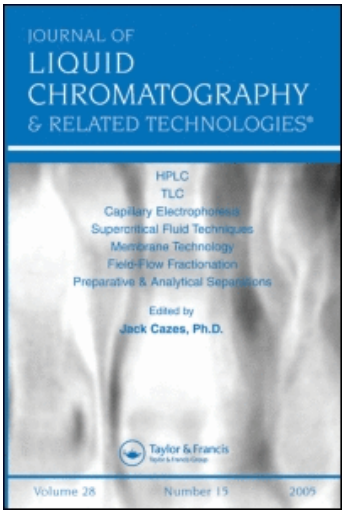


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# Plasma Sample Cleanup for Catecholamines Analysis Using Weak Cation Exchange Solid Phase Extraction

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# PLASMA SAMPLE CLEANUP FOR CATECHOLAMINES ANALYSIS USING WEAK CATION EXCHANGE SOLID PHASE EXTRACTION

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ABSTRACT

Norepinephrine and epinephrine were isolated from human plasma at picogram levels by solid phase extraction (SPE), on a proprietary weak cation exchange silica based packing. Plasma samples were spiked with catecholamines at two concentrations to simulate body response to varying levels of stress. Recoveries of norepinephrine and epinephrine at both concentrations exceeded 95%.

An analysis of catecholamines by ion pair high-performance liquid chromatography with amperometric detection is described.

## INTRODUCTION

Interest in catecholamines has increased since the discovery of a link between body levels of these compounds and

adrenal and neuronal tumors associated with endocrinological and neurological disorders (1). Elevated urine and plasma levels have been shown to be excellent indicators of the presence of these tumors. Urinary catecholamine levels, however, represent an integrated response over a long period of time. This can make it difficult to draw conclusions on the effects of various body stimuli on catecholamine production. Plasma catecholamine levels, on the other hand, respond more rapidly to stimuli, and thus are a more accurate indicator of the relationship between catecholamine production and body stimulus (2).

Several analytical methods have been developed for estimating catecholamine levels in plasma, including radioenzymatic assay (3), gas chromatography (4), gas chromatography-mass spectrometry (5), and high-performance liquid chromatography (HPLC) with ultraviolet (6), fluorescence (7) or electrochemical (8) detection. For routine plasma analyses, extensive sample cleanup is necessary with each of these analytical methods. The most common sample cleanup procedure involves a batch extraction of the catecholamines on alumina (9-11). This method is hampered by relatively poor recovery (approximately 70%) of the catecholamines from the alumina. Other cleanup methods, involving ion-exchange columns used in conjunction with an alumina extraction (12), suffer from the same recovery problems observed when using alumina alone.

We have developed a simple, rapid, plasma cleanup technique utilizing weak cation exchange solid phase extraction (SPE). Catecholamine recovery from the plasma extraction is greater than 90%. Thus, catecholamines analyses by ion pair HPLC with electrochemical detection can be performed on 500 µl plasma samples.

## MATERIALS AND METHODS

### Standards and Reagents

Norepinephrine and epinephrine standards, and the internal standard 3,4-dihydroxybenzylamine, were obtained from Sigma Chemical Co. (St. Louis, MO). Reagent grade ammonium hydroxide, hydrochloric acid, acetic acid, phosphoric acid and perchloric acid, and certified A.C.S. grade citric acid (anhydrous), sodium phosphate (dibasic) and disodium ethylenediamine tetraacetate, were obtained from Fisher Scientific (Pittsburgh, PA). The ion pairing reagent, HPLC grade 1-octanesulfonic acid (sodium salt), was obtained from Eastman Kodak Co. (Rochester, NY).



All aqueous solutions were prepared from water obtained from a Milli-Q water purification system (Millipore Co., Bedford, MA).

All volumetric flasks used in the preparation of standards were silanized with SYLON-CT (SUPELCO, Inc., Bellefonte, PA) prior to use, to prevent absorption of the catecholamines on the borosilicate glass.

A stock catecholamine standard, containing 20.5 mg of norepinephrine (NE) and 10.3 mg of epinephrine (E) in 100 ml of a 1% (v/v) acetic acid in water solution, was prepared in a volumetric flask.

A stock internal standard solution, containing 20.0 mg of 3,4-dihydroxybenzylamine (DHBA) in 50 ml of a 1% (v/v) acetic acid in water solution, was prepared in a volumetric flask.

A dilute catecholamine standard solution was prepared by diluting 10 µl of the stock catecholamine standard solution to 100 ml with water in a volumetric flask.

A dilute internal standard solution was prepared by diluting 10 µl of the stock internal standard solution to 100 ml with water in a volumetric flask.

Working catecholamine standards were prepared by diluting an appropriate amount of the dilute catecholamine standard to 500 µl with water.

