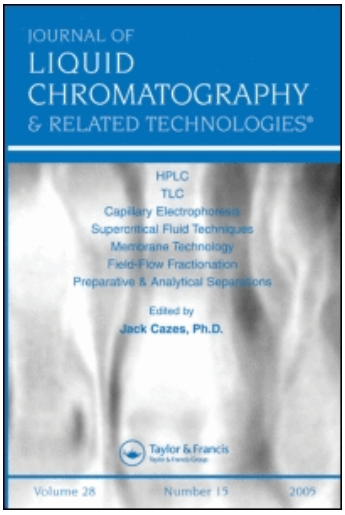

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Simultaneous Determination of Dopamine, Norepinephrine, Tyramine and Octopamine by Reverse-Phase High Performance Liquid Chromatography with Electrochemical Detection

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SIMULTANEOUS DETERMINATION OF DOPAMINE, NOREPINEPHRINE, TYRAMINE AND OCTOPAMINE BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A method is described for the simultaneous separation and estimation of the catecholamines, norepinephrine and dopamine and their monohydroxy-equivalents, octopamine and tyramine. The method employs high-performance liquid chromatographic separation of the compounds on a C18 reverse-phase column with a mobile phase containing methanol as the organic modifier, octane sulphonate as an ion-pair reagent and acetic acid/ammonium hydroxide buffer. The influences of electrode potential and solvent pH on detector response were studied, and the optimal conditions identified as detector potential of 0.95 volts and pH 6.0. The technique of post-column mixing was introduced to provide optimal pH conditions for detector response without the constraint of on-column oxidation of catecholamines. The effects of buffer ionic strength on retention factors and detector response were also investigated and, on the basis of the results obtained, the optimal buffer strength was identified as 0.08-0.09 molar. The described procedure can be used for simultaneous estimation of catecholamines and monohydroxyphenolamines at concentrations between 200-5000 pg.

INTRODUCTION

The monohydroxyphenolamines, tyramine and octopamine have been implicated in a number of nervous disorders including migraine, epilepsy, Parkinsonism, schizophrenia and hepatic encephalopathy (1), and octopamine has been identified also as a putative neurotransmitter in invertebrates (2). The obvious biomedical importance of monohydroxyphenolamines has resulted in the development of several analytical procedures to determine concentrations in nervous tissue. These diverse procedures include the use of radioenzymatic methods (3), gas chromatography mass spectrometry (4), fluorometric techniques (5), and reverse-phase liquid chromatography with detection of derivatised compounds by fluorescence (6) or using amperometric detection of natural compounds (7,8). The relative merits and constraints of the various analytical procedures have been discussed (4,9) and it is apparent that the utility of any technique depends upon the analytical requirements of the particular experimental procedure. One shortcoming in the existing arsenal of analytical techniques for monohydroxyphenolamines is the lack of a procedure that permits rapid, estimation of monohydroxyphenolamines and the equivalent dihydroxy compounds in a single sample.

The present study examines high-performance liquid chromatographic separation of the catecholamines dopamine and norepinephrine and the monohydroxy-equivalents tyramine and

octopamine using an isocratic aqueous mobile phase flowing over a bonded octadecylsilane solid phase with electrochemical detection of the eluate. The report describes the effects of electrode potential, solvent pH and buffer ionic strength on the separation and detection of the standard compounds and defines optimal chromatographic conditions for estimation of these amines in a single sample.

MATERIALS AND METHODS

Chemicals

Norepinephrine bitartrate, Dopamine HCl and Tyramine HCl were purchased from Calbiochem (La Jolla, Ca., U.S.A.). Octopamine HCl was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and other chemicals, except when otherwise indicated, were also obtained from this source and were of reagent grade. Stock solutions of standards were prepared in 0.1 M $HClO_4$, 5.0 mM sodium bisulfite at a concentration of 40 µg/ml and stored at 4°C. Working standard solutions were prepared freshly each week by diluting stock solutions in methanol to give a final concentration of 200 ng/ml for each compound.

