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USE OF NATIVE FLUORESCENCE MEASUREMENTS AND STOPPED-FLOW SCANNING TECHNIQUE IN THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CATECHOLAMINES AND RELATED COMPOUNDS

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SUMMARY

Simultaneous separation of catecholamines and tryptophan metabolites has been carried out using a reversed-phase partition mode of high-performance liquid chromatography (HPLC). Compounds are detected and measured via their native fluorescence emitted with an excitation wavelength of 285 nm and the emission cut-off filter of 340 nm. Sample preparation is minimized and the assay is selective and well-suited for routine analyses. The sensitivity of the method is in the nanogram range. The identity of chromatographic peaks is confirmed by their excitation spectra, obtained by the stopped-flow fluorescence scanning method.

This method is applied to the direct analysis of rat brain and heart extracts as well as human serum samples.

We believe that the well known separation power of HPLC, combined with the current state of the art in fluorescence monitoring will circumvent some of the problems presently encountered in the analysis of these compounds of great biological importance.

INTRODUCTION

Cellular and extracellular fluids of all living organisms usually contain complex mixtures of biogenic amines, and currently there is great interest in the development of rapid separations and sensitive detection methods to monitor these compounds at very low levels.

Close chemical similarity of biogenic amines, their precursors and metabolites have challenged analytical chemists to devise separation methods which allow determination of the individual compounds in the presence of other related compounds. Although many techniques have been applied in the analysis of biogenic amines and related compounds, the major difficulty has been, and still remains, in the detection of the exceedingly low levels of these compounds in biological extracts.

Several methods have been used for the separation of biogenic amines: gas–liquid chromatography of derivatized compounds[1–5], thin-layer chromatography[6,7],

radio-enzymatic assays[8], double-isotope derivative[9], differential double-pulse voltametry[10], and more recently, high-performance liquid chromatography (HPLC)[7,11–17]. The latter method has a tremendous potential as a separation technique, however, until recently the major problem was in the lack of adequately sensitive detection techniques. The use of the electro-chemical detector[18] is a novel approach and it has been applied in the analysis of biogenic amines in urine and tissue samples. This detection method exploits the 2-electron oxidation of catecholamines to the corresponding quinones.

The commonly used UV absorbance detectors for HPLC, with single (254 nm) or double wavelength measurements (254 nm/280 nm), are not suitable for the analysis of these compounds at the levels at which they occur naturally. Therefore, the use of fluorescence detection is almost mandatory.

In order to enhance the fluorescent yield of biogenic amines and related compounds, several derivatization techniques have been suggested and used.

The most commonly used derivatization methods are the trihydroxyindole method (catechol oxidation and alkaline rearrangement)[17], derivatization with fluorescamine[11], dansylation[19] and derivatization with *o*-phthaldehyde[20]. Although these methods enhance the detectability, at the same time they introduce additional steps in the analysis and because of the non-specificity of the derivatization agents, the interpretation of the resulting spectra becomes more involved.

Catecholamines possess a native fluorescence arising from the presence of a characteristic catechol group. Measurements of their native fluorescence usually employ an excitation wavelength of 285 nm and the emission wavelength of 325–340 nm. These settings are, however, subject to changes depending on the pH and the type of solvent system used.

Because of the recent developments of highly sensitive fluorescence monitors, it is possible to detect biogenic amines and related compounds sensitively and accurately using their native or induced fluorescence.

The great need for rapid, sensitive and selective assay procedures for tryptophan and its metabolites, resulted in the development of a variety of separation and detection methods. Those include: colorimetry[21], fluorimetry[22–24]. UV spectrophotometry[25,26] and gas chromatographic methods[27,28]. The HPLC techniques have overcome many of the problems associated with the separation of closely related compounds. Fluorometric detection is well suited for the analysis of indoles, because the ring system is a good fluorophore. The excitation and emission maxima lie in the convenient regions of the UV spectrum where relatively few compounds have similar fluorometric characteristics.

Because of the great importance of catecholamines and the tryptophan metabolites, the use of the reversed-phase partition mode of HPLC and the state of the art of fluorimetric detection and stopped-flow scanning were investigated in the analysis of these compounds in rat brain and rat heart tissue as well as the human serum samples.

EXPERIMENTAL

Apparatus

A Model 6000 A solvent delivery system, Model 660 solvent programmer and

Model U6K universal injector (Waters Assoc., Milford, Mass., U.S.A.) were used in all determinations. The variable wavelength UV detector was a Spectroflow monitor, Model SF 770 (Kratos, Schoeffel Instrument Division, Westwood, N.J., U.S.A.); the fluorescence monitor was a Model FS 970 with a 5-μl flow cell and selectable monochromatic excitation wavelength, equipped with a SFA 339 wavelength drive and MM 700 memory module, all from Kratos, Schoeffel Instrument Division.

