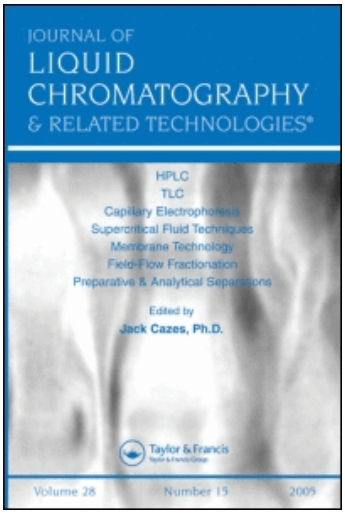

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Rapid Solid-Phase Extraction of Dopamine, Serotonin, and Their Acidic Metabolites for High-Performance Liquid Chromatography with Electrochemical Detection

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RAPID SOLID-PHASE EXTRACTION OF DOPAMINE, SEROTONIN, AND THEIR ACIDIC METABOLITES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A rapid solid-phase extraction procedure utilizing C18 reverse-phase mini-columns has been established for dopamine, 3,4-dihydroxyphenylacetic acid, serotonin, and 5-hydroxyindoleacetic acid in tissue perfusate samples. The method rapidly separates the analytes from protein in albumin-containing tissue perfusates. The analytes which are recovered in high yield are then determined by high-performance liquid chromatography with electrochemical detection. The mean interassay coefficient of variation for the recovery of dopamine, 3,4-dihydroxyphenylacetic acid, serotonin and 5-hydroxyindoleacetic acid was 12, 7, 13 and 8 percent, respectively.

INTRODUCTION

The measurement of biogenic amines and their metabolites in biological fluids has been performed by radioenzymatic assays (REA) (1), gas chromatography coupled with mass

spectrophotmetry (GC/MS) (2) and by high performance liquid chromatography (HPLC) with electrochemical detection (ED) (3-5) or fluorometric detection (FD) (6). Methods for sample preparation prior to assay depend upon the complexity of the sample matrix and the components to be measured. Samples with a relatively low protein content can often be directly analyzed without significant pre-treatment (7). On the other hand, serum samples with high protein concentrations are usually extracted with powdered alumina before assay (8).

We were interested in measuring dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and serotonin (5-hydroxytryptamine) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) by HPLC-ED in solutions of tissue perfusate containing large amounts of bovine serum albumin. The albumin had to be removed from the sample prior to injection on HPLC in order to avoid its retention and build-up on the analytical column. Several sample preparation methods, including alumina extraction (8), trichloroacetic acid (TCA) (9), perchloric acid (PCA) and ethyl acetate extraction (10), were attempted and found to be unacceptable. Alumina extraction failed to recover serotonin and 5-HIAA. Acid precipitation with TCA or PCA resulted in recoveries of less than 20%. Finally, extraction with ethyl acetate failed to recover dopamine, 5-HIAA and DOPAC. By significantly modifying an extraction procedure utilizing reverse-phase C18 mini-columns originally described by Minegishi and Ishizaki (11), we have been able to obtain good recoveries of serotonin, 5-HIAA, dopamine and DOPAC from perfusate samples containing high concentrations of albumin.

MATERIALS AND METHODS

Serotonin, 5-HIAA, DOPAC and n-acetylserotonin (NAS) were obtained from Sigma (St. Louis, MO, USA). Dopamine was from Aldrich (Milwaukee, WI, USA). Stock solutions of dopamine, DOPAC and serotonin were prepared in 0.1 M perchloric acid at a

concentration of 100 µg/ml. Stock solutions of 5-HIAA were prepared at 120 µg/ml. All other solutions were made with reagent grade chemicals. The perfusion buffer consisted of 135 mM sodium chloride, 3.5 mM potassium chloride, 1 mM magnesium sulfate, 20 mM sodium bicarbonate, 2.5 mM calcium chloride, 3.3 mM dextrose, 0.02 mM bacitracin, 0.006 mM dithiothrietol and 0.5% bovine serum albumin (5 mg/ml) at a pH of 7.0. Bond Elut C18 extraction columns (100 mg) were obtained from Analytichem International (Harbor City, CA, USA). BCA protein assay reagent was obtained from Pierce.