Equipment

Chromatographic separations were performed on a 15 cm x 3 mm I.D. stainless steel column packed with 5 µm Ultrasphere RP-18

ion pair material (Beckman). The mobile phase comprised acetic acid/ammonium hydroxide buffer containing 0.1 mM sodium EDTA, 2.5 mM sodium octane sulphonic acid (Aldrich Chem., Milwaukee, Wisc., U.S.A.; Bioanalytical Systems, Lafayette, Ind., U.S.A.; or Helix Associates, Newark, Del., U.S.A.) and 20% methanol (HPLC grade, Caledon Laboratories, Ont.). Prior to the addition of methanol, the buffer was filtered through a 0.22 μm millipore filter. The mobile phase was helium degassed and pumped at 2.0 ml/min by a Spectra Physics Model 740B pump through a Waters WISP 710A autosampler onto the analytical column. A Brownlee MPLC RP-18 SPHERI-5 guard column was installed between the injector and analytical column. Chromatographic separations were performed at ambient temperatures.

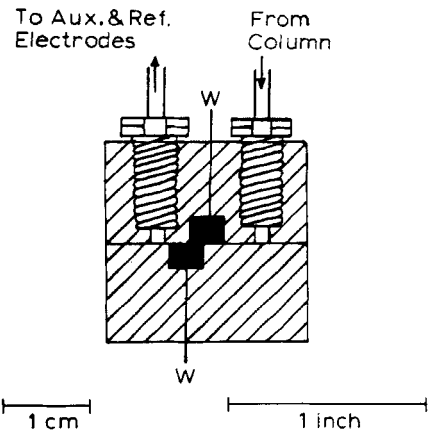

Figure 1. Modified thin-layer amperometric detector showing position of working electrodes.

Detection was accomplished electrochemically by means of a modified Bioanalytical Systems (Layfayette, Ind., U.S.A.) Model TL-5 glassy carbon detector cube and a Model LC-3 potentiostat. The electrochemical potential was adjusted to desired potentials against a silver-silver chloride reference electrode. The detector cube was modified to include a second glassy carbon working electrode surface as illustrated in Figure 1. This modification greatly increased the signal response without any appreciable increase in background current.

Peak height and area calculations of individual peaks were accomplished with the aid of a Spectra Physics Model 4100 computing integrator.

Experimental

Determination of optimal detector potential was achieved by making repeated injections of standard solutions at increasingly positive electrode potentials. This was continued until a maximum peak height for the component of interest was obtained. The current ratio at any potential was calculated by dividing the current (peak height) by that obtained at the most positive potential. The electrochemical response for monophenolamines at different pH values was determined by adjusting the buffer components, acetic acid and ammonium hydroxide, to a specific pH while maintaining the total ionic strength at approximately 0.06 Molar.

Changes in capacity factor (k') and detector response were analysed by altering the ionic strength of the buffer while maintaining a constant pH at 6.0.

Following the determination of the buffer conditions required to provide optimal response and capacity factors for all sample components, the linearity of the detector response was measured and approximate limits of detection for the sample components calculated.

RESULTS AND DISCUSSION

The estimation of catecholamines by HPLC/EC procedures is widely accepted (10), however the concomitant measurement of monohydroxyphenolamines has not yet been developed and, indeed, the application of amperometric detection to monohydroxyphenolamines separated by HPLC is limited to a few reports (7,8). An essential condition for detection of a compound electrochemically is that the electrode potential should be sufficiently high to effect electro-oxidation of the compound. The effect of electrode potential on the detector response to tyramine and octopamine is illustrated in Fig. 2. It is apparent that octopamine is not detected when the applied potential of the electrochemical cell is less than 0.7 volts whereas a slight response for tyramine is evident at this potential. The observed response for tyramine at detector potential of 0.7 volts appears to be at variance with a previous report which indicates no

