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Journal of Chromatography B, 653 (1994) 131–138

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection

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(First received September 9th, 1993; revised manuscript received December 6th, 1993)

Abstract

Several modifications of an HPLC–electrochemical assay method for plasma levels of norepinephrine (NE), epinephrine (EPI), dopamine (DA), dihydroxyphenylglycol (DHPG), dihydroxyphenylalanine (DOPA) and dihydroxyphenylacetic acid (DOPAC) that improve the accuracy and reliability of DHPG, DOPA, and DOPAC measurements are described. In batch alumina extractions, increasing the amount of alumina decreased analytical recoveries of DHPG, DOPA, and especially DOPAC, and increasing the strength of the eluting acid increased recoveries of these catechols, without affecting recoveries of the amines NE, EPI and DA. Refrigeration (4°C) until injection stabilized DOPAC in aqueous solution and therefore improved the reproducibility of plasma DOPAC measurements. Circulation of chilled water (15°C) around the column using a water jacket decreased variability in retention times of the catechols and thereby facilitated identification of peaks, while enhancing separation of DHPG from the solvent front. Use of 6-fluoro-DOPA and 6-fluoro-DOPAC as internal standards did not improve inter-assay reliability. We recommend that in assays of plasma catechols including DOPAC, small (5 mg), precisely measured amounts of alumina be used, with a relatively strong eluting solution (*e.g.* 0.04 *M* phosphoric acid–0.2 *M* acetic acid, 20:80, v/v), and that the samples be refrigerated until injection, with column temperature held constant at less than 20°C.

1. Introduction

Combined measurements of plasma concentrations of the catecholamines – norepinephrine (NE), epinephrine (EPI) and dopamine (DA) – and of the other endogenous catechols – dihydroxyphenylglycol (DHPG), dihydroxyphenylalanine (DOPA), and dihydroxyphenylacetic acid (DOPAC) – enable comprehensive assessments of sympathetic nervous function. Simultaneous measurements of plasma NE and DHPG levels can be used to examine the release, reuptake, and turnover of NE, the sympathetic neurotransmitter [1–4]. Concurrently obtained plasma DOPA levels appear to reflect tyrosine hydroxylation, the enzymatic rate-limiting step in catecholamine synthesis [5–7].

Plasma concentrations of the DA metabolite, DOPAC, may provide further information about catecholamine synthesis and metabolism in

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SSDI 0378-4347(93)E0430-X

sympathetic nerves. Because of rapid conversion of DOPA to DA, rapid deamination of axoplasmic DA to form DOPAC, and rapid extraneuronal O-methylation of DOPAC to form homovanillic acid (HVA), plasma DOPAC probably reflects neuronal metabolism of DA. Thus, in animals treated with reserpine to block vesicular translocation of catecholamines and thereby NE synthesis, plasma DOPAC levels increase markedly [7], and interference with tyrosine hydroxylation produces rapid, persistent decreases in plasma DOPAC levels [6]. Patients with pure autonomic failure usually have low plasma DOPAC levels, whereas patients with absence or deficiency of DA-β-hydroxylase have high plasma DOPAC levels [8,9], probably indicating decreased tyrosine hydroxylation in the former group and increased tyrosine hydroxylation in the latter group.

Current methods for simultaneous measurements of plasma levels of catechols use a batch alumina extraction step, followed by HPLC with electrochemical detection [10–12]. Alumina adsorption partially purifies the catechols, enabling injection of the eluate into a chromatographic system after a single cleanup step. This approach works well for measurements of plasma NE, EPI, and DA; however, recoveries of DHPG, DOPA and especially DOPAC are typically lower than those of the catecholamines and of the dihydroxybenzylamine (DHBA) internal standard. DOPAC recoveries may be less than 15% [10]. The poor accuracy and reliability of plasma DOPAC determinations has presented an obstacle to the use of levels of this metabolite to assess catecholamine synthesis and metabolism.

