CHROM. 13,920

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BIOGENIC AMINES

II. COMPARISON OF DETECTION METHODS

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(First received February 20th, 1981; revised manuscript received April 14th, 1981)

SUMMARY

With acidic aqueous mobile phases on silica, detection by electrochemical oxidation on a carbon paste working electrode was compared with detection by the native fluorescence by excitation at 280 nm of catechols and indoles. Based on evaluations of sensitivity, reproducibility, stability, selectivity and simplicity of handling, the fluorescence detector was considered superior to the voltammetric detector. Preliminary results from direct injections of deproteinized serum indicated that at levels of at least 10 μg/l the content of some indoles in biological fluids could be determined without further sample purification.

INTRODUCTION

In the development of procedures for the high-performance liquid chromatographic (HPLC) determination of catecholamines and indoles in biological fluids, the choice of the detector is crucial, with requirements such as high sensitivity, high stability and preferably some specificity. The high sensitivity is needed especially in the analysis of blood samples, as normal serum concentrations of 0.1–25 μg/l are not unusual[1]. At the 0.1 μg/l level, with no pre-concentration, a 10-μl injection of deproteinized serum would contain less than 1 pg of the component of interest. At present this is not sufficient for any HPLC detector and pre-concentration is required. After adsorption on alumina of 200-ml samples of urine, Mell and Gustafson[2] used a UV detector to determine catecholamines at levels down to 5 μg/l. The method was not sufficiently sensitive for serum analysis. Grushka and Kikta[3] used UV detection to determine tryptophan in urine from patients with excessive levels. Mainly with catecholamines, but also with some indoles, electrochemical detectors (EICDs) have been used on standard solutions[4–7], blood[8–10], urine[11–13] and tissue[14–18]. The detection limits reported are not consistent: the lowest detectable amount of injected dopamine has been reported to be 500[18], 200[7], 100[30], 53[15] and 15 pg[17].

Fluorescence detection of catecholamines has mainly been connected with the formation of derivatives, using *o*-phthalaldehyde[19–21], fluorescamine[22] or Dns

chloride[23]. Utilization of the native fluorescence of catecholamines and indoles, as shown for serotonin by Udenfriend *et al.*[24], has not had much success until spectrofluorimetric HPLC detectors became commercially available. After Graffeo and Karger[25] reported a detection limit of 5–15 ng of indoles and concluded that the sensitivity was sufficient for the analysis of tissue extracts, but not for urine; little was published until recently, when several reports appeared which included the use of fluorescence detection of indoles[26–28] and catecholamines[29–33]. Apparently the many problems associated with the stability of the electrochemical detectors have induced renewed interest in the field of native fluorescence.

With the aqueous silica system[34] the chromatographic conditions, with few solvent interferences and high solvent conductivity, seemed promising for both electrochemical and fluorescence detection. With this system the sensitivity, stability and applicability of the native fluorescence of a selected group of catechols and indoles have been compared with voltammetric oxidation on a carbon paste working electrode. The carbon paste electrode of the ElCD was chosen in order to avoid the cumbersome polishing procedures necessary with glassy carbon electrodes. For comparison, UV detection was partially included.

EXPERIMENTAL

Waters 6000A pumps and a U6K injector were connected to a Spherisorb S5 silica (5 μm) column (250 × 4.6 mm I.D.) and a Waters 440 UV detector, a fluorescence detector or an electrochemical detector. A Kontron SFM 23 LC spectrofluorimeter was equipped with a 150-W xenon lamp (WGS), the scanning option and a 20-μl flow cell. The bandwidths of excitation and of emission were 10 and 20 nm, respectively. The voltammetric detector with a carbon paste working electrode and a silver reference electrode in a PTFE cell has been described in detail elsewhere[35].

The mobile phase consisted of solutions of perchloric acid, monochloroacetic acid or acetic acid in water, depending on the pH and ionic strength desired[34]. If not otherwise indicated the mobile phase contained perchloric acid (0.01 M) and sodium perchlorate (0.04 M) in water. The flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

UV detection

Dopamine and serotonin were chosen as a representative catecholamine and indoleamine, respectively. In 0.1 M perchloric acid + 0.04 M sodium perchlorate, the absorbance maxima were determined to 280 and 270 nm, respectively. The serotonin absorbance at 280 nm was only slightly less than that at 270 nm. With 10-μl injections, with the Waters 440 detector at 280 nm, the detection limits (determined as four times the baseline noise) were 3 ng of dopamine and 1.5 ng of serotonin. This is a good sensitivity, but the usefulness of UV detection depends strongly on the sample preparation, as the selectivity is inferior to that of electrochemical and fluorescence detection.

Electrochemical detection

Aqueous solvents containing completely dissociated ions are generally the op-

timal solvents for EICDs, owing to the high conductivity. Such solvents also make the best combination with carbon paste working electrodes, owing to the negligible solubility of the dispersive oil of the paste. A mobile phase of 0.01 M perchloric acid + 0.04 M perchloric acid in water should therefore give a system of high stability. On the other hand, the pH of this solvent is not optimal.