A prepacked, stainless-steel column, μBondapak C_{18} was obtained from Waters Assoc.

For the preparation of tissue homogenates, a Fisher sonic dismembrator, Model 300, with a 1/2-in. titanium tip probe assembly (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was used.

Reagents

All reagents used were of the highest purity (A.C.S. certified grade). All buffers and aqueous solutions were prepared in glass distilled-deionized water.

Potassium dihydrogen phosphate was purchased from Mallincrodt (St. Louis, Mo., U.S.A.), methanol distilled in glass from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.), sodium meta bisulphate and perchloric acid from Aldrich (Milwaukee, Wisc., U.S.A.), and trichloroacetic acid (TCA) and tris(hydroxymethyl)-aminomethane (Trizma base) from Sigma (St. Louis, Mo., U.S.A.).

The following reference compounds were purchased from Sigma: norepinephrine (NE), epinephrine (EN), dopa (DOPA), dopamine (DA), methyldopa (methyl DOPA), deoxyepinephrine (dEN), tyramine (TYM), isoproterenol (ISO), serotonin (5-HT), tryptophan (TRP), 5-hydroxytryptophan (5-OH TRP), tryptamine (T), 5-hydroxyindole-3-acetic acid (5-OH IAA), indoleacetamide (IA), indole-3-lactic acid (ILA), indole-3-propionic acid (IPA), anthranilic acid (AA), indole (I), kynurenine (KYN), tyrosine (TYR), hypoxanthine (HYP), xanthosine (XAO), inosine (INO), guanosine (GUO), 3,7-dimethylxanthine (3,7-Me_2XAN), 1,3-dimethylxanthine (1,3-Me_2XAN) and caffeine.

Solutions of catecholamines and the indole compounds were prepared in 0.1 *M* perchloric acid which was also 0.4 m*M* in $NaHSO_3$ (antioxidant). All solutions were kept frozen and on ice while in use.

Chromatographic conditions

Samples were analyzed using a reversed-phase partition mode of HPLC. For the analysis of catecholamines and related compounds, the low concentration eluent was a 0.02 *M* KH_2PO_4, pH 3.7; the high concentration eluent was a mixture of anhydrous methanol–water (6:4, v/v). Eluents were filtered through Millipore membrane filters, Type HA, pore size 0.45 μm (Millipore, Bedford, Mass., U.S.A.) and degassed under vacuum before use.

A linear gradient from 0 to 100% of the high concentration eluent in 35 min was used. The flow-rate was 1.5 ml/min, and the temperature was ambient in all cases.

Modified conditions were used for the analysis of serum constituents; the low concentration buffer was a solution of 0.02 *M* KH_2PO_4, pH 5.5, and the high concentration eluent was a mixture of anhydrous methanol–water (6:4, v/v). A linear gradient from 0 to 60% of the high concentration eluent in 35 min was used. All other conditions were the same as in the analysis of catecholamines.

Sample preparation

Rat brain extracts. Male Sprague-Dawly rats (Hormone Assay, Chicago, Ill., U.S.A.), weighing 200–400 g were anesthetized with chloral hydrate and barbiturate, the scalp and cranium were opened, the brain was removed quickly and rinsed with Krebs-Ringer bicarbonate buffer. The brain was then placed on its dorsal surface on a glass plate over ice. Specific sections were removed by standard dissection procedures, using a sharp razor blade. The average weights of the hypothalamus samples were approximately 40 mg. Individual slices were weighed and transferred to a test tube to which 500 μl of a 0.4 *M* $HClO_4$ containing $NaHSO_3$ were added. Samples were then homogenized for 20–30 sec and centrifuged for 10 min at a speed of 630 *g*. The catecholamine-acid supernatant was withdrawn from the cellular debris and transferred to a prechilled test tube. Samples were then stored on ice and analyzed as soon as possible. Periodically, samples were re-run to ensure that no decomposition was taking place. It was observed that they were stable for at least four weeks of storage.

Rat heart extracts. Following the heart attack which is indiced by a subcutaneous injection of an isoproterenol solution, rat hearts were quickly removed from the animals using clamps frozen in liquid nitrogen. Heart samples were prepared by homogenization in 0.4 *M* $HClO_4$, using identical method as in the case of catecholamine analysis.

Serum samples. To obtain serum samples, freshly drawn blood was collected in a tube without anticoagulant. The blood was allowed to clot spontaneously for 10 to 15 min at room temperature. The protein in the supernatant fluid was precipitated by adding 1 ml of cold, 6% by weight TCA to each ml of serum. Excess acid was neutralized with Trizma base.

Identification of peaks in biological extracts

Initial identification of chromatographic peaks was accomplished by comparing their retention times with known reference compounds and by co-injection with the reference compounds.

