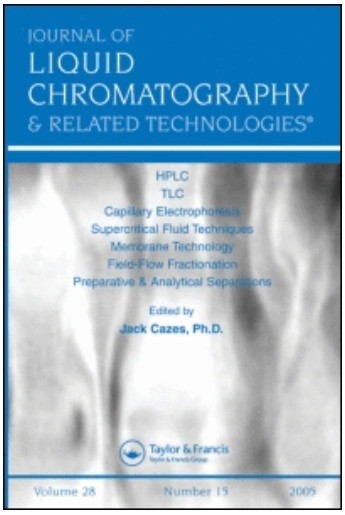

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S. Javidan[a]; M. J. Cwik[b]
[a] College of Pharmacy, Tehran Medical University, Tehran, Iran [b] Clinical Research Laboratory, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois

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DETERMINATION OF CATECHOLAMINES IN HUMAN PLASMA BY HPLC WITH ELECTROCHEMICAL DETECTION

S. Javidan,[1] M. J. Cwik[2]

[1]College of Pharmacy
Tehran Medical University
Tehran, Iran

[2]Clinical Research Laboratory
College of Pharmacy
University of Illinois at Chicago
833 S. Wood
Chicago, Illinois, 60612.

ABSTRACT

An assay procedure is described to quantitate low picogram concentrations of catecholamines in human plasma using reversed phase ion-pair HPLC with electrochemical detection. Optimization of chromatographic conditions allows use of samples as small as 0.25 mL. The overall recovery of 78% offers high precision and accuracy at low concentrations. The lower limit of detection is 0.87 pg/mL for epinephrine, 3.5 pg/mL for norepinephrine and 8.3 pg/mL for dopamine.

INTRODUCTION

The exact relationship between dose of intravenously administered dopamine (DA) and hemodynamic effect produced in children is unclear.[1-5] Pharmacokinetic and pharmacodynamic studies on DA and its metabolites epinephrine (E) and norepinephrine (NE) in this area are thus necessary. Such studies in the pediatric population, however, are often difficult to perform due to the limited sample volume available. Low basal concentrations of catecholamines (CA) coupled with small sample size require an assay having high sensitivity and selectivity. Current HPLC assays for CA generally require 1 to 4 mL of sample, limiting their use in pediatric studies.

Radioenzymatic assays offer the ability to measure CA in sample volumes less than 1 mL, but the complexity and poor reproducibility of this technique prevent its widespread use.[6] The combination of electrochemical detection (ECD) with HPLC for the analysis of CA was introduced by Kissinger et al.[7] Hallman et al.[8] applied this technique to cation exchange chromatography for the determination of plasma CA. Introduction of reversed phase ion-pair separations improved the efficiency, sensitivity and versatility of the technique.[9] Various methods of sample enrichment have been developed. Boronyl compounds have been used for sample preparation. Extraction of CA onto boronic acid gel[10] or extraction into organic solvent using diphenylborate as a complexing agent has been reported.[13] Dihydroxyborylsilica has been used for on-line sample preparation.[14] Extraction with organic solvents or adsorption of CA onto cationic exchange columns followed by extraction into organic solvent has been reported.[11] The micro-batch alumina absorption method originally used by Anton and Sayre[12] remains the most simple, economical and widely used technique. According to a recent review,[15] the analytical recovery by this method has been less than 50% for published assays.

We present a method for the determination of CA using alumina extraction. Improved recovery allows sample volumes as small as 0.25 mL.

MATERIAL

Norepinephrine bitartrate, epinephrine bitartrate, dopamine hydrochloride, 3,4-dihydroxybenzylamine hydrobromide (DHBA), Trizma® base (tris[hydroxymethyl]aminomethane), sodium metabisulfite, 1-octane sodium sulfonic acid and Na_2EDTA were obtained from Sigma Chemical Co. (St. Louis, Missouri). Hydrochloric acid, perchloric acid and phosphoric acid were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey).

Acid-washed aluminum oxide (AAO) was purchased from Bioanalytical Systems (West Lafayette, Indiana). Microfilters were from Rainin Instruments (Woburn, Massachusetts).

The HPLC system consisted of a Model 6000A Solvent Delivery System (Waters Associates, Milford, Massachusetts), a Model 7125 injection valve fitted with a 100 μL sample injection loop (Rheodyne Inc., Cotati, California) and a Resolve™ C_{18} (150 x 3.9 mm) column packed with 5 μm spherical particles (Waters Associates, Milford, MA). Column eluant was monitored by an LC-4 amperometric detector equipped with a TL-5 glassy carbon electrode assembly (Bioanalytical Systems, West Lafayette, Indiana). Detector signal was monitored by an HP-3390A electronic integrator (Hewlett-Packard, Corvallis, Oregon).

