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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND DETECTION OF CATECHOLAMINES AND RELATED COMPOUNDS

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SUMMARY

Four sets of chromatographic conditions are described for the separation and identification of selected catecholamines and related chemicals by high-performance liquid chromatography. Three mobile phases, three different columns and three detection systems, including ultraviolet absorption, fluorescence and electrochemical detection are reported. The use of detection response ratios as an additional means of identification is discussed and demonstrated. Nineteen compounds were studied and the retention times and detector responses are reported.

INTRODUCTION

The recent literature contains a large number of reports dealing with the analysis of catecholamines and related compounds. Of those involving high-performance liquid chromatography (HPLC), several investigators have employed precolumn radiolabelling of the compounds of interest, with liquid scintillation counting of collected fractions[1–3], fluorescence detection with pre-column derivatization[4] or oxidation[5], ultraviolet (UV) absorbance detection[6–8], or electro-chemical detection[9–14]. Other compounds related to catecholamines have also been assayed by HPLC[4–6,12]. None of the papers cited includes the separation of all of the compounds reported in this investigation. This report includes several sets of chromatographic conditions with which selected groups of nineteen compounds can be separated and detected. Response of these catecholamines and related chemicals to UV absorption, fluorescence and electrochemical detection is reported and the value of multiple detection and detector response ratios is illustrated.

EXPERIMENTAL

Materials

The compounds investigated were obtained from Calbiochem (Los Angeles, Calif., U.S.A.) or Sigma (St. Louis, Mo., U.S.A.) as various salts or as free compounds and used without further purification. The compounds investigated and their abbreviations are listed in Table I.

TABLE I

RELATIVE RETENTION TIMES AND DETECTION OF CATECHOLAMINES AND RELATED COMPOUNDS

+, − = was (not) detected by this system; UV = ultraviolet absorbance detection at 280 nm; FL = fluorescence detection at excitation 280 nm, emission 315 nm; EIC = electrochemical detection at +0.72 V *vs.* Ag–AgCl. Mobile phases: M-SDS-PA = methanol, 5% (v/v) in aqueous 0.01% sodium dodecyl sulfate and 0.04% H_3PO_4; SDS-PA = aqueous 0.01% sodium dodecyl sulfate and 0.07% H_3PO_4; and C-P-SDS = 300 ml 0.05 *M* citric acid + 160 ml 0.05 *M* Na_2HPO_4 + 15 mg sodium octyl sulfate, maintained at 40°. Columns: ODS = ODS-Sil-X-1, 250 × 2.6 mm I.D. (Perkin-Elmer); HC = ODS-HC-Sil-X-1, 250 × 2.6 mm I.D. (Perkin-Elmer); and RP = LiChrosorb RP-18 10 μm, 250 × 4.0 mm I.D. (Merck).

Compound	*Abbreviation*	*Detection*			*Relative retention times* for selected mobile phase–column systems*			
		UV	*FL*	*EIC*	*M-SDS-PA:ODS*	*SDS-PA:ODS*	*C-P-SOS:HC*	*C-P-SOS:RP*
Creatinine	CRT	+	−	−	1.41	−**	−	***
Epinephrine	EPI	+	+	+	1.40	1.34	1.93	1.98
3,4-Dihydroxy benzylamine (internal standard)	DHBA	+	+	+	−	−	2.31	2.47
3,4-Dihydroxy mandellic acid	DOMA	+	+	+	0.34	0.42	0.48	0.62
3,4-Dihydroxy phenethylamine	DA	+	+	+	2.13	2.01	4.53	4.83
3,4-Dihydroxy phenyl acetic acid	DHPAC	+	−	+	0.44	0.49	2.73	4.70
3,4-Dihydroxy phenyl alanine	DOPA	+	+	+	1.08	1.11	1.24	1.50
3,4-Dihydroxy phenyl glycol	DHPG	+	+	+	0.33	0.43	0.61	0.82
Homovanillic acid	HVA	+	+	+	0.67	0.69	0.87	0.95
m-Hydroxy phenyl acetic acid	MHPAC	+	−	−	0.54	−	−	−
p-Hydroxy phenyl acetic acid	PHPAC	+	+	−	0.53	−	−	−
Metanephrine	MN	+	+	+	2.36	2.95	6.50	8.10
3-Methoxy-4-hydroxy phenyl glycol	MHPG	+	+	+	0.42	0.49	1.53	2.02
α-Methyl DOPA	MD	+	+	+	2.12	2.26	3.94	5.15
β-O-Methyl epinephrine	OME	+	+	+	3.33	3.89	−	−
Norepinephrine	NE	+	+	+	1.00	1.00	1.00	1.00
Normetanephrine	NMN	+	+	+	2.00	1.83	3.01	3.47
Serotonin (5-hydroxy tryptamine)	5HT	+	+	+	6.67	−	−	***
Vanillyl mandellic acid	VMA	+	+	+	0.38	0.46	0.80	1.17

* Retention times relative to NE.

** Was not attempted.

*** Did not elute within 60 min under these conditions.

The individual compounds were dissolved in 0.1 *M* HCl at a concentration of 100 μg/ml. These stock solutions were kept frozen and aliquots were diluted with water to appropriate concentrations, refrigerated, and used within two days.