Conclusive identification was done by means of excitation spectra obtained using the stopped-flow scanning techniques. Excitation spectra were obtained for all the reference compounds and used for comparison with the scans of the unknown peaks. In order to obtain excitation spectra, first a blank gradient was run and the flow stopped at the point where the compound of interest elutes. The excitation spectra were scanned from 200 to 320 nm at which point the emission filter starts to transmit. This background is stored in the memory unit and later automatically subtracted from the repeated scan to give a corrected spectrum of the compound under study.

Next, the compounds were chromatographed under the same conditions, the flow stopped at the top of each peak and the excitation spectrum scanned.

RESULTS AND DISCUSSION

For the simultaneous analysis of catecholamines and related compounds, a reversed-phase mode of HPLC was used and the separation achieved using gradient elution. Fig. 1 shows the HPLC separation of catecholamines and tryptophan metabolites detected by (a) measuring their native fluorescence with the excitation wavelength of 285 nm and the emission cut-off filter of 340 nm, (b) UV absorption at 254

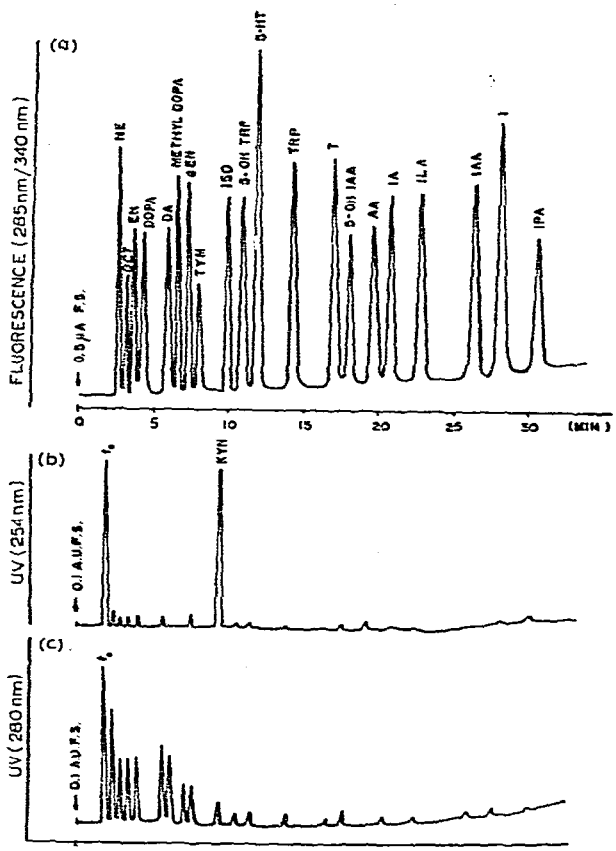

Fig. 1. HPLC separation of standard catecholamines and tryptophan metabolites detected by (a) their native fluorescence (285 nm ex., 340 nm, cut-off filter), (b) UV absorption at 254 nm and (c) UV absorption at 280 nm. Conditions: column, μBondapak C_{18}; eluents, (low concentration) 0.02 *M* KH_2PO_4, pH 3.7; (high concentration) anhydrous methanol–water (6:4, v/v); gradient, linear from 0 to 100% of the high concentration eluent in 35 min; flow-rate, 1.5 ml/min; temperature, ambient. Peaks: NE, 11.8 nmole; OCT, 18.8 nmole; KYN, 19.8 nmole; EN, 15.9 nmole; DOPA, 9.2 nmole; DA, 20.4 nmole; methyl DOPA, 12.2 nmole; dEN, 18.2 nmole; TYM, 17.2 nmole; ISO, 18.5 nmole; 5-OH TRP, 3.9 nmole; 5-HT, 0.92 nmole; TRP, 4.4 nmole; T, 3.9 nmole; 5-OH IAA, 3.1 nmole; AA, 8.2 nmole; IA, 4.0 nmole; ILA, 3.9 nmole; IAA, 4.0 nmole; I, 3.2 nmole and IPA, 4.5 nmole.

nm and (c) UV absorption at 280 nm. It is clear that the UV absorbance at either wavelength is not sensitive enough for practical purposes. KYN is the only compound which exhibits significant absorption at 254 nm and practically no fluorescence under the conditions used. A slightly higher absorbance of catecholamines is observed at 280 nm as compared to 254 nm, while the indole compounds do not absorb at either wavelength. Indoles fluoresce very efficiently in the UV region of the spectrum (285 nm ex., 340 nm em.), and their emission is an excellent basis for a sensitive and selective fluorometric assay. It is obvious from Fig. 1 that in the case of both classes of compounds, fluorescence measurements are mandatory in the analysis of biological samples containing exceedingly low levels of these compounds.