Extraction Procedure

Bond Elut C18 extraction columns were placed in 16 x 125 mm borosilicate disposable culture tubes. They were activated with 1 ml of 100% methanol and washed with 1 ml of hplc-grade water. The columns were then centrifuged at 1600 x g for 15 sec (23°C) to remove excess water. One nanogram (4.58 pmol) of the internal standard NAS was added to each 0.2 ml volume of sample. Samples (0.2 ml) were applied to the Bond Elut C18 extraction columns and centrifuged as before. The columns were removed from the waste collection tubes and inserted onto 0.25 ml microcentrifuge tubes (Fisher #05-407-8). Methanol (0.2 ml) was added to the columns and the analytes were removed from the column by centrifugation as described above. The microcentrifuge tubes containing eluents were evaporated to dryness in a Savant Speed-Vac for 45 min (<0.5 TORR at -106°C). The samples were then reconstituted in 200 µl of HPLC mobile phase and 50-100 µl were injected onto the HPLC column. Using this procedure, twenty samples could be extracted in 30 min.

High-Performance Liquid Chromatography

The chromatographic system consisted of a Waters 6000A pump, a Waters WISP 710B automatic sample processor, an LC-4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN) and Chromatochart-PC software (Interactive

Microware, Inc., State College, PA) used for data acquisition and integration. The detector was a thin-layer analytical cell containing a glassy carbon dual-electrode in parallel with an Ag/AgCl gel reference electrode. Data was acquired from a single electrode which had an applied potential of +800 mV. Full scale was set at 2 nanoamperes (nA) with an offset of 0 to 0.5 nA. Separations were carried out on a 100 x 3.2 mm VeloSep RP-18 reverse-phase column (Brownlee Labs). The mobile phase consisted of 114 mM monochloroacetic acid, 200 mM sodium hydroxide, 0.09 mM disodium ethylenediamine tetraacetate, 1.6 mM sodium heptanesulfonate, 0.6% tetrahydrofuran (THF), pH 4.00. The THF was added to the mobile phase only after the other components had been filtered through a 0.2 μM filter and degassed under vacuum for 20-30 minutes. The mobile phase was run at a flowrate 1 ml/min and was not recycled.

RESULTS AND DISCUSSION

When large amounts of protein are injected onto a C18 analytical HPLC column, it soon looses efficiency and retention characteristics. This was observed when 100 μl of spiked perfusion buffer containing 500 μg of albumin was directly injected several times onto a VeloSep C18 column (Figure 1). In the first injection, shown in chromatogram 1A, DOPAC, dopamine, 5-HIAA and serotonin are clearly resolved with retention times of 3.59, 4.06, 6.23 and 10.73 min, respectively. However, after 20 injections, as shown in chromatogram 1B, the separation has deteriorated with retention times for the same four standards being 3.17, 3.38, 5.37 and 8.54 min. The accumulation of albumin on the column caused decreased retention times which soon made it difficult to resolve dopamine from DOPAC. Even after washing the column with water and a variety of organic solvents, there was no improvement (not shown) in column separation. However, when albumin was removed by extraction on the C18 Bond Elute

