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Short communication

Reversed-phase high-performance liquid chromatography of catecholamines and indoleamines using a simple gradient solvent system and native fluorescence detection☆

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Abstract

A reversed-phase HPLC method using a C_{18} column and a two-mobile-phase gradient elution system containing only volatile components has been developed for separation of norepinephrine, octopamine, epinephrine, dopamine, dihydroxyphenylalanine, tyramine, tyrosine, serotonin, 5-hydroxytryptophan, N-acetyl-serotonin and tryptophan. Mobile phase A contains 0.05% aqueous trifluoroacetic acid and methanol (97.5:2.5, v/v) and mobile phase B contains 0.05% aqueous trifluoracetic acid and methanol (40:60, v/v). This method has the advantage that the mobile phase can be removed completely, without salt residues, from the eluted fractions thus simplifying further analytical procedures on isolated fractions. The elution profile of standards is related to structural characteristics allowing prediction of retention times of known compounds and insight into possible structural characteristics of unknown components in a mixture. Detection is via native fluorescence using excitation at 220 nm and emission at 320 nm and under the conditions described has a sensitivity range from 2.5 to 25 pmol, although the sensitivity range can be extended depending on the emission wavelength used.

Keywords: Catecholamines; Indoleamines; Biogenic amines

1. Introduction

Analysis of catecholamines and indoleamines, or biogenic amines as they are often called, is of continued interest due to the role they play in the central nervous system of numerous organisms [1–3]. A variety of methods to separate and detect specific catechol or indole amines present in samples of interest has been developed. Different types of HPLC columns including reversed-phase, ion-exchange, and microbore HPLC columns have been used in the separation of biogenic amines, with reversed-phase methods becoming dominate [4–7]. Reversed-phase HPLC analysis using fluorescence detection has become a major method for analyzing biogenic amines [4,5,8–10] with electrochemical detection also being used extensively [6,11–13]. A variety of mobile phases has been developed which provide good resolution for complex mixtures of both catechol and indole amines. These mobile phases usually contain one or more of the following components: buffers of phosphate, acetate or citrate;

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sodium octyl sulphate; EDTA and sodium chloride [14–17]. However, the analysis of catechol and indole amines is often carried out on complex mixtures which contain components other than the standards at hand [18,19]. The ability to separate, elute and collect unknown fractions from a complex mixture for further analysis would be advantageous. However, complex mobile phases used in chromatographic separation of biogenic amines make isolation of a partially purified unknown component difficult.

The reversed-phase HPLC analysis of catecholamines and indoleamines described below employs a gradient elution system using two mobile phases that are volatile and contain no salts or other nonvolatile components. The volatile solvent system is ideal for analysis of complex mixtures that may contain unknown compounds that require isolation and further structural characterization. In addition, the elution profile of standards is related to structural characteristics of the compounds. The separation method was used to investigate the native fluorescent components present in blood of molting tiger prawns, *Penaeus monodon*.

2. Experimental

2.1. Materials and reagents

Norepinephrine, epinephrine, octopamine, dopamine, DOPA, tyramine, tyrosine, serotonin, 5-hydroxytryptophan, N-acetylserotonin, tryptophan, epinine, metanephrine and the disodium salt of EDTA, were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol and trifluoroacetic acid were obtained from Mallinckrodt (Phillipsburg, NJ, USA). Water from a Milli-Q purification system was used to make all solutions and dialysis filters were obtained from Millipore (Bedford, MA, USA).

2.2. Instrumentation

A refrigerated benchtop Jouan MR1822 centrifuge (Saint Herblain, France) was used for blood sample preparation.

The HPLC system comprised a ICI LC 1440 systems organizer, LC 1150 HPLC pump, LC 1650 advanced autosampler, and LC 1255 fluorodetector (GBC, Melbourne, Australia). Data were processed on an IBM 2248 computer.

2.3. Standard and sample preparation

Stock solutions of each standard at 1.25 m*M* were prepared in 0.1 *M* HCl. The stock solutions were stored in the dark at 4°C. Fresh HPLC standards were prepared each day by taking aliquots from stock solutions and diluting them with water to make solutions ranging from 0.25 to 2.5 μ*M*. These solutions were stored on ice.

Blood samples of ≈220 μl were removed by cardiac puncture of a prawn using a 1-ml syringe equipped with a 16G (0.5×16 mm) hypodermic needle. The blood was transferred to a cold 1.5-ml centrifuge tube. A 200-μl aliquot of the freshly removed blood was then combined with 200 μl of ice cold 2% EDTA in filtered seawater. The blood–EDTA samples were either stored at −70°C and centrifuged at a later date, or centrifuged in prewashed Ultrafree MC filters (Cat. No. UFC3LGCNB) with a 10 000 molecular mass (M_r) cut-off for 60 min at 5000 *g*. The filtrates were stored at −70°C. Filtrates were analyzed directly by HPLC.