## Initial Plasma Preparation

Plasma was prepared from fresh blood drawn daily from healthy volunteers. The blood was drawn into heparinized tubes and centrifuged for 10 minutes at 2500 g at 4°C. The plasma was then removed from the centrifuge tube and stored at 4°C for up to one day.



## Plasma Cleanup

A 1 ml size Supelclean LC-WCX SPE tube (SUPELCO Inc., Bellefonte, PA) was conditioned with 500 µl of 0.5 M hydrochloric acid in water, followed by 1 ml of water to remove the excess acid from the packing. The sample, consisting of a 1:1 dilution of a 500 µl plasma sample with water, was passed through the extraction tube at a slow dropwise rate. Dilution of the plasma with water allowed plasma components to pass through the ion-exchange packing, without interfering with the extraction of catecholamines.

A three step wash procedure was used to remove the more strongly retained interferring plasma components from the extraction tube. The tube was first washed with 1 ml of water. This removed both residual plasma left on the tube and many of the polar impurities that otherwise appeared in the solvent front during HPLC analysis. The tube was then washed with 250 µl of 1.0 M ammonium hydroxide in water, to remove impurities otherwise seen as shoulders on the catecholamine

peaks during analysis. A second 1 ml water wash was then used to remove both the residual ammonium hydroxide solution and the rest of the polar impurities.

A 30 µl aliquot of the dilute internal standard solution was added to the tube and washed onto the ion-exchange packing with 250 µl of water at a slow dropwise rate. The internal standard was added at this point, to ensure that absolute recovery of the internal standard exceeded 90%. When the internal standard was added earlier, approximately 40% was lost when the tube packing was washed.

The catecholamines and internal standard were eluted from the tube with 250 µl of 0.2 M perchloric acid in water. The eluate was collected in a small glass vial, silanized with SYLON-CT (SUPELCO) to prevent adsorption of the catecholamines on the borosilicate glass.

Recoveries of the two catecholamines, norepinephrine and epinephrine, from the Supelclean LC-WCX tubes, were determined by standard addition. All extractions were done in triplicate. The human plasma was spiked at two different catecholamine levels to stimulate body response to varying degrees of stress.



## Chromatography

A Varian Instrument Group (Palo Alto, CA) 5000 Series liquid chromatograph, equipped with an LC/4B amperometric detector (Bioanalytical Systems, West Lafayette, IN), was used to analyze the plasma extracts. The catecholamines were separated on a SUPELCOSIL® LC-18-DB HPLC column (15 cm x 4.6 mm, 5 µm particles) (SUPELCO). A Supelguard LC-18-DB guard column (2 cm x 4.6 mm, 5 µm particles) (SUPELCO) was employed to prolong the life of the analytical column. The mobile phase (13) consisted of 0.025 M citric acid, 0.025 disodium

phosphate, 0.05 mM disodium EDTA and 34 mg/L of the 1-octanesulfonic acid ion pairing reagent (pH adjusted to 3.4 with 85% phosphoric acid). It was prepared fresh daily and passed through a 0.2 μm filter and degassed for 20 minutes under vacuum before use. The mobile phase was passed through the column at a flow rate of 1.5 ml/min. at ambient temperature. The amperometric detector was used in the oxidative mode with an applied potential of +650 mV, a range of 1.0 nA and a filter setting of 0.1 Hz.

## RESULTS

Figure 1a shows an extract from human plasma spiked with 205 pg of norepinephrine and 103 pg of epinephrine. Figure 1b shows the working standard prepared at the same catecholamine concentrations. The differences in peak heights between the spiked and the standard plasma samples represent catecholamines originally present in the plasma sample, as seen in Figure 1c.

Catecholamine recovery was determined from peak height relative to the internal standard, DHBA, using Equation 1.

EQUATION 1

$$\text{Percent Relative Recovery} = \frac{\left(\frac{(H)x}{(H)std}\right)_S - \left(\frac{(H)x}{(H)std}\right)_B}{\left(\frac{(H)x}{(H)std}\right)_{WS}} \times 100\%$$

(H)x = height of catecholamine peak
(H)std = height of internal standard peak
S = spiked plasma sample
B = blank plasma sample
WS = working standard