These limitations led us to test several modifications to improve the analytical recovery and reproducibility of assays for simultaneous measurements of plasma levels of DOPAC and of other endogenous catechols. Different amounts of alumina were used, in order to identify the optimum amount for reproducible and maximal recoveries. Various mixtures of phosphoric and acetic acids as the eluting solution were tested, to determine whether use of a stronger acid mixture would improve analytical recovery. Effects of holding the column temperature constant were compared with those when the column was exposed to ambient room temperature, since alterations in chromatographic retention times could lead to misidentification of peaks, and manipulation of the column temperature could affect differentially the retention times of the compounds of interest. To examine the possible influences of temperature-dependent degradation before injection, effects of refrigeration of aqueous standards and alumina eluates were assessed. Finally, results using internal standards for DOPA (6-fluoro-DOPA) and DOPAC (6-fluoro-DOPAC) were compared with those using external standards.

2. Experimental

2.1. Apparatus

Alumina extracts were injected onto a 5-μm, 250 × 4.6 mm I.D. ODS Ultrasphere (No. 235329) HPLC column (Beckman Instruments, Fullerton, CA, USA) using a Waters Model 710B (WISP) automated sample processor (Waters Associates, Milford, MA, USA).

The mobile phase was pumped isocratically at 1.0 ml/min by a Waters Model 590 programmable solvent delivery system. Quantification of the catechols was by electrochemical detection using a coulometric detector (Model 5100A; Environmental Science Associates, Bedford, MA, USA) and triple electrode system (Models 5021 and 5011; Environmental Science Associates). The first electrode in the series was set at 0.30 V, the second at 0.16 V, and the third at −0.35 V. The output of the last electrode was recorded on a Waters Model 730 Data Module.

Column temperature was controlled using a refrigerated water circulator (Lauda K-2/R; Brinkman Instruments, Westbury, NY, USA) that pumped a 50:50 mixture of chilled water and ethylene glycol through a glass water jacket (Beckman) surrounding the column.

2.2. Plasma

Plasma was obtained from healthy workers in our laboratory who were not taking any medications and who had fasted overnight. An an-

tecubital venous catheter was inserted precutaneously, and blood samples were obtained through the catheter after at least 20 min of supine rest. The blood was drawn through the indwelling catheter into prechilled, glass, heparinized tubes and kept on ice until the plasma was separated by refrigerated centrifugation (3000 *g*, 20 min) and transferred immediately to plastic cryotubes for storage at −70°C. Thawed plasma samples were centrifuged before alumina extraction.

2.3. Modifications

Amount of alumina

Scoops calibrated to contain 2.5, 5, 10, 20, or 50 mg alumina were fabricated from the cut tips of plastic limited volume inserts (Waters). Analytical recoveries of catechol standards (DHPG, NE, DOPA, EPI, 6-fluoro-DOPA, DHBA, DOPAC, DA, and 6-fluoro-DOPAC) from aqueous solutions and plasma were calculated for each amount of alumina, by comparison with heights of chromatographic peaks corresponding in retention time to those of the directly injected standards.

Mixtures of eluting acids

Four mixtures of eluting acids were examined: 0.2 *M* acetic acid; 0.04 *M* phosphoric acid–0.2 *M* acetic acid (20:80, v/v); 0.04 *M* phosphoric acid–0.2 *M* acetic acid (40:60, v/v); and 0.2 *M* phosphoric acid–0.2 *M* acetic acid (20:80, v/v). Since eluates after treatment of alumina with phosphoric acid often contained contaminating substances that interfered with the chromatographic recordings of the catechols, we wished to use only the minimum effective amount of phosphoric acid in the mixture of eluting acids. Chromatographic results were compared after the above acids were used to elute catechols from the alumina after extraction of aqueous standards or of aliquots of plasma.

Column temperature

Retention times of catechols were compared with or without enclosure of the column in the water jacket (15°–30°C). A heating jacket was not used, because increasing the column temperature shortened the chromatographic retention time of DHPG, forcing the peak into the solvent front.