Minimum detection limits

The high efficiency of the HPLC separation of catecholamines and TRP metabolites combined with the sensitivity of fluorometric monitoring affords detection of several nanograms. Indoles and 5-HT have lower detection limits than the catechol-containing compounds. This sensitivity was found adequate for the analysis of these compounds in tissue extracts.

Linearity

Linear responses were obtained for all the compounds under study over the entire working range. The linear regression coefficients ranged between 0.989 and 1.00.

Analysis of catecholamines and related compounds in biological samples

Rat brain tissue. Using the HPLC method described, rat brain extracts were chromatographed and the chromatographic peaks detected by measuring native fluorescence with an excitation wavelength of 285 nm and an emission cut-off filter of 340 nm. A typical chromatogram of a rat brain extract is shown in Fig. 2. By connecting

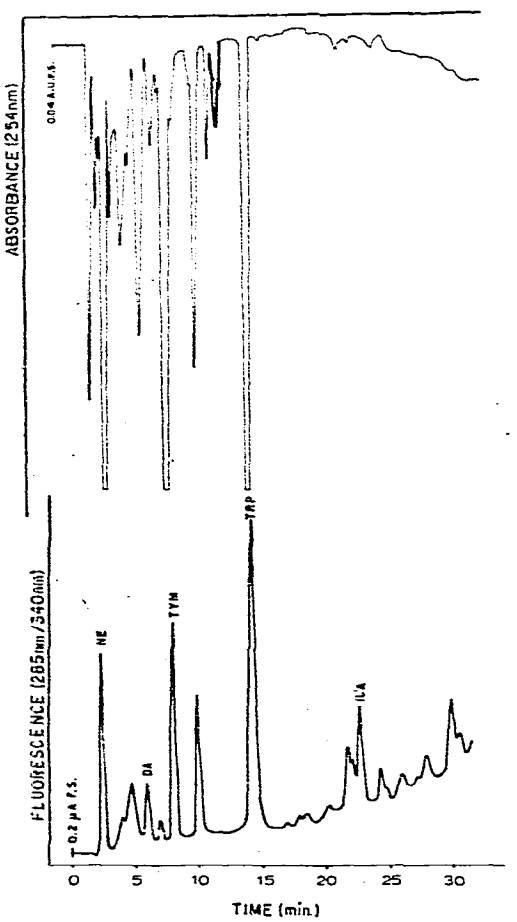

Fig. 2. Chromatogram of a rat brain extract detected by fluorescence and UV absorption. Chromatographic conditions same as in Fig. 1.

a UV detector in series with the fluorometer, it is possible to simultaneously monitor the UV absorption and fluorescence. As shown in Fig. 2, relatively few compounds exhibit native fluorescence as compared to the number of UV-active compounds. This makes fluorescence monitoring not only more sensitive, but also more selective than the absorption of UV radiation.

In order to confirm initial identification based on retention characteristics and co-injection with the reference compounds, excitation spectra were also obtained using the stopped-flow fluorescence scanning technique. Spectra of the excitation wavelength were scanned from 200 to 320 nm and the light emitted measured with cut-off filter of 340 nm. Since the spectra obtained from dilute samples at high sensitivities are distorted by the spectral background of the mobile phase, flow cells and the photomultiplier, it is necessary to use the memory unit which stores the background spectrum and subtracts it from a repeated scan to give a corrected spectrum. The corrected background spectrum for the stated wavelength region, with no compound arrested in the sample cell is shown in Fig. 3. The effectiveness of the correction afforded by the memory module is illustrated with TRP (Fig. 3).