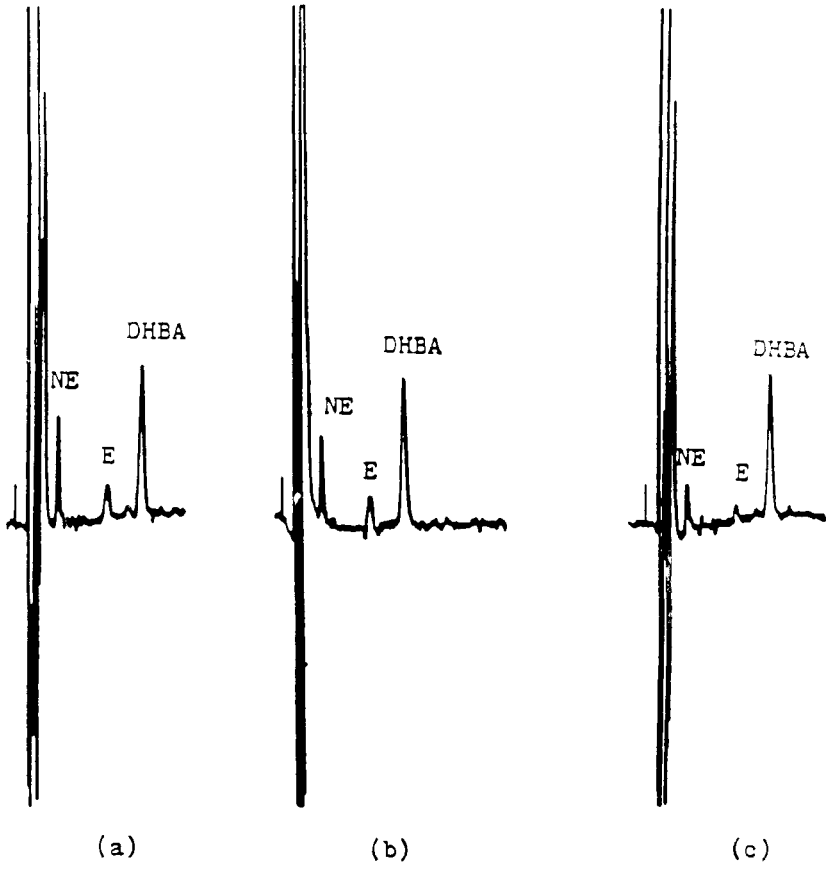


Figure 1: (a) Human plasma spiked with 205 pg NE, 103 pg E and 1200 pg DHBA. (b) Catecholamine working standard containing 205 pg NE, 103 pg E and 1200 pg DHBA. (c) Normal catecholamine levels found in human plasma spiked with 1200 pg DHBA. NE - norepinephrine E - epinephrine DHBA - 3,4-dihydroxybenzylamine (internal standard)

TABLE 1

Catecholamine Recovery from Human Plasma

| Catecholamine | spike (pg) | relative recovery 1 | 2 | 3 | Mean ± S.D. |
|---|---|---|---|---|---|
| NE | 205 | 92.8 | 102 | 95.9 | 96.9 ± 4.7 |
| E | 103 | 95.8 | 89.5 | 108 | 97.8 ± 9.4 |
| NE | 615 | 110 | 100 | 109 | 106 ± 5.5 |
| E | 309 | 98.1 | 90.7 | 102 | 96.9 ± 5.7 |



Recovery data is summarized in Table 1. Absolute recovery of norepinephrine and epinephrine, at the concentrations shown in Figure 1, was 91.1 ± 4.4% and 91.9 ± 9.2% respectively. These values are slightly lower than the relative recovery reported in Table 1. Absolute recovery of norepinephrine and epinephrine from plasma spiked with 615 pg and 309 pg, respectively, was 102 ± 6.5% and 93.1 ± 7.3%.

## DISCUSSION

Plasma catecholamine analysis has taken on greater importance, as the role of catecholamines as indicators of stress related illnesses, adrenal tumors and neuronal tumors has become more defined. The rapid analysis time and high degree of sensitivity provided by high-performance liquid chromatography, when coupled with amperometric detection, is rapidly making this technique the method of choice in many laboratories (14).

The choice among sample cleanup methods is not as well defined. Characteristic concentrations in the picogram per milliliter range require a cleanup method that is fast and reproducible, and will provide maximum sample recovery.

Published results from the alumina batch extraction method commonly used for catecholamine analysis show recovery values ranging from 45% (15) to 74% (16). The method is also rather time consuming, requiring approximately 45 minutes per sample. Other methods, involving a batch extraction of the catecholamines on a boric acid gel (16), provide good recovery of the catecholamines (in excess of 91%). But these methods have sample preparation times of at least 15 minutes, with an additional 15 minute turnaround time between each analysis to reactivate the gel.



Extraction of plasma catecholamines on a single, disposable Supelclean LC-WCX SPE tube allows complete plasma cleanup in less than 10 minutes, with no turnaround time between analyses. The method also provides relative catecholamine recovery values greater than 95%. Absolute recovery was 91.1 $\pm$ 4.4% and 102 $\pm$ 6.5%, respectively, for plasma samples spiked with 205 pg and 615 pg of norepinephrine. Absolute recovery of epinephrine was 91.9 $\pm$ 9.2% for 103 pg and 93.1 $\pm$ 7.3% for 309 pg. Absolute recovery of the internal standard, DHBA, was 95.0 $\pm$ 1.8%.

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