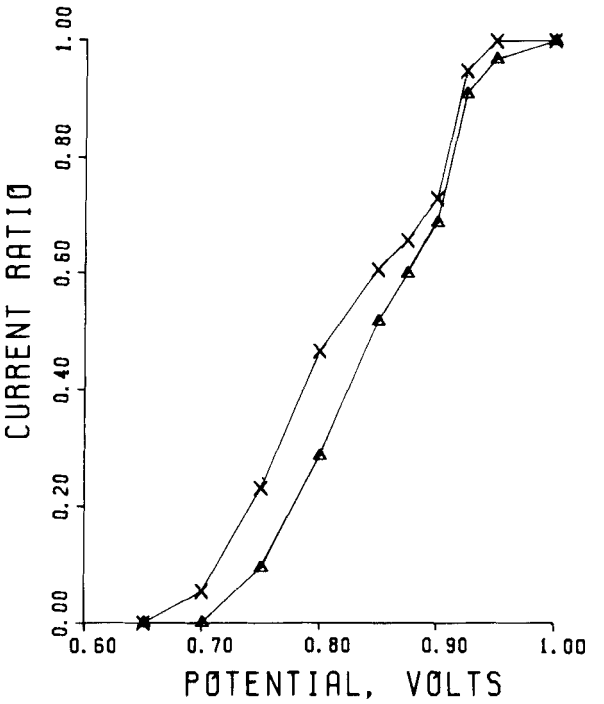

Figure 2. Hydrodynamic voltammograms of tyramine (x) and octopamine (Δ) standards.

detector response for this compound at 0.7 volts (11), although the overall pattern of the hydrodynamic voltammograms in the two studies are very similar. The slight difference in the minimum electrode potential that is required for detector response may be explained by the different pH employed in the two investigations. The pH (6.0) used in the present study facilitates oxidation at the electrode surface and enables octopamine and tyramine to be detected at a lower detector potential than under more acidic conditions. The results presented in Fig. 2 indicate also that

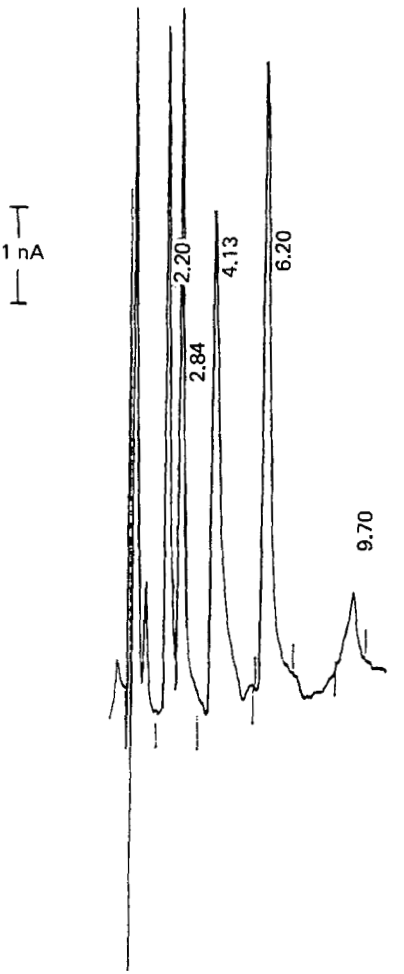

Figure 3. High-performance liquid chromatogram of a standard solution containing 5 ng norepinephrine (RT:2.20), octopamine (RT:2.84), dopamine (RT:4.13), tyramine (RT:6.20). Chromatographic conditions are described in the text.

the maximal detector response for octopamine and tyramine is obtained with an applied potential of 1.0 volts. However, for practical purposes it is convenient to operate the detector potential at 0.95 volts. Reduction of the potential from 1.0 volt to 0.95 volts results in an appreciable decrease in background current and noise, and also eliminates the necessity for frequent cleaning of the detector surface that is apparent at 1.0 volts.

A typical chromatogram for separation of 5 ng samples of dopamine, norepinephrine, tyramine and octopamine with detector potential set at 0.95 volts is shown in Fig. 3. The buffer used to obtain this chromatograph contained 0.04 M acetic acid and 0.04 M ammonium hydroxide and was adjusted to give a final pH of 6.0. The peak appearing at 9.70 minutes is a contaminant associated with sodium octane sulphonic acid and was present in material obtained from several manufacturers.