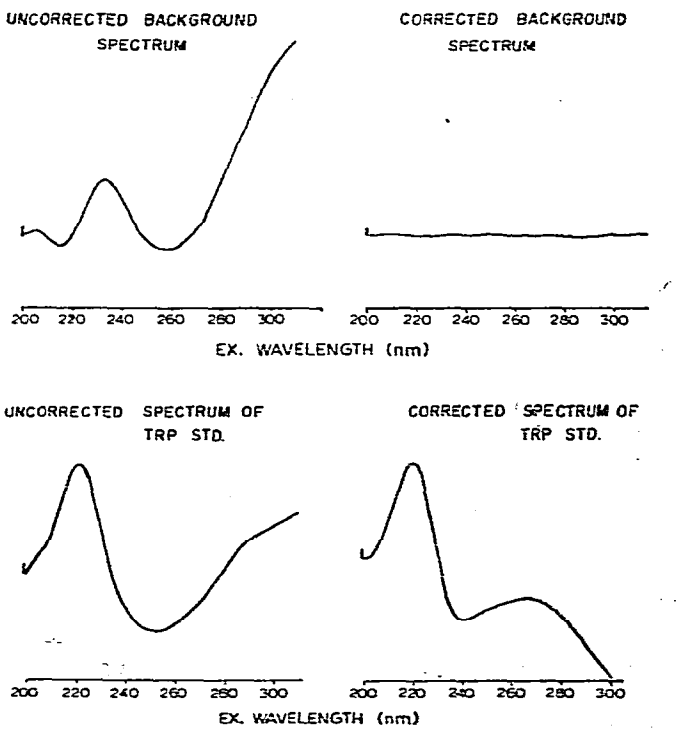

Fig. 3. Corrected *vs.* uncorrected spectra. Upper part of the illustration corrected *vs.* uncorrected spectral background of the solvent, flow cells and photomultiplier; lower part: uncorrected *vs.* corrected spectra of tryptophan standard. Scanning rate: 100 nm/min; range: 0.1 μA f.s.

The uncorrected TRP spectrum consists of its own excitation spectrum superimposed on the background spectrum, If, however, the background spectrum is stored in the memory unit and subtracted from the scan for TRP, a corrected spectrum of TRP can be obtained (Fig. 3).

The same technique was used in the identification of the peaks in the rat brain extract (Fig. 4). Background spectrum was stored in the memory unit and subtracted from each peak scan to give a corrected spectrum of the compound under study. It should be noted that the effect of the solvents on the background spectra obtained at different points along the gradient curve, is quite negligible within the solvent system used. Therefore, the same spectral background can be used for all the peaks regardless of their elution time. Also, because of the low solute diffusivity in the mobile phase, diffusion effects are not significant even if the mobile phase flow is arrested for several hours. The excitation spectra for the seven compounds in the extract from the hypothalamus region of the rat brain, which were tentatively identified on the basis of their retention characteristics, are shown in Fig. 5. The marked similarity between these spectra and the spectra of the suspected compounds confirms the identity of the endogenous compounds in the brain tissue sample.

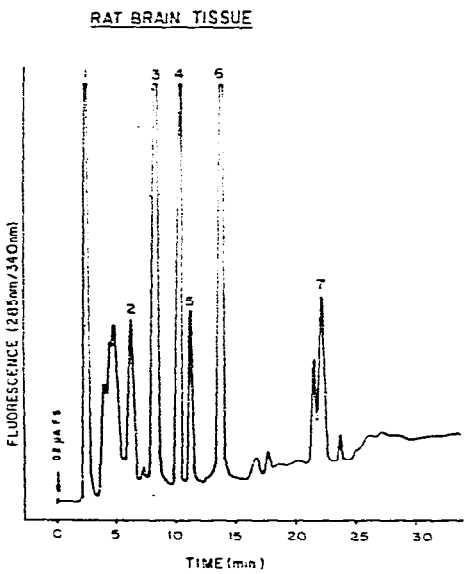

Fig. 4. Chromatogram of a rat brain extract (hypothalamus). Chromatographic conditions same as in Fig. 1.

Rat heart samples. The same identification method was also applied to the analysis of rat heart extracts (Fig. 6). The excitation spectra of the six compounds in the rat heart and the spectra of the corresponding reference compounds are shown in Fig. 7. The presence of exogenous isoproterenol results from the induction of the heart attack with this compound. Based on the combined evidence accumulated from retention times, co-injection with the reference compounds and the excitation spectra, the peaks in the heart extract were identified as NE, DA, TYM, ISO, TRP, and ILA.

Human serum extract. In order to achieve an adequate separation of the UV absorbing constituents, TCA extracts of human serum were analyzed using modified chromatographic conditions. The separation of some catecholamines and related compounds as well as some of the naturally occurring UV-absorbing compounds is