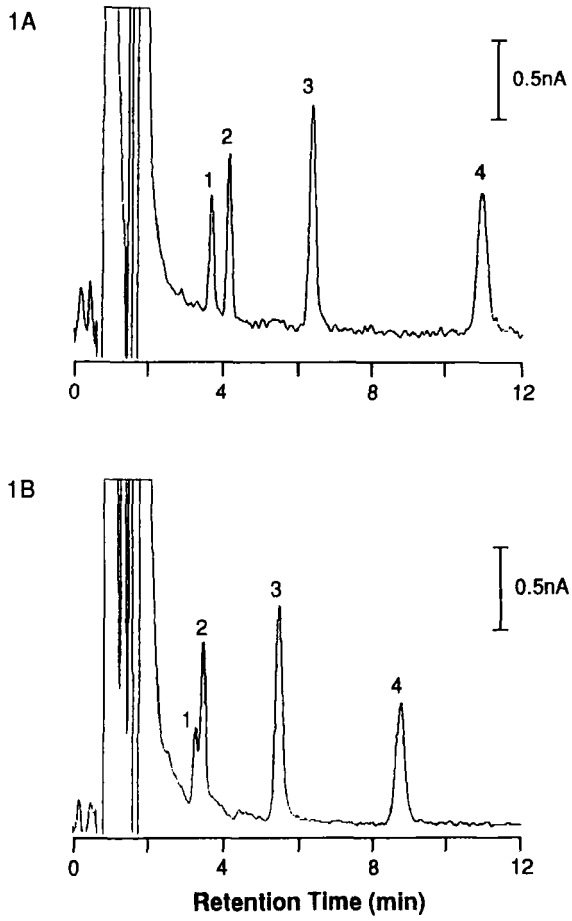

FIGURE 1. High-performance liquid chromatographic separation of standards determined by amperometric electrochemical detection without prior extraction. The perfusate sample (100 µl), containing 500 µg BSA, 250 pg DOPAC (1), 250 pg dopamine (2), 300 pg 5-HIAA (3) and 250 pg serotonin (4), was injected directly into the HPLC. Figure 1A represents the first sample in a series; Figure 1B represents the 20th injection in the same series.

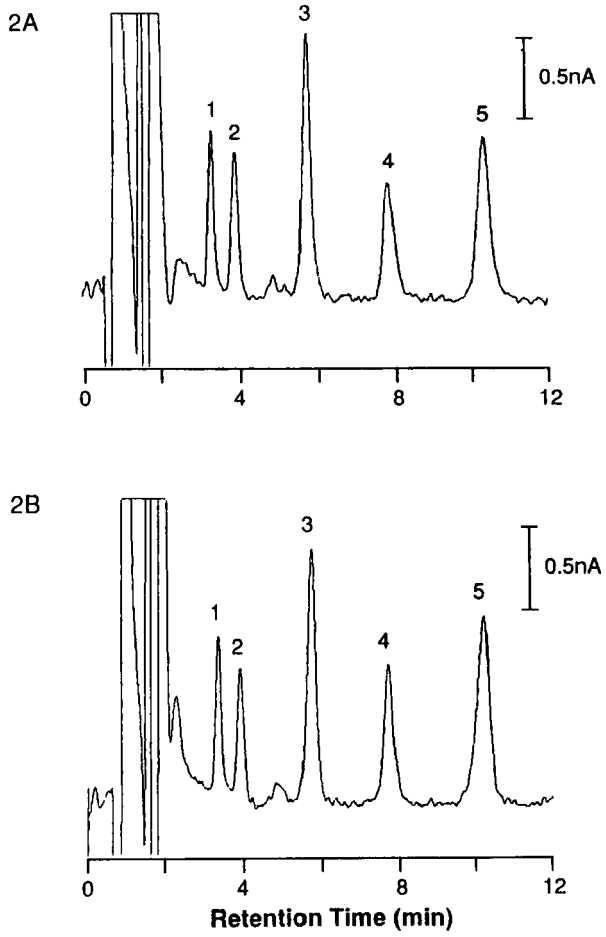

FIGURE 2. High-performance liquid chromatographic separation of standards after Bond Elut C18 extraction. The perfusate sample (200 µl), containing 1000 µg BSA, 1000 pg DOPAC (1), 1000 pg dopamine (2), 1200 pg 5-HIAA (3), 1000 pg NAS (4) and 1000 µg serotonin (5), was applied to a Bond Elut C18 column, centrifuged and the analytes eluted with 200 µl of methanol. The eluent was dried and reconstituted with 200 µl HPLC mobile phase of which half (100 µl) was used for HPLC sample injection. Figure 2A represents the first sample in a series; Figure 2B represents the 30th injection in the same series.