Apparatus

The chromatographs used were a Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 601 liquid chromatograph and an instrument assembled by the investigators consisting of a Milton-Roy (Laboratory Data Control Division, Riviera Beach, Fla., U.S.A.) mini-pump Model 396, pulse damper, pressure gauge and a Rheodyne (Laboratory Data Control) Model 7010 loop injector. This instrument has been previously described in detail[11,13,14].

The HPLC columns employed were: a pre-packed Perkin-Elmer ODS-Sil-X-1, 250 × 2.6 mm I.D. (ODS); a pre-packed Perkin-Elmer ODS-HC-Sil-X-1, 250 × 2.6 mm I.D. (HC); and LiChrosorb RP-18 10 μm (RP) (E. Merck, Elmsford, N.Y., U.S.A.) column which was packed by the investigators using the slurry technique in a 250 × 4.0 mm I.D. stainless-steel column. The column packing apparatus was obtained from Micromeritics (Norcross, Ga., U.S.A.). A slurry of the RP packing material in isopropyl alcohol was pumped into an empty column, maintaining the back pressure at or near the pressure limit of the pump, which is 5000 p.s.i. After the pressure remained constant with no further adjustments of flow-rate, the pumping was continued for an additional 15 min. The column was then washed with *ca.* 100 ml 1 *M* NaH_2PO_4 prior to use in order to obtain a low background signal with electrochemical detection.

The mobile phases consisted of reagent-grade chemicals in triple-distilled water and the following were utilized: methanol, 5% (v/v) in an aqueous solution of 0.01% sodium dodecyl sulfate and 0.04% H_3PO_4 (M–SDS–PA); an aqueous solution of 0.01% sodium dodecyl sulfate and 0.07% H_3PO_4 (SDS–PA); and 300 ml 0.055 *M* citric acid + 160 ml 0.05 M Na_2HPO_4 + 15 mg sodium octyl sulfate, maintained at 40° (C–P–SOS).

Three systems of detection were employed: UV detection using a Perkin-Elmer Model LC-55 spectrophotometer set at 280 nm; fluorescence detection utilizing a Perkin-Elmer Model 204 (fluorescence spectrophotometer) with excitation at 280 nm and emission measured at 315 nm; and electro-chemical detection using a Bioanalytical Systems (West Lafayette, Ind., U.S.A.) Model LC-2A detector with the electrochemical potential set at + 0.72 V *vs.* Ag–AgCl.

RESULTS AND DISCUSSION

As can be seen from Table I, any of the mobile phase–column combinations listed can be employed to chromatograph selected groups of compounds. Also in Table I is an indication of the detection methods which can be used for each compound.

One of the primary objectives of this investigation was the analysis of the four biogenic amines NE, DOPA, EPI and DA. When employing an ODS column, the mobile phase M–SDS–PA was not able to separate NE and DOPA, while EPI and DA were well resolved. Mobile phase SDS–PA was able to separate NE and DOPA, although DOPA was eluted at the same time as EPI.

Mobile phase C–P–SOS was developed and used in combination with an HC column. With this set of conditions, the majority of the compounds investigated were well resolved. A typical chromatogram of a mixture of standards is shown in Fig. 1.

The use of surfactants for reversed-phase ion-pair HPLC gives good separations, but results in problems with column stability and reproducibility. Short column life was also noted in our laboratory, and, in order to reduce the cost and time lost in securing replacement columns, we elected to pack our own columns with a material which would have retention characteristics similar to those of commercially available, pre-packed columns. The packing procedure is described briefly under Experimental. The RP columns we prepared gave similar retention data to that of the commercial HC column as is evident by comparing the relative retention times in the last two columns of Table I. The data are similar but not identical. The RP column did not effectively separate HVA and NE or MD, DA and DHPAC.

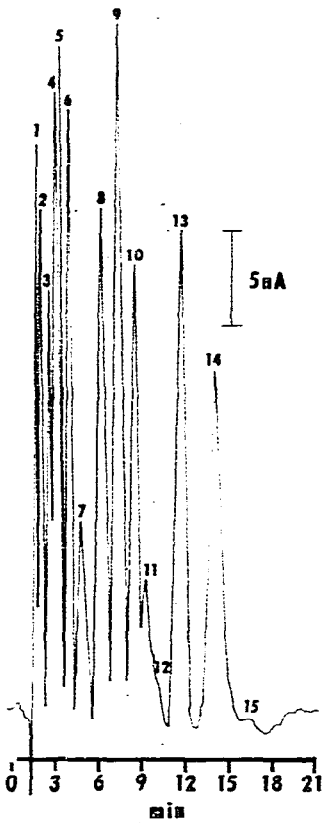

Fig. 1. Typical chromatogram of catecholamines and related compounds. Peak identification with amount injected: 1 = DOMA, 5 ng; 2 = DHPG, 5 ng; 3 = VMA, 10 ng; 4 = HVA, 50 ng; 5 = NE, 10 ng; 6 = DOPA, 10 ng; 7 = MHPG, 50 ng; 8 = EPI, 10 ng; 9 = DHBA, 25 ng; 10 = DHPAC, 5 ng; 11 = NMN, 150 ng; 12 = unknown, 13 = MD, 25 ng; 14 = DA, 10 ng; 15 = MN, 150 ng. Column: Perkin-Elmer HC-ODS-Sil-X-1, 250 × 2.6 mm I.D. Mobile phase: 300 ml 0.05 *M* citric acid + 160 ml 0.05 *M* Na_2HPO_4 + 15 mg sodium octyl sulfate (C–P–SOS). maintained at 40°, flow-rate 0.52 ml/min. Detection: electrochemical at +0.72 V *vs.* Ag–AgCl.