Sample refrigeration

Aqueous standards, alumina eluates from extracted mixtures of the standards, and alumina eluates from human plasma were placed in the carousel of the WISP™ (Waters Intelligent Sample Processor) and injected during a 24-h period at room temperature. Alternatively, the samples were kept in a refrigerator until just before injection.

Fluorinated internal standards

6-Fluoro-DOPA and 6-fluoro-DOPAC were provided by Dr. Kenneth Kirk, Laboratory of Chemistry, NIDDK, NIH. Analytical recoveries of 6-fluoro-DOPA and 6-fluoro-DOPAC were compared with those of DOPA and DOPAC external standards. Inter-assay coefficients of variation were calculated from 10 separate assays of pooled plasma.

Data analysis

Statistical testing consisted of independent-means *t*-tests.

3. Results

Normal values for plasma concentrations of DOPAC were similar to those of DOPA and averaged *ca.* 100 times those of DA (Table 1).

Analytical recoveries of DOPAC, DOPA, and DHPG using the current method exceeded those using the method of Eisenhofer *et al.* [10], and inter-assay coefficients of variation for DOPAC and DOPA levels using the current method were less than those using the method of Eisenhofer *et al.* [10] (Table 2). In contrast, application of the two methods yielded similar average analytical and similar coefficients of variation for NE, EPI, and DA.

Recoveries of DHPG, DOPA, and DOPAC declined with increasing amounts of alumina (Fig. 1). The decreases in recovery were especially prominent for DOPAC. Recoveries of catecholamines varied little as a function of the

Table 1
Concentrations of catechols (pg/ml) and inter-assay coefficients of variation for pooled plasma of healthy humans

Catechol	Concentration (mean ± S.D., $n = 10$) (pg/ml)	C.V. (%)
DHPG	1168 ± 55	5
NE	348 ± 10	3
DOPA[a]	1607 ± 74	5
EPI	48 ± 2	4
DOPAC[a]	1604 ± 234	15
DA	15 ± 2	9
DOPA[b]	1596 ± 47	3
DOPAC[b]	1304 ± 59	4

[a] DOPA and DOPAC calculations from recoveries of 6-fluoro-DOPA and 6-fluoro-DOPAC.
[b] DOPA and DOPAC calculations from recoveries of DOPA and DOPAC in extracted mixture of standards.

amount of alumina. Addition of phosphoric acid to the acid eluting mixture improved the recoveries of DHPG, DOPA, and DOPAC, with less apparent effects on recoveries of catecholamines (Fig. 1). When 0.2 *M* phosphoric acid–0.2 *M* acetic acid (20:80, v/v) was used, the chromatographic recordings included extra peaks that interfered with endogenous catechols – especially DHPG (Fig. 2).

Recovery of DOPAC averaged 11% using the old alumina extraction method (10 mg alumina, 0.2 *M* acetic acid) and 52% using the new method (5 mg alumina, 20:80 mixture). Recovery of DOPAC increased further to 66% using the 0.2 *M* phosphoric acid–0.2 *M* acetic acid; however, chromatographic recordings obtained from plasma were prone to contamination with additional peaks.

When the aqueous standards or alumina eluates were exposed to ambient room temperature until injected, the peak heights of DOPAC decreased markedly and variably during the 24-h period of injections of eluates from aqueous standards (Fig. 3). Refrigeration of the samples eliminated this variability. With the column exposed to ambient room temperature, retention times of DOPAC and the other catechols fluctuated, declining as room temperature increased (data not shown). Chromatographic retention times decreased with increasing water temperature in the column jacket (Fig. 4). The extent of the changes in retention time varied among the catechols, with especially pronounced changes in retention times for DOPAC and DOPA. Enclos-

Table 2
Comparison of analytical recoveries and coefficients of variation between standard mixtures extracted with previous [10] and current method