At pH 2 the ring-formation step of the catecholamine oxidation scheme[36] is too slow to give a full four-electron transfer (Fig. 1). A limiting current was obtained with all of the catechols examined by using a voltage scan of 0.40–1.10 V. For dopamine, epinephrine and N-acetyldopamine the limiting current was obtained at 0.60 V and for normetanephrine at 0.80 V (Fig. 2). A current maximum at 0.60 V was obtained with homovanillic acid (Fig. 2).
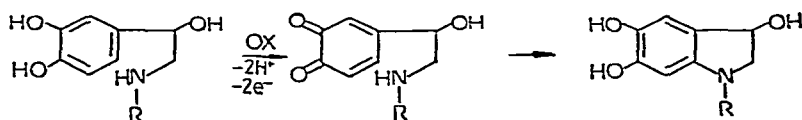

Fig. 1. Two-electron oxidation of catecholamines followed by the pH-dependent formation of dihydroindoles.
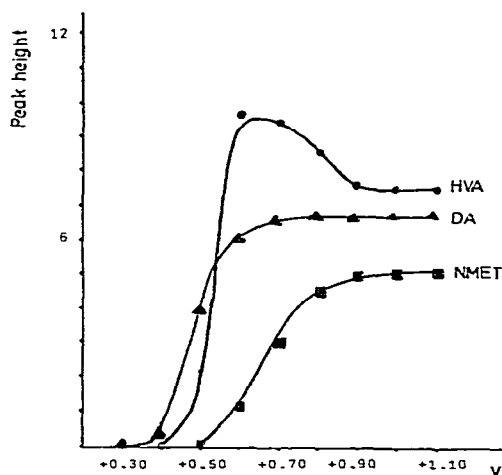

Fig. 2. Voltammograms of homovanillic acid (HVA), dopamine (DA) and N-metanephrine (NMET).

Large day-to-day variations in the background noise resulted in problems in determining absolute detection limits (injected in a volume of 25 μl). With a signal-to-noise requirement of better than 3:1, the minimal detectable amount of catecholamines varied between 0.1 and 1 ng (injected). At the 50-ng level a short-term relative precision of better than 1 % was obtained, but with amounts less than 1 ng at the highest sensitivity the reproducibility was not satisfactory for quantitative analysis. The relative precision was determined as the percentage standard deviation of the mean.

Electrochemical oxidation of indoles is reported to involve both N-oxidation and oxidation of the phenolic group of 5-hydroxy compounds[37]. A voltage scan of 5-

hydroxy compounds verified the dual oxidation scheme. 5-Hydroxytryptamine (sero-tonin), 5-hydroxytryptophan and 5-hydroxyindoleacetic acid showed a current plateau at 0.60 V and a rising current on further increase in potential. Compounds without the phenolic group, such as tryptamine and tryptophan, gave no limiting current at potentials up to 1.10 V, as shown in Fig. 3. Higher potentials resulted in too much noise to be useful for any practical purpose. As the oxidation of the 5-hydroxy group started at a lower potential than the N-oxidation, a nearly selective detection of the 5-hydroxy compounds was obtained at 0.60 V (Fig. 4). As the limiting current of the oxidation of indoles could not be obtained within the voltage range of the carbon paste electrode, the detector response is strongly dependent on the potential. This also means that for quantitative analysis good potential control is required.
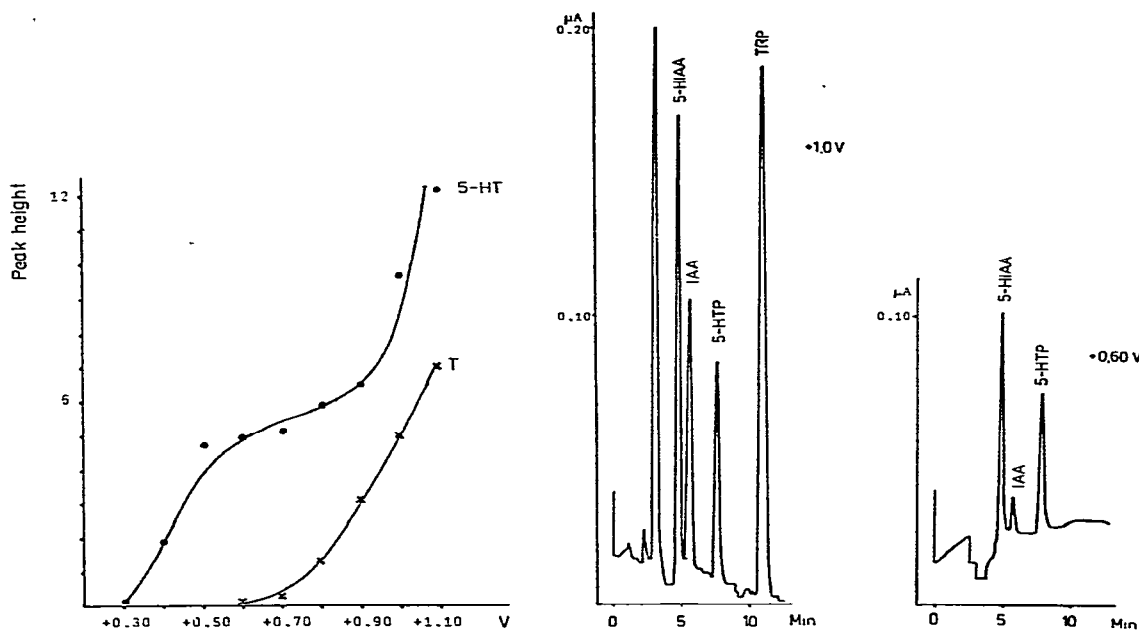