Initially, columns which were used with buffer–surfactant mobile phases and were not regularly washed had useful lives of less than one month. Washing columns at the end of each day with water followed by 50% methanol required the buffer-surfactant to be pumped through the column for several hours to attain column equilibrium and stable retention times, and was discontinued. Currently, RP columns are washed at the end of the day with water containing 2 drops toluene/l. This procedure increases column usefulness to a minimum of two months, and column equilibrium with stable retention times can rapidly be attained upon introduction of the buffer-surfactant into the column. Only when columns are to be stored for 2 days or more are the washed with 50% methanol.

The carbon paste electrodes used for electrochemical detection were stable, reliable, sensitive and free from noise for 2–3 weeks when aqueous solvents were used. The electrode life was shortened by the introduction of an alcohol or other organic solvent into the solvent system. Detector stability was adversely affected by interfering ionic materials in the solvent system and air bubbles trapped in the flow cell.

The three detection systems employed vary greatly in sensitivity. Table II provides the absolute detection limit for each of the four major biogenic amines by each of the three detection systems. For all the compounds in this study, the on column detection limit was approximately 5 ng for UV and approximately 2 ng by fluorescence detection. The detection limit using electrochemical detection varied considerably, for example, from 1 ng for NMN to 10 pg for DOMA.

TABLE II

DETECTION LIMITS OF FOUR BIOGENIC AMINES BY THREE DETECTION SYSTEMS

Compound	*Detection limit (ng)* *		
	UV	*Fluorescence*	*Electrochemical*
NE	5	2	0.05
DOPA	10	5	0.10
DPI	5	2	0.15
DA	5	5	0.20

* Detection limit is the smallest amount injected on column which gave a quantitatable peak.

The use of several detection systems is not unique to this report. The use of dual-wavelength UV[15], consecutive UV and fluorescence[16] and dual electrochemical[9] detection have all been examined. A new and powerful tool for both identification and quantitation is the consecutive monitoring of the eluent of a single injection by UV, fluorescence and electrochemical detection. The following example will illustrate the importance of this system of detectors.

Using the solvent system C–P–SOS with an RP column (see Table I), DHPAC, DA and MD cannot be distinguished from each other when using only UV detection since the relative retention times are so similar. However, when using UV and fluorescence detection, DHPAC can be identified due to the fact that it does not fluoresce while DA and MD are fluorescent under the conditions employed. The latter two compounds can be distinguished if they are not present as a mixture, based

on their relative detection responses. This data for DA and MD is presented in Table III. As can be seen, different detector responses do occur for each of the three detection systems, and relative response ratios can be established to assist in identification. For maximum accuracy and reproducibility, UV response is measured in microabsorbance units (μa.u.), and electrochemical response is measured in nanoamperes (nA). Fluorescence can only be measured in arbitrary units or millivolts (mV) at a fixed fluorimeter setting sensitivity (control 7 and selector ×1 for the PE 204).

TABLE III

RELATIVE DETECTION RESPONSE OF DOPAMINE AND α-METHYL DOPA

FL = Fluorescence; ElC = electrochemical detection.

Compound	*Detector response**			*Relative response ratios***		
	UV (μa.u./ng)	*FL (mV/ng)****	*ElC (nA/ng)*	*UV/FL*	*FL/ElC*	*VU/ElC*
DA	133.40 ± 2.02	0.6040 ± 0.0112	3.2860 ± 0.0280	220.86	0.1838	40.60
ND	5.427 ± 0.061	0.0256 ± 0.0003	0.1077 ± 0.0015	211.99	0.2377	50.39§

* Each value has been determined from the mean ±S.D. of three injections at a single concentration.

** Each value is the mean ±S.D. of five injections at a single concentration.

*** Fluorometer settings at sensitivity control 7 and selector 1 for PE 204 fluorescence spectrophotometer.

§ $p < 0.01$ as compared to DA.

A detection system which includes more than one detector offers a significant increase in the range of detection and the quantitation of compounds of interest. The system described herein provides a detection and quantitation range of over three orders of magnitude for the compounds listed in Table II.

CONCLUSIONS

(1) Selected catecholamines and related compounds can be separated on several different columns with several different mobile phases by reversed-phase, ion-pair HPLC.

(2) The use of two or more detection systems simultaneously can provide a greater range of detection and quantitation and greater confidence in the identification of a particular compound.

(3) Retention times and detector responses for nineteen compounds are reported.

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