Catechol	Previous method		Current method	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
DHPG	57	12	75	8
NE	78	7	83	9
DOPA	47	12	69	8
EPI	76	6	81	10
DHBA	78	5	85	9
DOPAC	11	31	52	9
DA	84	8	83	10
6-Fluoro-DOPA	–	–	66	9
6-Fluoro-DOPAC	–	–	46	13

Note: Previous method, 10 mg of alumina and 0.2 *M* acetic acid. Current method, 5 mg of alumina and 0.04 *M* phosphoric acid–0.2 *M* acetic acid (20:80, v/v). DHPG, dihydroxyphenylglycol; NE, norepinephrine; DOPA, dihydroxyphenylalanine; EPI, epinephrine; DHBA, dihydroxybenzylamine; DOPAC, dihydroxyphenylacetic acid; DA, dopamine.

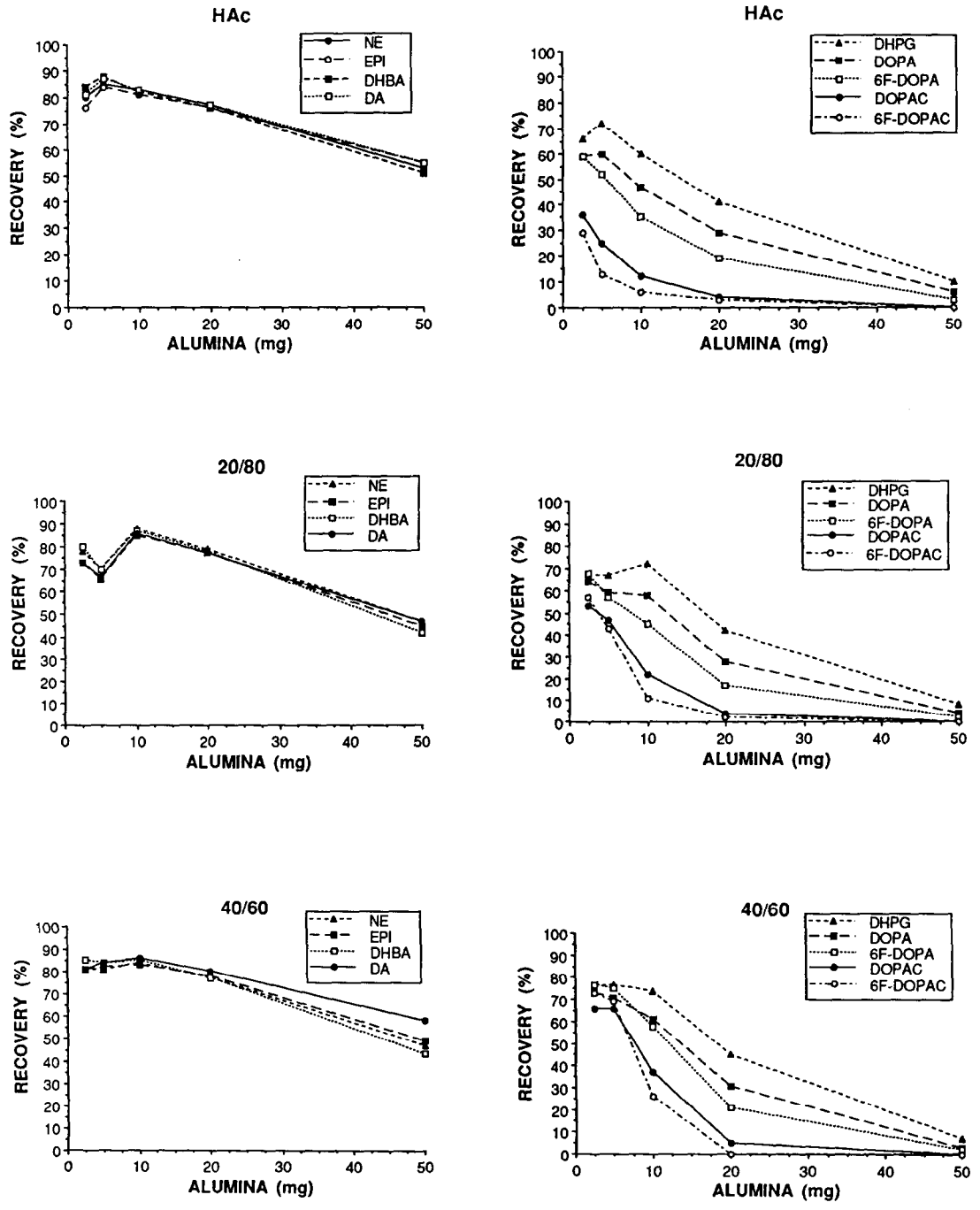

Fig. 1. Percent recoveries of catechols after alumina extraction of a mixture of aqueous standards: (DOPA, dihydroxyphenylalanine; DA, dopamine; NE, norepinephrine; EPI, epinephrine; DOPAC, dihydroxyphenylacetic