2.4. Chromatography

Standards and samples were analyzed at room temperature using either a 5-μm Alltech Hypersil BDS C_{18} (150×4.6 mm I.D.) column or a 5-μm Alltima C_{18} (150×4.6 mm I.D.) column (Alltech, IL, USA). Mobile phase A consisted of 0.05% aqueous trifluoroacetic acid (TFA)–methanol (97.5:2.5, v/v) and mobile phase B consisted of 0.05% aqueous TFA–methanol (40:60, v/v). The mobile phases were prepared as needed and degassed by helium sparging prior to use and by maintaining a positive pressure head of helium over the solvent during chromatography. Injection volumes of both standards and blood–EDTA samples were 10 μl. A flow-rate of 1.0 ml min^{-1} was used over 20-min with the following gradient: 0.00 min, 100% A; 1.00 min, 100% A; 16.00 min, 50% A and 50% B (linear gradient from 1 to 16 min); 16.05 min, 100% A to return column to initial conditions by 20 min.

Detection was by native fluorescence using excitation at 220 nm and emission at 320 nm.

3. Results and discussion

The HPLC elution profile of eleven standards illustrates the resolution obtained with the gradient described (Fig. 1). Linearity of all standards, except serotonin and N-acetylserotonin, was obtained from 2.5 pmol to 25 pmol using excitation at 220 nm and emission at 320 nm (Table 1). Linearity for serotonin and N-acetylserotonin was obtained from 2.5 to 10 pmol under the conditions used. The maximum emission range, using excitation at 220 nm, for each of the standards is given on Table 1. Altering the emission wavelength of detection would allow expansion of the concentration–response curve either to smaller amounts (detection in the maximum emission range) or greater amounts (detection further from the maximum) of any of the standards.

Fig. 2 illustrates the elution profiles of three samples: prawn blood taken 1 day after molt; prawn blood taken 1 day after molt spiked with five standards; and five standards. The DOPA standard coeluted with the first major blood sample peak, however, the asymmetry of the peak in the blood profile did not allow conclusive characterization or quantification of the blood component as DOPA. The tyrosine and tryptophan standards coeluted with major peaks 2 and 5 of the blood sample. The blood sample had two peaks that have yet to be identified.

Two aspects of this reversed-phase HPLC separation of catecholamines and indoleamines are noteworthy. First, the mobile phase of the system is

Table 1
Standard compound's R^2 values from a linear regression analysis of peak area (emission at 320 nm) versus 2.5–25 pmol of each standard (except where indicated), and the λ_{max} range of fluorescence emission under chromatographic conditions using 220 nm excitation; $n=17$ except where indicated

Standard	R^2	Range of λ_{max} (nm)
Norepinephrine	0.999	320–326
Octopamine	0.999	305–311
Epinephrine	0.999	320–326
Dopamine	0.986	320–326
Dopa	0.974	311–317
Tyramine	0.994	310–316
Tyrosine	0.991	305–311
Serotonin	0.988[a]	338–344
5-OH-Tryptophan	0.992	344–338
N-Acetylserotonin	0.998[a]	341–347
Tryptophan	0.993	344–350

[a] $n=11$ and linearily is from 2.5 to 10 pmol under the conditions used.

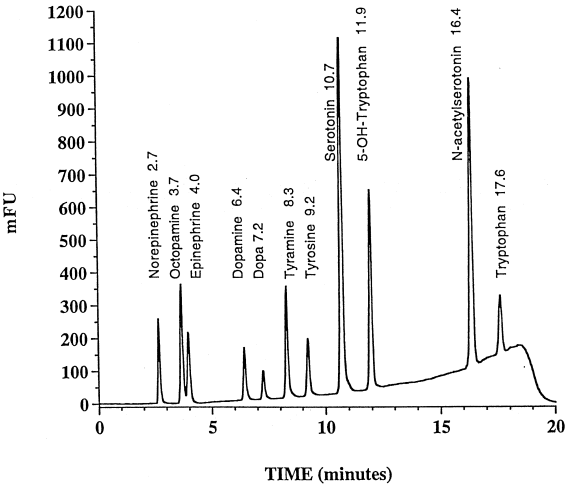

Fig. 1. HPLC elution profile of 10-pmol samples of eleven standards. Retention times are in min. Column: 5-μm Alltima C_{18} (150×4.5 I.D.).