Monohydroxyphenolamines have a higher oxidation potential than their dihydroxy-equivalents, and this difference is reflected in the effect of pH on detector response to the compounds under investigation. Fig. 4 demonstrates the effect of pH on detector response, as measured by peak height, and indicates increased oxidation of octopamine and tyramine at the electrode surface with increasing pH. The reduced signal for norepinephrine and dopamine at a pH above 5.75 is also apparent and confirms an earlier report by Moyer and Jiang (12). The

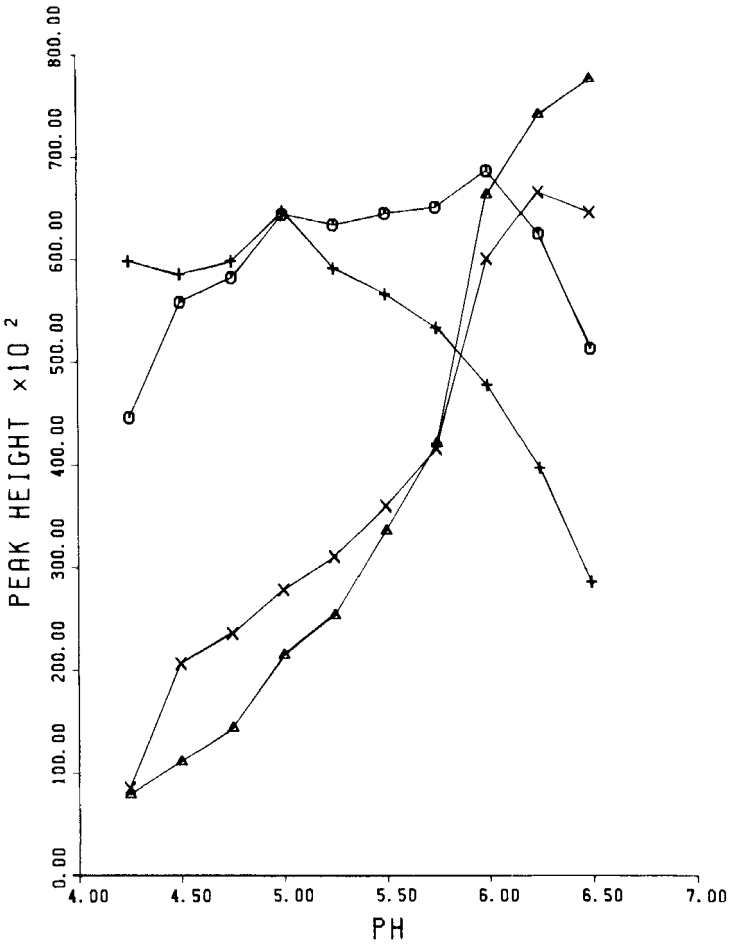

Figure 4. Effect of solvent pH on detector response to 5 ng samples of norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) following separation by HPLC. Coefficients of variation were calculated for the 7-10 determinations that comprise each point and range from 0.445-2.898 (norepinephrine), 1.044-7.329 (octopamine), 0.528-2.371 (dopamine) and 1.082-5.791 (tyramine).

reduction in detector response for catecholamines at higher pHs may be due to auto-oxidation of the compounds prior to their arrival at the detector cell. The data presented in Fig. 3 indicate that the optimal solvent pH at which to obtain an acceptable level of detector response for all compounds included in this study is pH 6.0; however, if only monohydroxyphenolamines are being estimated the solvent pH may be increased above 6.0.

In addition to promoting on-column oxidation of catecholamines the use of more alkaline solvents also increases the amount of detector background current. These constraints can be overcome by the use of post-column mixing, a technique that has previously been described by Kissinger et al. (13). This requires assembly of a three-way T-union between the analytical column and the detector cell. In the present system one inlet port admits a solvent with a higher pH than that of the mobile phase buffer, which enters through the second inlet port following passage through the column. The two solvents mix in a short teflon mixing coil prior to their entry into the detector cell. A pulse-free pumping source for the post-column solvent is provided by an adapted reservoir with flow restrictor attached to a pressurised nitrogen cylinder. The use of post-column mixing permits the pH for chromatography and electrochemical detection to be optimised separately.

The influence of ionic strength on the retention of catechol compounds has been demonstrated (12,14), and the theoretical