acid; and DHPG, dihydroxyphenylglycol). Results are shown for assays involving 2.5, 5, 10, 20, and 50 mg of alumina and eluting acid solutions containing 0.2 *M* acetic acid (HAc), 0.04 *M* phosphoric acid–0.2 *M* HAc (20:80, v/v), and 0.04 *M* phosphoric acid–0.2 *M* HAc (40:60, v/v).

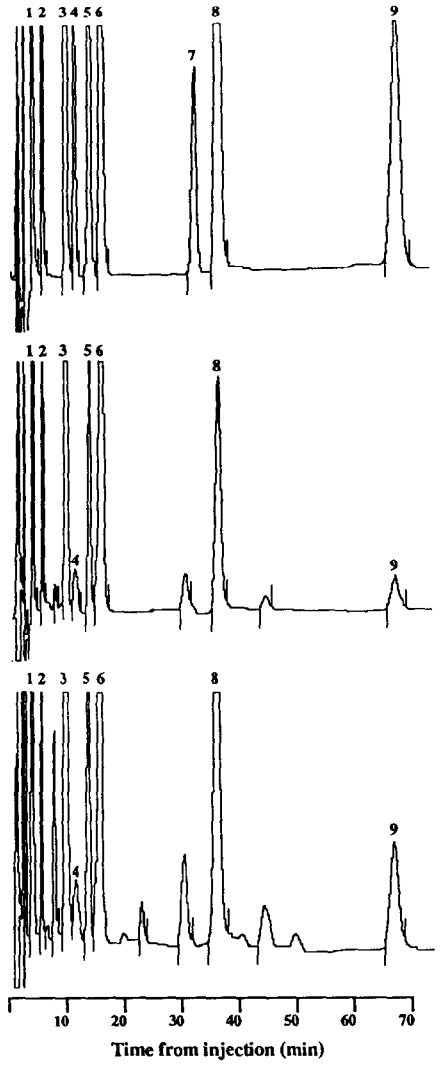

Fig. 2. Chromatographic recordings for (top) an aqueous mixture of standards, (middle) for pooled human plasma, with eluent 0.2 *M* acetic acid, and (bottom) for pooled human plasma, with eluent 0.04 *M* phosphoric acid–0.2 *M* acetic acid (20:80, v/v). Peak identification: 1 = dihydroxyphenylglycol; 2 = norepinephrine; 3 = dihydroxyphenylalanine; 4 = epinephrine; 5 = 6-fluoro-dihydroxyphenylalanine; 6 = dihydroxybenzylamine; 7 = dopamine; 8 = dihydroxyphenylacetic acid; 9 = 6-fluoro-dihydroxyphenylacetic acid.

