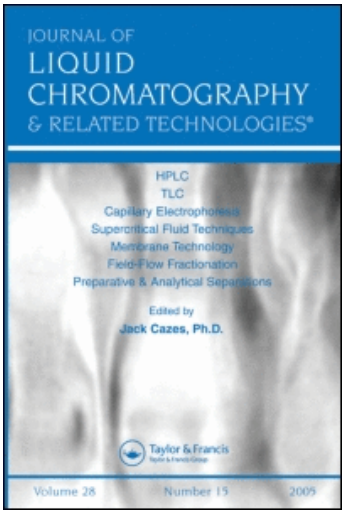

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Fast Analysis of Tissue Catechols Using a Short, High-Efficiency (3 µm) LC Column and Amperometric Detection

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FAST ANALYSIS OF TISSUE CATECHOLS USING A SHORT, HIGH-EFFICIENCY (3 μm) LC COLUMN AND AMPEROMETRIC DETECTION

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ABSTRACT

Tissue catecholamines were analyzed in less than 8 minutes per sample, using a 5 cm LC column, packed with a 3 μm particle size reverse phase sorbent, under conditions which permit fast analysis of tissue extracts of up to 70 μl with no loss in resolution or theoretical plate height. The sensitivity of amperometric detection makes this method suitable for analyzing small amounts of catechols in tissues such as rat brain prefrontal cortex.

INTRODUCTION

The formation and function of catecholamines in biological tissues have been the subject of intense investigation which has resulted in the introduction of several approaches to the quantitative determination of catecholamines. The newest of these involves liquid chromatography with electrochemical detection (1-8). Since the application of electrochemical detection (1),

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the single most important development has been the improvement in chromatographic columns.

Until recently, chromatographic columns have measured 25 to 30 cm in length and have been packed with octadecylsilane particles 5 to 10 microns in diameter. For the analysis of catecholamines with such columns, the determination of noradrenaline (NA) has exemplified the conflict between minimal time per analysis and adequate resolution. Fast analyses were obtained only at the price of allowing NA to be eluted with the solvent front (4,6). On the other hand, if the NA was retained long enough to allow adequate resolution, the time per analysis became excessive (5,8).

One partial solution to this speed-resolution conflict was the use of 5-cm columns packed with 3-micron diameter particles (9). However, such columns were also reported to require minute sample injection volumes (less than 5 microliters (9)), thus limiting the quantity of sample that could be applied to the column and, in consequence, the utility of the approach.

These limitations have now been overcome through the use of a 5-cm 3-micron column, under conditions which yield fast, reproducible analyses of six different catecholamines. In these analyses, NA is resolved from both the solvent front and 3,4-dihydroxyphenylalanine (DOPA). The separation requires less than 1100 psi applied pressure and is complete in less than 8 minutes. As much as 70 µl of sample may be injected without loss of either column efficiency or detector linearity.

MATERIALS AND METHODS

The liquid chromatograph (LC) system uses a Milton-Roy mini-pump equipped with a Waters high-pressure filter, a Waters intelligent sample processor (WISP® 710B; Waters Associates, Milford, MA), an amperometric detector (Bioanalytical Systems Inc., W. Lafayette, IN), and a Kipp & Zonen 2-pen recorder (Rainin Instrument Co., Woburn, MA). An in-line 0.5 µm solvent filter (Upchurch Scientific, Seattle, WA) was placed between the injector and the column. The column used for all of the separations was a 5 x 0.5 cm "Little Champ"® column (Regis Chem. Co., Morton Grove, IL) packed with a 3 µm spherical octadecylsilane (ODS) sorbent.

The mobile phase for separation of catechols was 0.1 M monochloroacetic acid (pK = 2.86, buffered to pH 3.0 with 10 N NaOH), containing 0.3 mM Na^+ octylsulfate 0.1 mM EDTA and 1% (v/v) acetonitrile. The mobile phase was pumped at 1.0 ml/min (~1000 psi).

Catechols were extracted from tissues and prepared for LCEC analysis by a previously described procedure (11), with modifications described as follows: tissues were sonically disrupted in 0.4 to 1.0 ml of 0.1 M $HClO_4$ containing 5 mM cysteine (as an antioxidant) and 2.7 µM 3,4-dihydroxybenzylamine (DHBA as an internal standard for catechol degradation and recovery). The homogenates were centrifuged at 30,000 x g x 10 min; 300 µl aliquots of the clear supernatant were retained for analysis. Catechols were adsorbed onto miniature alumina columns composed of Eppendorf® pipet tips with a pledget of Pyrex® wool and ~20 mg of acid washed alumina (Crout, 1961).

