*[13] with "Affi-Gel 601" boric acid gel in place of alumina. A chromatogram obtained with cation-exchange LC appears in Fig. 3. An interference peak or peaks coincided with EPI, making it impossible to quantitate. Recovery of NE was decreased. Only the DA peak was similar to that in Fig. 2. Recoveries, determined in the same manner as for alumina isolation, gave 11% for NE, 34% for DA, and 27% for DHBA.

DISCUSSION

Numerous analytical options for determining the plasma catecholamines exist. Alumina extraction has been the isolation procedure of choice, and reversed-phase

liquid chromatography with electrochemical detection is becoming increasingly popular. Problems with alumina isolation include recoveries of less than 70%, interference from common compounds such as uric acid, and difficulty in concentrating the sample into small volumes. Several alternate approaches have been described[10,13]. The use of boric acid gel as tested here does not appear promising, in contrast to these other reports. Further work is investigating the cause of these discrepancies.

Difficulties with reversed-phase LC include interference from coffee[14], instability in columns with ion-pairing reagent, and a relatively high background signal using the electrochemical detector. Several advantages of the cation-exchange system include fewer peaks eluting near the void volume, reducing associated noise, fewer voids (or "negative peaks"), reduced baseline noise, improved separation for EPI leading to lower detection limits and greater precision in the crucial reference range, fewer late-eluting peaks giving increased throughput, and slightly improved detection of DA. In conclusion, the cation-exchange approach is an option that must be considered by any laboratory especially interested in determining EPI. Additional work must be done to clarify whether interferences observed with other techniques are consistently avoided using cation-exchange chromatography.

ACKNOWLEDGEMENT

We are grateful to Steve Binder of Bio-Rad Labs. in Richmond, CA, U.S.A., for the kind gift of Affi-Gel 601.

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