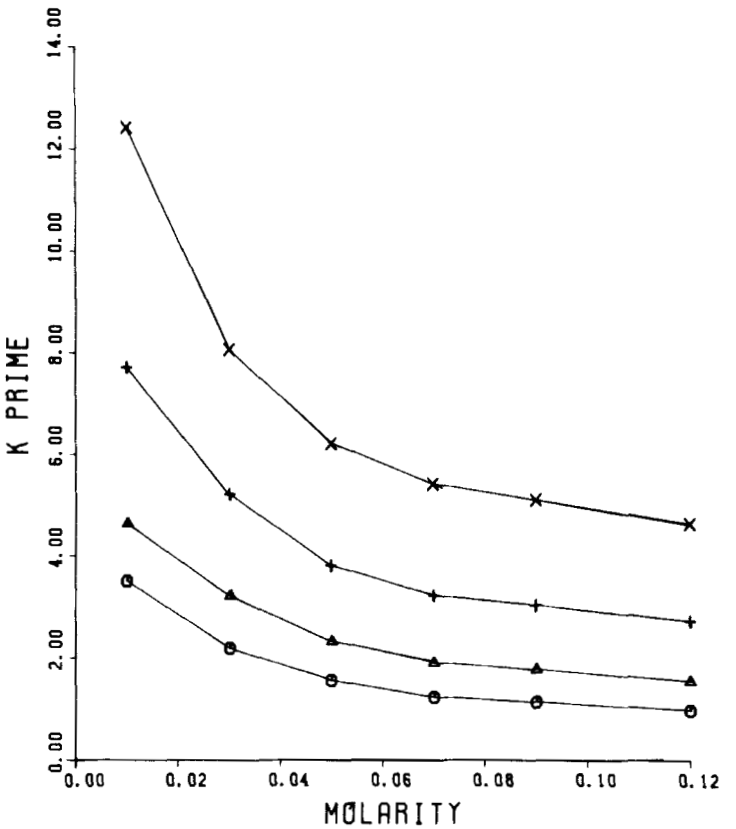

Figure 5. Effect of molarity of solvent buffer on retention of norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) during HPLC separation on RP-C18 column. Coefficients of variation were calculated for the 6-10 determinations that comprise each point and range from 0.441-1.390 (norepinephrine), 1.096-3.986 (octopamine), 0.681-2.840 (dopamine) and 1.004-2.975 (tyramine).

implications of solvophobic interactions have been discussed (15). In the present study, the effect of changes in molarity of acetic acid and ammonium hydroxide on the capacity factor (k') was investigated and the results are illustrated in Fig. 5. It is evident that k' tends to decrease as the molarity of the buffer increases, and this effect may be explained in terms of alterations in hydrophobicity of solute molecules due to changing amounts of acetic acid in solution. Thus, the results presented in Fig. 5 should not be equated with earlier studies on absolute ionic strength (13,15).

Fig. 6 illustrates the effect of molarity on detector response as indicated by measurement of peak height. The data presented in this figure must be considered in association with that of Fig. 5, which demonstrates the greater retention time of the standard compounds at low molarities. The increased retention time results in broadening of peaks and reduced peak height, and also increases the possibility of on-column auto-oxidation at pH 6.0; thus, the observed reduction in detector response at low molarities may be due in part to the change in k'.

On the basis of the data contained in Figs. 5 and 6 the buffer strength selected to achieve optimal chromatographic conditions was between 0.08 and 0.09 molar. At this molarity the capacity factor for each amine is within the optimal limits of between 1 and 6 (Fig. 5) and a high detector response is also