The 300 µl tissue extract was rapidly adjusted to pH 8.6 by adding 75 µl of 2 M Tris-hydroxymethylaminomethane (TRIS), pH 8.6. The alkaline extract was poured atop the columns which were then centrifuged at 50 x g (500 rpm), to drive the extract through the column. The effluent fraction can be acidified with 25 µl of 8 M $HClO_4$ and saved for the assay of indoles and homovanillic acid (11). The columns were washed with ~400 µl of water was discarded) and eluted into Waters limited volume WISP inserts with 75 µl of 0.1 M monochloroacetic acid (unbuffered). The limited volume inserts were placed directly into WISP vials for injection onto the LC.

The catechol standards were obtained from the Regis Chemical Co. (Morton Grove, IL). Acetonitrile (non-UV) was purchased from Burdick & Jackson (Muskegon, MI). All other reagents were of the highest purity, obtained commerically.

Capacity factors (k') and resolution (Rs) were calculated using the following formulas (12):

$$k' = \frac{\text{Retention Time of Peak - Retention Time Solvent Front}}{\text{Retention Time of Solvent Front}}$$

$$Rs = \frac{2\ (\text{Retention Time A - Retention Time B})}{\text{Peak Width A + Peak Width B}}$$

RESULTS

Preparation of samples for LC on miniature alumina columns has been described previously (11). The current modification of eluting with 0.1 M monochloroacetic acid (MCAA) has resulted in a shorter, simpler preparation. The recovery of several catechols

TABLE 1

Compound	Response (pA/pmol)	R.T. (min)	k'	Recovery From Alumina
noradrenaline	500	2.22	1.92	58.1%
3,4-dihydroxyphenylalanine	442	2.76	2.61	46.4%
adrenaline	421	3.67	3.76	55.0%
3,4-dihydroxybenzylamine	416	4.48	4.76	59.2%
3,4-dihydroxyphenylacetic acid	353	5.48	6.15	43.6%
dopamine	280	7.83	9.07	57.2%

R.T. = retention time
k' = capacity factor

is reproducible, as shown in Table 1. Because a high proportion of the sample can be applied to the 5-cm 3-micron column, more than 35% of the tissue catecholamines can actually be injected onto the column. Additional data relating to the separation and quantitation of catechols are presented in Table 1. The void volume of the columns used in these studies was estimated by multiplying the retention time for the inflection of the solvent front by the flow rate. The capacity factor for noradrenaline, the earliest eluting catechol, was nearly 2.0.

The present method appears to be more sensitive than earlier methods (3,5,8) (see later remarks, under DISCUSSION). The detector response, expressed as pA/pmol, is given in Table 1.

A representative chromatogram is depicted in Figure 1a. To illustrate the applicability of this method to a biological sample, a chromatogram of catecholamines in rat brain prefrontal cortex is also shown (Figure 1b). The retention of NA, in the tissue

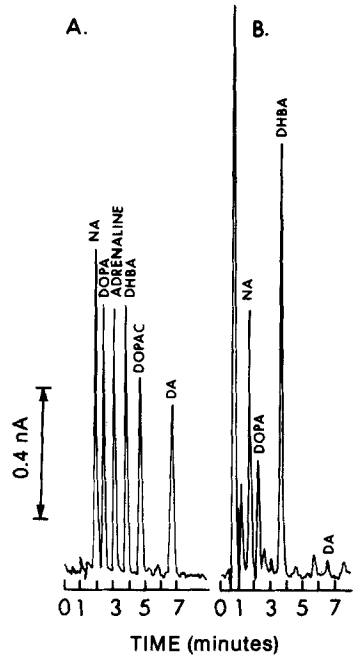

FIGURE 1. Chromatogram of catechols: A. standards (2 pmol each); B. 10 µl of a 75 µl alumina column eluate of rat brain prefrontal cortex from an animal which had been injected with an inhibitor of aromatic amino acid decarboxylase (m-hydroxybenzylhydrazine 0.72 mmol/kg i.p.) 30 minutes earlier.

extract, is sufficiently resolved from the solvent front to permit accurate analysis; the dopamine peak eluted in approximately 7 minutes.

To determine the maximum volume of sample which the column could accept without loss of efficiency, we examined the relationship between injection volume, the height of an experimental theoretical plate (HETP), and resolution (Rs). Figure 2 demonstrates that both HETP and Rs are independent of injection volumes until volumes greater than 70 µl are injected.