The flow rate through the column was 1.0 mL/min. The detector electrode was set at 0.65 V vs Ag/AgCl. The noise filter was set at 0.3 Hz. The mobile phase consisted of buffer mixed with methanol. The buffer contained 1 mM Na_2EDTA, 1 mM 1-octane sulfonic acid and 75 mM NaH_2PO_4, adjusted to pH 3.1 using 85% phosphoric acid. The buffer was filtered through a 0.22 μm nylon filter. Methanol was added (4% v/v) and the mobile phase was degassed by sonication under vacuum. The methanol concentration was adjusted to give stable retention times as the column aged. After equilibration, the mobile phase was recycled through the system. This practice gave a more stable baseline and conserved mobile phase. A single batch of mobile phase (4 L) could be used for about one month before being replaced.

All CA stock solutions were prepared in 0.1 N HCl containing 0.1 mM Na_2EDTA. These solutions were stored at 4° C protected from light. They were stable for approximately two months under these conditions. Standard solutions were prepared from the stock solutions daily.

Internal standard stock solution (DHBA, 1.0 mg/mL) was prepared and stored under the same conditions as the CA stock solutions. This solution was stable for approximately 6 months. The working internal standard solution was prepared weekly from the stock.

Tris buffer (1.5 M) was prepared by dissolving 45 g Trizma® base and 5 g Na_2EDTA in 0.250 L filtered deionized water. The pH was adjusted to 8.7 with 3 N HCl. The solution was stored at 4° C until use. Sodium metabisulfite solution (1 mg/mL) was prepared daily.

METHODS

Blood samples (0.5-1 mL) were withdrawn in the supine position via an indwelling catheter into heparinized tubes containing about 3 mg Na_2EDTA per tube. The sample tubes were stored at 4° C until centrifugation within one hour of collection. Centrifugation was performed at room temperature for 15 min at 2500 rpm. The separated plasma was transferred into tubes containing sodium metabisulfite crystals (approximately 3 mg/mL plasma). The tubes were stored at -70° C until assayed.

Samples were protected from light during the extraction procedure. Aluminum oxide (AAO 10 mg) was placed into a 5 mL Reactivial®. Tris buffer containing Na_2EDTA (1 mL) was added and the vials were vortex mixed for 30 seconds. Sodium metabisulfite solution (0.1 mL) was added, followed by 40 µL internal standard and 0.25 mL thawed plasma or standard solutions. The vials were mixed on a rotary mixer (Ernest, Model SA7-2424, Lester, Pennsylvania) for 15 minutes.

The AAO was separated from the supernatant by centrifugation at 2500 rpm for 15 minutes. The supernatant was discarded and the AAO was washed twice with cold water on the rotary mixer and separated from the supernatant as above. The water was discarded after each wash. The AAO was then transferred to a microfilter and dried under vacuum.

Perchloric acid (120 µL, 0.1 M) containing 40 µg/mL sodium metabisulfite was added to the AAO with mixing and the samples were incubated at room temperature for 5 minutes. The filters were capped and vortex mixed for 30 seconds. They were centrifuged at 3500 rpm for 7 minutes at 4° C. The filtrate was transferred again to the AAO and centrifuged a second time. The collected filtrate was stored on ice until analysis. Sample injection volume was 40 µL. Retention times for NE, E, DA and I.S. were 2.6, 4.6, 9.3 and 5.7 minutes respectively.

RESULTS

The total run time for the determination of CA in plasma was 11 minutes. The reproducibility of the HPLC step was determined by injecting a standard mixture containing NE, E and DA repeatedly for 15 days. The coefficient of variation for these samples was 2.5% for NE at 1.0 ng/mL, 8% for E at 0.5 ng/mL and 5.8% for DA at 2.4 ng/mL.

Table 1

Reproducibility of Catecholamine Assay Using HPLC-ECD From Plasma Spiked With Known Amounts of Catecholamines

Catecholamine	Concentration (pg/mL)	Within-run C.V. (n = 3)	Between-day C.V. (n = 7)
NE	1000	2.4	0.8
	500	3.6	1.3
	100	4.2	1.8
	20	5.4	2.1
	10	6.0	2.9
E	500	7.0	5.0
	200	7.6	5.5
	50	8.2	5.8
	10	9.1	6.1
	2.5	9.7	6.3
DA	2400	4.0	5.4
	1200	4.8	5.7
	400	6.9	6.0
	100	8.0	6.5
	25	9.0	7.2

Within-run and between-day variation were determined by analyzing plasma samples to which a known amount of CA was added. Five concentrations of each CA were analyzed in triplicate over 7 days. Within-run variation ranged from 2.4 to 9.7% and between-day variation was from 0.8 to 7.2%. The results for the individual analytes are presented in Table 1.

Calibration curves constructed for each compound were linear, unbiased and reproducible. Correlation coefficients were all greater than 0.99. The linearity of the detector response was confirmed by injection of standard solutions of known concentration. Response for each compound was linear over the range 0-10 ng/mL for NE, 0-5 ng/mL for E and 0-72 ng/mL for DA. Sensitivity of the assay determined as signal-to-noise ratio of 5 was 1.3 pg/mL NE, 0.3 pg/mL E and 3 ng/mL DA.