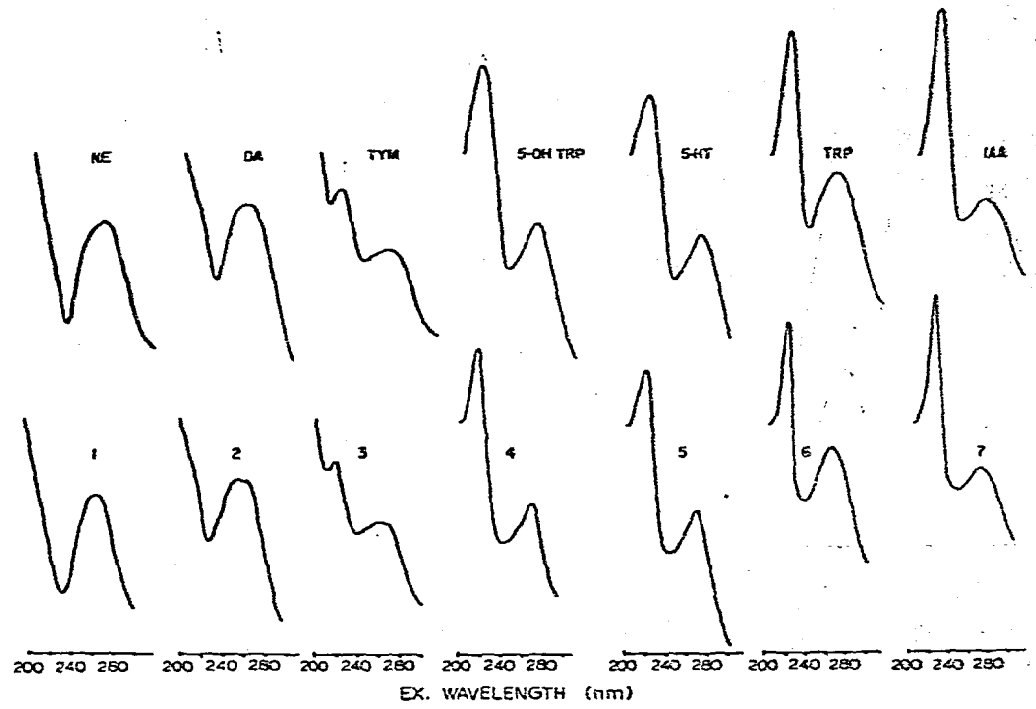

Fig. 5. Corrected excitation spectra of some reference compounds and chromatographic peaks in the rat brain extract (hypothalamus). Scanning rate: 100 nm/min; range: 0.1 μA f.s.

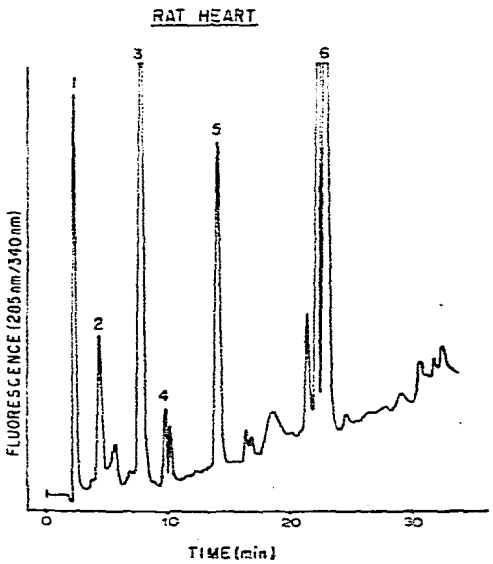

Fig. 6. Chromatogram of a rat heart extract. Chromatographic conditions same as in Fig. 1.

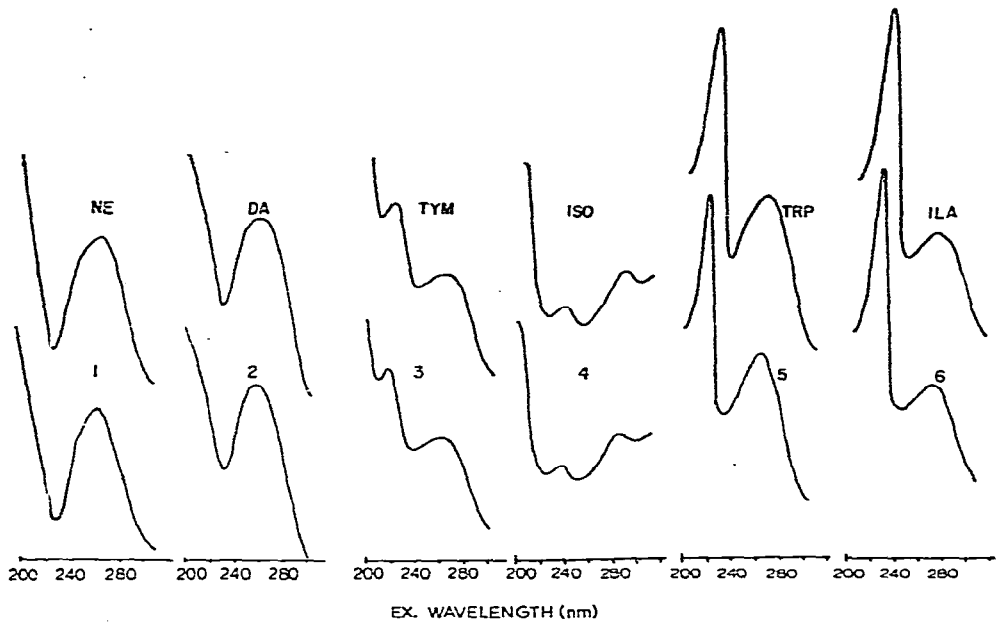

Fig. 7. Corrected excitation spectra of some reference compounds and chromatographic peaks in the rat heart extract. Scanning rate: 100 nm/min; range: 0.1 μA f.s.