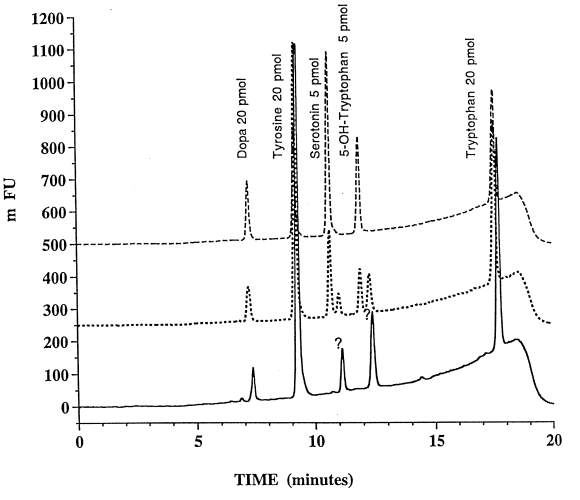

Fig. 2. HPLC elution profile of 10 μl of a prawn blood–EDTA sample (solid lower line), profile of prawn blood–EDTA spiked with five standards (1:1, v:v) (dotted middle line), and profile of five standards at pmols indicated (dashed upper line).

volatile. Because of this, components that are separated, eluted and collected using the system can be isolated easily by simply evaporating the solvent. The components are then ready for derivatization, if desired, or for analysis using another analytical method, if necessary.

Depending on the original complexity of the sample being analysed, the gradient may be altered to include a 0.05% TFA in methanol wash. In the case of filtered prawn blood, elution of all components was accomplished without a methanol wash. If a complex sample has hydrophobic components which require elution with a methanol wash, the following gradient maybe used: 0.00 min, 100% A; 1.00 min, 100% A; 16.00 min, 50% A and 50% B (linear gradient from 1 min to 16 min); 20.00 min, 50% A and 50% B; 20.05 min, 100% C (C being 0.05% TFA in methanol); 24.00 min, 100% C; 24.05 min, 100% A to return column to initial conditions by 28 min.

Reversed-phase HPLC allows prediction of relative retention times for structurally related compounds. For example, with this solvent system as with others [14,15,17] the secondary amine, epineprine, has a longer retention time than its structurally analogous primary amine, norepinephrine (Fig. 1). One can then predict that the secondary amine, epinine, will have a longer retention time than its primary amine structural analogue, dopamine (Figs. 1 and 3). Metanephrine, a methoxy analogue of the phenol, epinephrine, elutes after epinephrine (Figs. 1 and 3). However, the second aspect of this solvent system worth note is that amine–amino acid structurally related pairs produce a consistent elution pattern compared to the pattern from other solvent systems [14,15,17]. Pairs of structurally related amines and amino acids elute with a difference in retention times ranging from 0.8 to 1.2 min: dopamine prior to DOPA; tyramine before tyrosine; and serotonin before 5-OH-tryptophan (Fig. 1).

Consistent relative retention times of several structurally related pairs of compounds provides insight into the character of unknowns even though exact structural identification of peaks with retention times different from standards is not possible. For example, Fig. 2 indicates two unknown fluorescent components present in prawn blood. From the difference in retention times it is possible that the components are

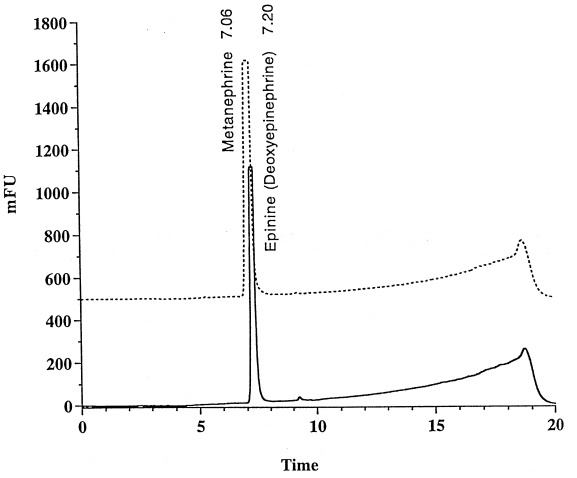

Fig. 3. HPLC elution profiles of 250 pmol each or epinine (deoxyepinephrine) (solid lower line) and metanephrine (dashed upper line).

a structurally related amine–amino acid pair or a primary amine–secondary amine pair.

The simplicity and volatility of the mobile phase components, the resolution obtained for a mixture of catecholamines and indoleamines, and the fact that the difference in retention times of several structurally related pairs of standards is consistent, makes this C_{18} reversed-phase HPLC system particularly suited for investigation of unknown mixtures present in complex samples.

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