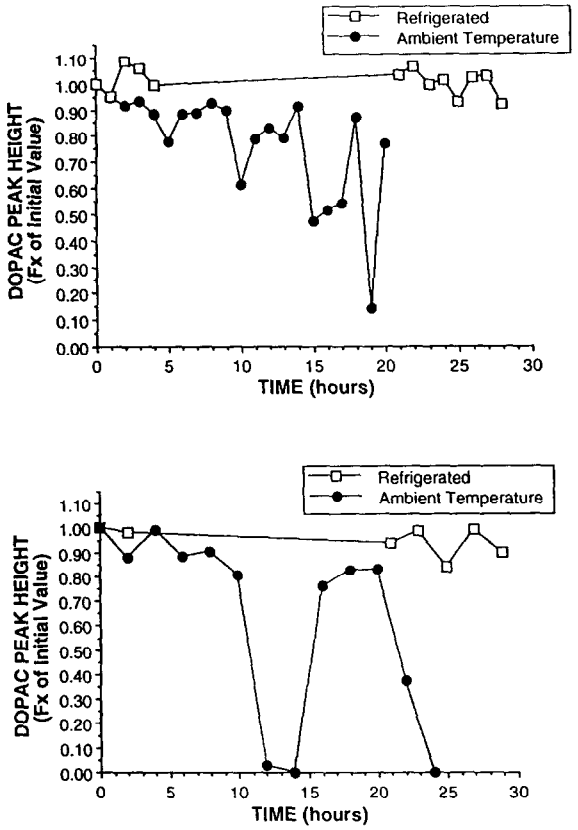

Fig. 3. Chromatographic peak heights of dihydroxyphenylacetic acid (fraction of initial value) at ambient room temperature or refrigerated until injected into the HPLC-electrochemical system. (Top) extracted mix; (bottom) eluates of plasma.

ing the column in the jacket virtually eliminated fluctuations in the retention times of all the catechols. This allowed more accurate identification of peaks of interest. Additionally, column temperatures below *ca.* 20°C facilitated separation of DHPG from the solvent front.

Analytical recoveries of 6-fluoro-DOPA and 6-fluoro-DOPAC through the alumina extraction step were slightly but consistently less than those of DOPA ($p < 0.001$) and DOPAC ($p < 0.001$), when 5 mg alumina and 0.04 *M* phosphoric acid–0.2 *M* acetic acid (20:80, v/v) were used (Fig. 1). When the amounts of alumina and the constitution of the eluting acid were varied, recoveries of 6-fluoro-DOPA and 6-fluoro-DOPAC changed generally similarly to those of DOPA and DOPAC. The inter-assay coefficient of variation for plasma DOPAC levels, however, was *ca.*

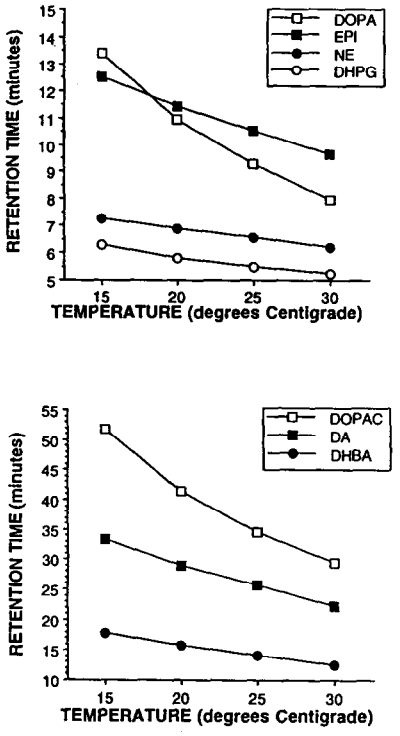

Fig. 4. Retention times of catechols as a function of water temperature in the column jacket. (Top) retention times for early-eluting compounds; (bottom) retention times for late-eluting compounds.

three-fold larger when the 6-fluoro-DOPAC internal standard was used than when the DHBA recovery and recovery of DOPAC in an extracted mixture were used (Table 1).