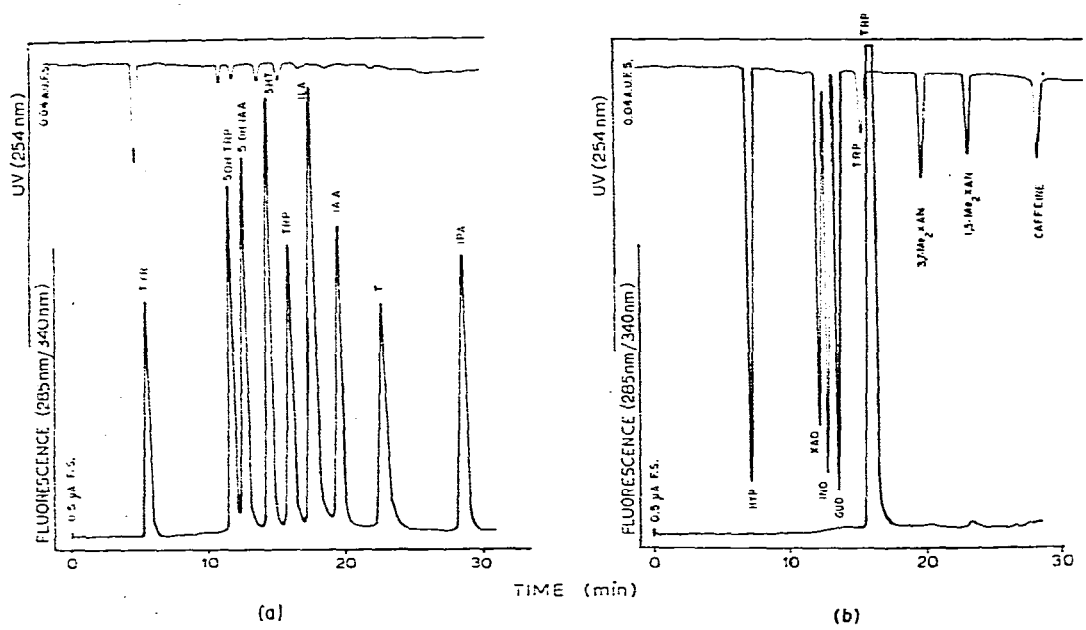

Fig. 8. Separation of some standard catecholamines and related compounds (a), and (b) some naturally occurring UV-absorbing compounds. Chromatographic conditions: column, μBondapak C_{18}; eluents: (low concentration) 0.02 M KH_2PO_4, pH 5.5; (high concentration anhydrous methanol–water (6:4, v/v); gradient, from 0 to 60% of the high concentration eluent in 35 min; flow-rate, 1.5 ml/min; temperature, ambient.

shown in Fig. 8. Fig. 8a illustrates that the UV absorption of catecholamines and related compounds is negligible even at the highest sensitivity settings. Among the UV absorbing species, TRP us the only compound which exhibits strong fluorescence (Fig. 8b).

The application of the modified conditions is illustrated with a human serum from a normal subject (Fig. 9). Only two fluorescent compounds were detected. In order to confirm their identity, after the intial identification based on their retention times and co-injection with the reference compounds, excitation spectra were obtained (Fig. 10). Agreement between the fluorescence spectra of the peaks in serum and the TYR and TRP gave conclusive confirmation of their identity.

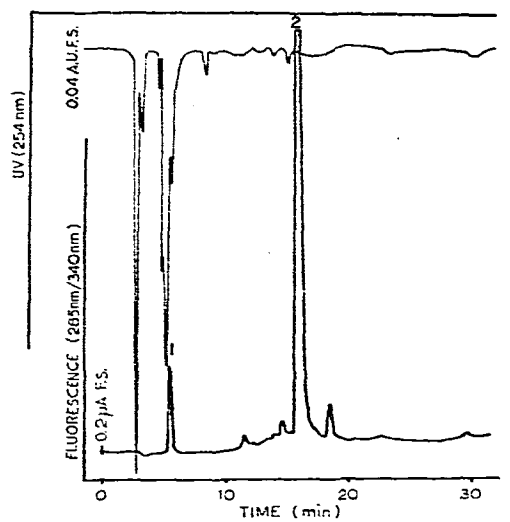

Fig. 9. Chromatogram of a normal human serum sample. Chromatographic conditions same as in Fig. 8.