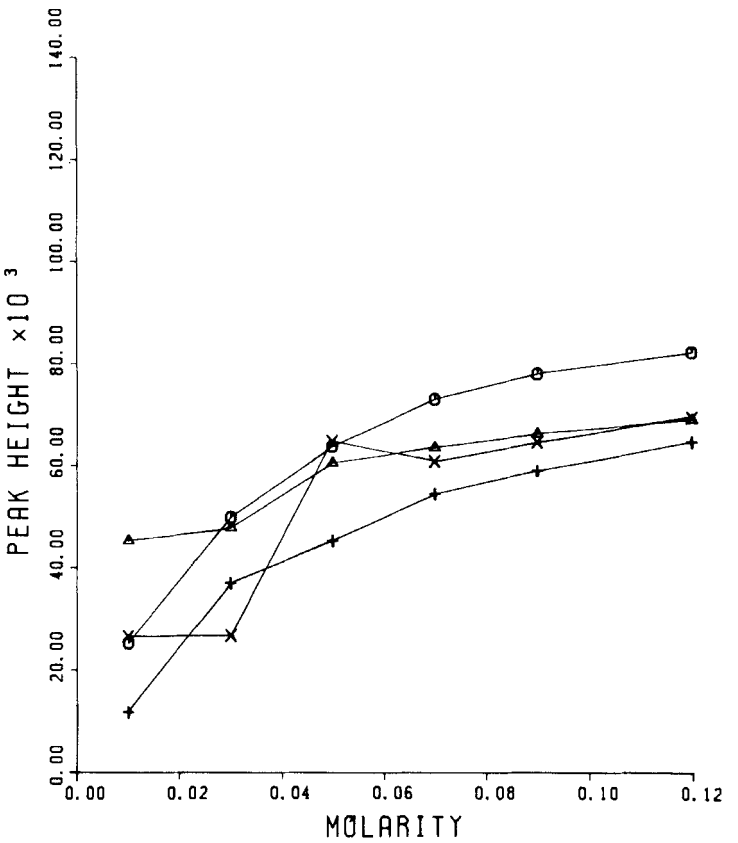

Figure 6. Effect of molarity of solvent buffer on detector response to norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) as measured by peak area following separation by HPLC. Coefficients of variation were calculated for the 7-10 determinations that comprise each point and range from 1.073-1.884 (norepinephrine), 2.149-4.615 (octopamine), 2.479-5.334 (dopamine) and 1.246-6.295 (tyramine).

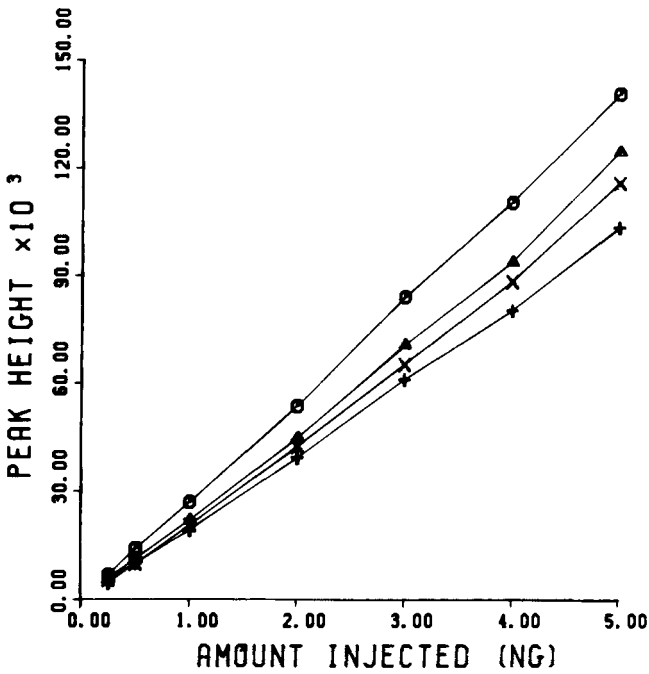

Figure 7. Linearity of estimation of norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) by HPLC with electrochemical detection. Linear regression analyses are presented in Table 1.

obtained (Figs. 6). The detector response continues to increase at molarities above 0.1 molar, but associated with the increased response is an excessive increase in background current.

Fig. 7 demonstrates the linearity of detector response to the four test amines using concentrations ranging from 250 to 5000 picograms. Linear regression analyses of these data are given in Table 1 and confirm that the chromatographic procedure described in this report permits accurate and simultaneous

TABLE 1

Linear regression analysis of effect of amine concentration on electrochemical detection following separation by high-performance liquid chromatography.

COMPOUND	SLOPE ± S.E.	Y-INTERCEPT ± S.E.	CORRELATION COEFFICIENT (R SQUARE)	APPROXIMATE DETECTION LIMIT (PG)*
Norepinephrine	2.826 ± 0.025	-0.069 ± 0.065	.999	131
Octopamine	2.469 ± 0.052	-0.172 ± 0.136	.997	191
Dopamine	2.074 ± 0.023	-0.086 ± 0.059	.999	186
Tyramine	2.306 ± 0.043	-0.155 ± 0.112	.998	197

*limit established with signal:noise ratio of 4:1.

determination of catecholamines and monohydroxyphenolamines at concentrations below 200 picograms. Indeed, as the present experiments were conducted with detector sensitivity at only 5 nA full scale, the potential for even greater sensitivity is considerable. The application of this procedure to extracted biological samples will be reported in a subsequent publication.

ACKNOWLEDGEMENTS

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