4. Discussion

Whereas many reports describe HPLC methods for plasma levels of catecholamines, only several reports have described methods for simultaneous measurements of plasma levels of catecholamines and DHPG [1–4]; even fewer have included levels of DOPAC [10,11]. Recent results of studies with laboratory animals and with humans have indicated that simultaneous measurements of plasma levels of DOPAC with levels of other catechols provide information about related but distinct aspects of sympathoneural function [1,13]. Inadequacies in the previous assay method for plasma DOPAC incited the present testing to improve the method.

The present report shows how adoption of several modifications markedly improves the reliability of simultaneous HPLC determinations of plasma catechols – especially DOPAC. Table 3 summarizes the modifications.

Inclusion of a relatively small (5 mg), reproducible amount of alumina and an eluting acid mixture (0.04 *M* phosphoric acid–0.2 *M* acetic acid, 20:80, v/v) improved the analytical recovery of DOPAC, and other non-amine catechols, without interfering with the chromatographic results. In assays for plasma levels of catechols, the smallest practicable amount of alumina should therefore be used, and the eluting solution should be stronger than 0.2 *M* acetic acid.

The results also support the routine use of a refrigerated automatic injector for HPLC assays of plasma concentrations of DOPA, DHPG and especially DOPAC. Alternatively, to minimize the time alumina eluates are exposed to ambient temperature, samples should be kept refrigerated and loaded on the injector carousel in small batches. Otherwise, peak heights of these compounds will begin to fluctuate chaotically after several hours at ambient temperature in the carousel.

The column temperature should be held constant, since this minimizes the likelihood of misidentifying peaks due to temperature-dependent changes in chromatographic retention times. Additionally, one may vary column temperature to improve chromatographic separation of DOPA from EPI, DOPAC from DA, and DHPG from the solvent front.

The use of 6-fluoro-DOPA and 6-fluoro-DOPAC as internal standards does not improve inter-assay reliability, compared with the use of DOPA and DOPAC as external standards and DHBA as the internal standard, because analytical recoveries of 6-fluoro-DOPA and 6-fluoro-DOPAC were less than those of the non-fluorinated compounds, and the differences in recovery between the fluorinated and non-fluorinated compounds varied from sample to sample.

Table 3
Assay method for plasma levels of catechols

To 1 ml freshly thawed plasma in a plastic 1.5-ml sample tube add:
5 mg acid-washed alumina
500 μl TRIS/EDTA buffer, pH 8.6
DHBA internal standard (20 μl)

Shake vigorously for 30 min, then centrifuge for 1 min, aspirate and discard supernate.

Wash alumina with HPLC grade water, shake for 15 s, centrifuge for 1 min, aspirate and discard supernate, wash alumina again with HPLC grade water, shake for 15 s, centrifuge for 1 min, aspirate and discard supernate.

Elute catechols from alumina with 100 μl of *0.04 M phosphoric acid–0.2 M acetic acid (20:80, v/v)*. Shake vigorously for 5 min, then centrifuge for 1 min.

Transfer alumina eluate to WISP vial insert, load insert in injector carousel, *inject refrigerated eluate.*

HPLC column: Beckman No. 235329 stainless steel packed with reverse-phase 5-μm ODS. Column in chilled water jacket.
Mobile phase: To 1 l HPLC grade water add:
Sodium phosphate, monobasic (13.8 g)
Octanesulfonic acid (28–40 mg)
EDTA (50 mg)
Acetonitrile (0–5 ml)
Adjust pH to 3.2–3.3 using 85% phosphoric acid.

Changes from the method of Eisenhofer *et al.* [10] are indicated in italics.

The present results do not call for modifications of the previously published method [10] for assaying plasma levels of the catecholamines, NE, EPI, and DA.

Adoption of the recommended modifications should improve future studies using simultaneous measurements of plasma levels of catechols to examine aspects of catecholaminergic function in health and disease.

5. References

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