TABLE 1

Recovery of Standards from BSA-Containing Perfusate Samples after Bond Elut C18 Extraction

		DOPAC		DOPAMINE		5-HIAA		NAS		SEROTONIN	
Day	N[a]	%R[b]	%CV[c]	%R	%CV	%R	%CV	%R	%CV	%R	%CV
1	9	95	11	82	5	96	13	97	11	113	19
4	9	76	9	68	25	88	9	99	12	94	12
5	4	89	10	67	10	88	10	ND	ND	ND	ND
6	4	83	7	56	13	75	7	85	10	80	8
7	2	80	9	67	3	83	4	79	7	82	3
26	3	84	7	62	5	89	14	96	11	93	11
27	2	85	1	66	5	91	10	108	1	96	<1
28	3	93	6	62	14	96	4	99	7	93	11
33	3	87	4	57	4	86	11	87	6	85	5
34	2	89	3	57	<1	86	5	91	18	86	<1
35	3	88	11	62	13	91	9	95	4	86	4
40	3	74	15	56	18	76	13	77	15	74	20
41	3	75	14	54	20	77	14	80	12	63	22
42	3	77	5	52	9	76	3	85	6	76	3
47	3	81	12	67	33	76	13	89	5	93	13
55	2	85	7	59	17	91	4	95	<1	95	9
63	2	80	16	51	13	90	13	77	17	80	10
Average Mean		84	7	61	12	86	8	90	9	87	13

[a] N = Number of injections done on individual days
[b] %R = Percent recovery (mean)
[c] %CV = Percent coefficient of variation

columns prior to HPLC, retention times for dopamine, DOPAC, serotonin, 5-HIAA and the internal standard NAS were unchanged after more than 30 injections (Figure 2). The first injection after C18 extraction, chromatogram 2A, shows retention times for DOPAC, dopamine, 5-HIAA, NAS and serotonin as 3.27, 3.87, 5.68, 7.71 and 10.13 min, respectively; and, after 30 injections, chromatogram 2B retention times were 3.24, 3.80, 5.59, 7.50 and 9.88 min, respectively.

To determine the amount of albumin injected onto the HPLC after C18 column extraction, the protein content was measured in each step of the extraction procedure. In a 200 µl sample containing 1,000 µg of BSA, 82% (820 µg) of the BSA was removed from the sample after initial loading and centrifugation, approximately 9% (90 µg) remained on the extraction column, 4.5% (45 µg) was injected onto the HPLC, and 4.5% remained in the injection vial. Even though a small amount of BSA is still being injected onto the column, it did not present a problem with mutliple sample determinations.

Spiked perfusion buffer samples were extracted and assayed along with actual tissue perfusate samples. The recoveries and percent covariances of these spiked samples (n=60) were calculated for eighteen assay days over a three-month period. As shown in Table I, the mean recovery for DOPAC, 5-HIAA, NAS and serotonin were 84%, 86% 90% and 87%, respectively. Dopamine had a consistently lower mean recovery of 61%.

CONCLUSION

A rapid and simple method for the extraction of two biogenic amines, dopamine and serotonin, and their respective acidic metabolites, DOPAC and 5-HIAA, from albumin-containing tissue perfusate is described. The removal of albumin or other proteins from HPLC samples prior to injection is crucial in order to maintain the integrity of the analytical reverse-phase column. With this Bond Elut C18 extraction procedure, over 90% of the albumin was removed. This virtually eliminated the build-up of albumin on the HPLC column and allowed for many samples to be injected without any shift in retention times.

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