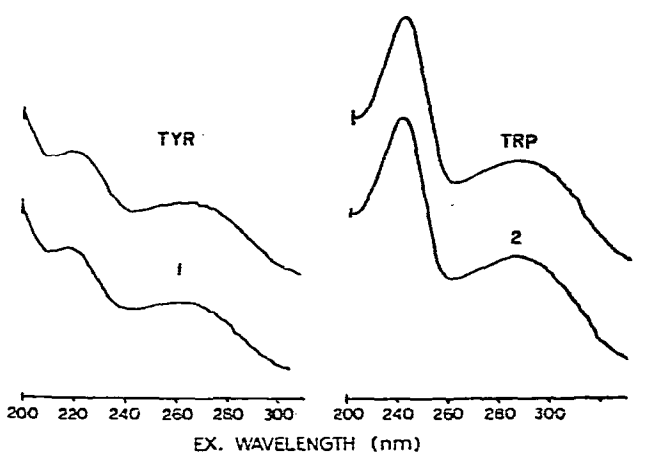

Fig. 10. Corrected excitation spectra of some reference compounds and peaks in human serum. Scanning rate: 100 nm/min; range: 0.1 μA f.s.

In summary, the described HPLC simultaneous separation of catecholamines and related compounds, coupled with the current state of the art of fluorescence monitoring, provides a rapid and sensitive method for the detection of these compounds in biological samples. The excitation spectra obtained by the stopped-flow fluorescence scanning proved to be a powerful aid in the identification of chromatographic peaks in biological extracts. Sample preparation is minimized and chromatographic separation optimized to give sharp peaks in order to improve their detectability. This method of analysis, we believe, is well suited for routine testing of these tremendously important biological compounds.

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REFERENCES

1 H. Ch. Curtius, M. Wolfensberger, B. Steinmann, U. Redweik and J. Siegfried, Jr., *J. Chromatogr.*, 99 (1974) 529.
2 F. Zambotti, K. Blau, G. S. King, S. Campbell and M. Sandler. *Clin. Chem. Acta*, 61 (1975) 247.
3 H. G. Lovelady, *Biochem. Med.*, 15 (1976) 130.
4 H. Miyezaki, Y. Hashimoto, M. Iwanoga and T. Kubodera, *J. Chromatogr.*, 99 (1974) 575.
5 T. C. Lhuguenot and B. F. Maume, *J. Chromatogr. Sci.*, 12 (1974) 411.
6 G. C. Boffey and G. M. Martin, *J. Chromatogr.*, 90 (1974) 178.
7 R. W. Stout, R. J. Michelot, I. Molnar, C. Horváth and T. K. Coward, *Anal. Biochem.*, 76 (1976) 330.
8 N. Ben-Jonathan and J. C. Porter, *Endocrinology*, 98 (1976) 6.
9 K. Engelman, B. Portnoy and W. Lovenberg, *Amer. J. Med. Sci.*, 255 (1968) 268.
10 R. F. Lane and A. T. Hubbard, *Anal. Chem.*, 48 (1976) 1287.
11 K. Imai, *J. Chromatogr.*, 105 (1975) 135.
12 R. M. Riggin and P. T. Kissinger, *Anal. Chem.*, 49 (1977) 2109.
13 G. Schwedt, *J. Chromatogr.*, 143 (1977) 463.
14 K. Imai, M. Tsukamoto and Z. Tamura, *J. Chromatogr.*, 137 (1977) 357.
15 I. Molnar and C. Horváth, *Clin. Chem.*, 22 (1976) 1497.
16 P. M. Plotsky, R. M. Wightman, W. Chey and R. N. Adams, *Science*, 197 (1977) 904.
17 E. Ueda, N. Yoshida, K. Nishimura, T. Joh, S. Autoku, S. Tsukada, S. Ganno and T. Kokubu, *Clin. Chem. Acta*, 80 (1977) 447.
18 P. T. Kissinger, C. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465.
19 M. S. F. Ross, *J. Chromatogr.*, 141 (1977) 107.
20 S. S. Simons and D. F. Johnson, *J. Amer. Chem. Soc.*, 98 (1976) 7098.
21 S. N. Mahaboob Basha and R. Michael Roberts, *Anal. Biochem.*, 77 (1977) 378.
22 A. N. Krstulovic, P. R. Brown, D. M. Rosie and P. B. Champlin, *Clin. Chem.*, 23 (1977) 1984.
23 A. J. Lewis, P. J. Holden, R. C. Ewan and D. R. Zimmerman, *J. Agr. Food Chem.*, 24 (1976) 1081.
24 J. H. Peters and B. J. Berridge, Jr., *Chromatogr. Rev.*, 12 (1970) 157.
25 S. S. Hassan, *Anal. Chem.*, 47 (1975) 1429.
26 R. A. Wapnir and J. H. Stevenson, *Clin. Chem. Acta*, 26 (1969) 203.
27 G. W. Gehrke and H. Takeda, *J. Chromatogr.*, 76 (1973) 77.
28 P. Felker, *Anal. Biochem.*, 76 (1976) 547.
29 D. D. Chilcote and J. E. Mrochek, *Clin. Chem.*, 18 (1972) 778.