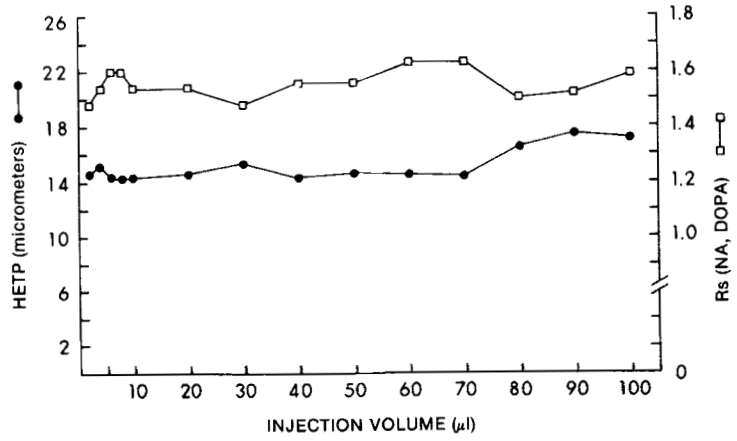

FIGURE 2. The effect of injection volume on theoretical plate height (HETP) and resolution (Rs). A 1 µM solution of catechols was injected in the volumes indicated; HETP and Rs were calculated as described in the text.

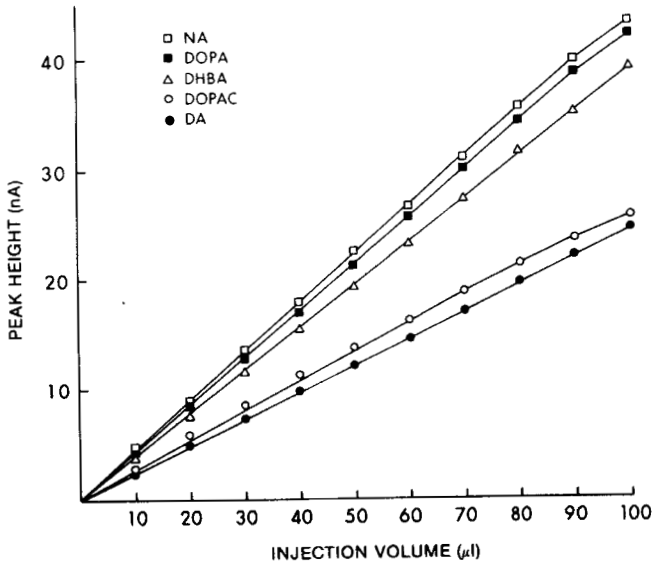

FIGURE 3. Standard curves for catechols as a function of injection volume. A 1 µM solution of catechols was injected at the volumes indicated.

The linearity of the present assay was similarly tested between injection volumes of 2 µl (2 pmol) and 100 µl (100 pmol). Again, detector response was linear for all the catecholamines up to 70 µl (70 pmol) of applied sample.

DISCUSSION

The present method provides conditions for rapid, sensitive, and automated analyses of catecholamines in biological samples. The alumina column step was judged necessary for two reasons: first, alumina elution reduced the number of early eluting peaks which follow the solvent front and obscure the catechol peaks of interest; and second, the preparation eliminated late-eluting peaks (e.g., 5-hydroxytryptamine) which could delay or interfere with the analysis of succeeding samples.

One feature of rapid analysis is an enhanced detector response. However, such enhancement should not be attained by increased oxidizing potential, since it increases both baseline noise and "ghost" peaks. Earlier methods, using longer columns, obtained values of 40 (3), 196 (8), and 300 (5) pA/pmol. However, the 300 pA/pmol response (5) was obtained at the price of an unfavorably high oxidizing potential of 0.9 V. By contrasts, the present method affords a signal of 500 pA/pmol at only 0.7 V oxidizing potential.

The present method also offers adequate resolution of all the catechols examined in less than 8 minutes. Earlier methods have achieved similar resolution, but with retention times of up to 1 hour (8). The present capacity factors, however, are comparable

to those obtained with methods which involve longer retention times (5).

The fast analysis in this case is determined by the combination of the shortness of the column and the high efficiency that results from the 3-micron diameter of the particles. The very small particle size also decreases the loss in column efficiency that typically results from increased, higher-than-optimum flow rates (9,12). Thus the separations shown can be maintained at flow rates even higher than those reported here, yielding these same separations in less than 3 minutes per sample.

FOOTNOTE

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