Recovery of the extraction procedure was determined by comparing the yields from a series of extracted samples to a solution containing known amounts of CA. Individual recoveries of 77% (NE), 78% (E), 88% (DHBA) and 71% (DA) were achieved. This degree of recovery has not been previously reported for micro-batch alumina extraction.

DISCUSSION

Pharmacokinetic and pharmacodynamic studies of dopamine in children require sensitive assays using small sample volumes. Present methods do not meet these criteria. Those assays using small sample volumes are not sensitive enough to measure baseline levels of circulating DA.[16-18]

HPLC coupled with electrochemical detection is the most commonly used technique for the determination of CA. The method is relatively simple and does not require special instrumentation or extensive chemical modification of the CA structure as do other techniques.

Non-chromatographic techniques such as radioenzymatic assay[6] and ELISA[19] have been employed for the determination of CA. These techniques require a separate assay for each CA of interest.

Microbore HPLC with electrochemical detection is becoming more popular because of both increased sensitivity and reduced sample volume.[20] It has also been combined with microdialysis for the preparation of plasma samples.[21] The current sample preparation method to increase sensitivity is compatible with this technique.

Separation of CA by HPLC followed by detection with post-column derivatization and detection by chemiluminescence[22,23] or fluorescence[24] has also been reported. These procedures introduce more variables into the determination of CA.

Gas chromatography with mass spectrometric detection has been reported,[25] but application of this technique is limited due to the small number of laboratories with this instrumentation available.

Oxidation of the catechols results in poor sensitivity of most assays for the determination of CA due to low recovery of analytes. In the current assay, sodium metabisulfite reduces oxidation of CA and increases analytical recovery from about 50% to almost 80%, resulting in increased sensitivity. Glutathione

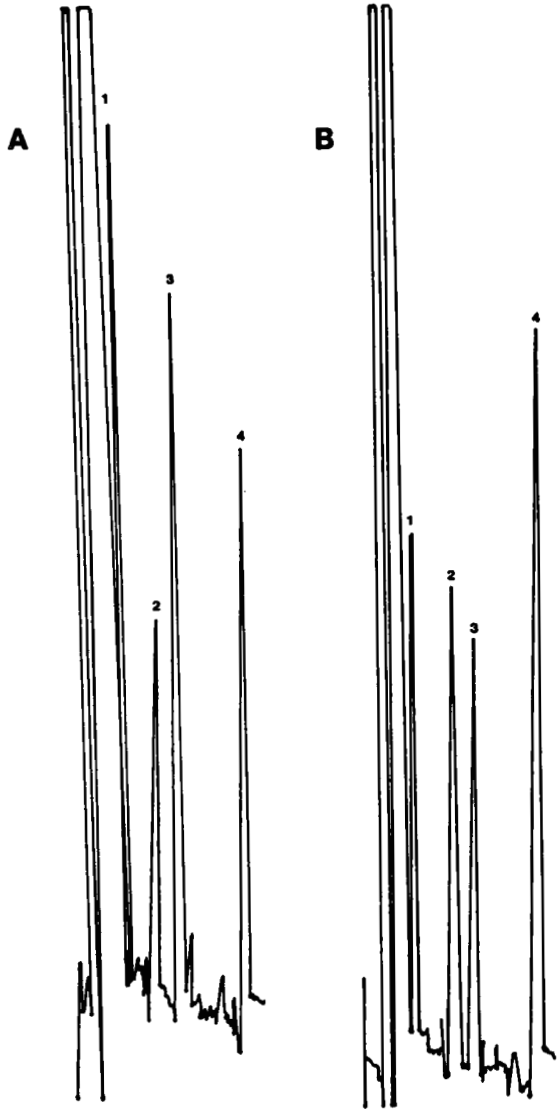

Figure 1. Chromatograms showing the extraction of catecholamines from human serum using AAO: A) with the addition of sodium metabisulfite and B) without the addition of sodium metabisulfite. Peaks: 1) NE; 2) E; 3) I.S., DHBA; 4) DA.

has also been reported to reduce oxidation of catechols during sample preparation.[26] Keeping samples cold during work-up by washing with cold water and storing extracted samples on ice while awaiting injection also helps slow oxidation. Figure 1 presents chromatograms of extracted plasma samples with and without the addition of sodium metabisulfite during the extraction procedure.

Goldstein and Feuerstein[27] suggest useful precautions to improve ECD performance in order to lower the detection limit of epinephrine using 1mL of plasma and injection of total elute. Among these are use of sodium metabisulfite during sample preparation and frequent cleaning or replacement of electrodes. They report a lower limit of 20 pg for E. In the present method, the lower limit of detection for E is 0.87 pg and uses only one third of the eluant from the AAO.

The improvements achieved in reduction of CA loss during the assay offers the opportunity of replicate injections or smaller sample size. The latter factor is especially important when dealing with pediatric patients.

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