Fig. 3. Voltammogram of serotonin (5-HT) and tryptamine (T).

Fig. 4. Selective determination of 5-hydroxyindolic compounds at 0.60 V.

A constant problem with electrochemical detection is the low stability and the many unidentifiable sources of noise at high sensitivities. Voltstats and an earthed Faraday cage were included, but had no significant effect on the noise. Effective deaeration of the solvent was found to be important for increasing the stability and reducing the noise. The solvents were degassed for several hours in an ultrasonic bath, but constant helium or argon degassing, which is required for electrochemical reduc-tion[35], is probably desirable also for oxidative processes. Addition of EDTA to the solvent, which is supposed to form stable complexes with trace amounts of metals in the solvents[15], had no effect, probably owing to the low stability constants of most metal–EDTA complexes at low pH.

Fluorescence detection

Catechols and indoles are known to exhibit native fluorescence on excitation at 280 nm[7,25]. Maximal emission after excitation at 280 nm was also obtained with this system for the catecholamines and indoles examined, except for serotonin, which showed a maximum at 305 nm. Only a minor difference in emission intensity was obtained, however, at 280 nm compared with 305 nm for serotonin. Excitation at 280 nm gave emission maxima at 315 nm for dopamine, epinephrine, metanephrine, homovanillic acid and dopa, at 335–340 nm for serotonin and at 350 nm for tryptamine, as shown in Fig. 5. With excitation at 280 nm and emission at 340 nm, the maximal sensitivity was determined to be 0.5–0.8 ng of catecholamines and 0.03–0.2 ng of indoles. An example of a 0.5-ng injection of tryptamine and serotonin is shown in Fig. 6. A strong suppression of the indole peaks was obtained by emitting at 315 nm (Fig. 7), where the intensity of the catecholamine emission was little affected. Thus, some selectivity could be introduced, which could prove helpful in the analysis of catecholamines in the presence of indoles, by emission at approximately 310 nm.
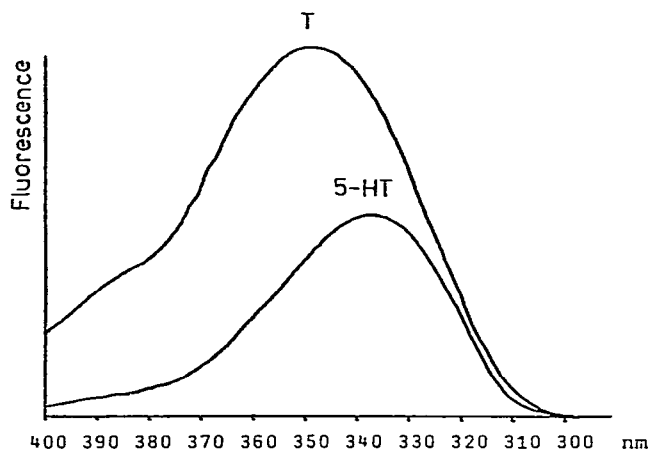

Fig. 5. Fluorescence emission spectra of serotonin and tryptamine from excitation at 280 nm in 0.01 M $HClO_4$ + 0.04 M $NaClO_4$.

A linear relationship was found between the amount injected and the peak height in the range 0.1–0.5 ng (Fig. 8) at the highest sensitivity.

The instrumental sensitivity control was linear at all levels, except when the photomultiplier potential was varied (Fig. 9). The relative precision for 0.3-ng injections of serotonin and tryptamine was better than 3 %. No stability problems were observed, even at the highest sensitivity.

The spectrofluorimeter was equipped with a lamp with a cut-off at 250 nm (ozone free). By using a deuterium lamp and excitation at 200 nm, Jackman *et al.*[31] found that 0.1–0.5 ng of catecholamines and 0.02 ng of serotonin could be determined. At this wavelength, however, the sensitivity towards other indoles was significantly lower, and no selectivity of catecholamines *versus* indoles was obtained.
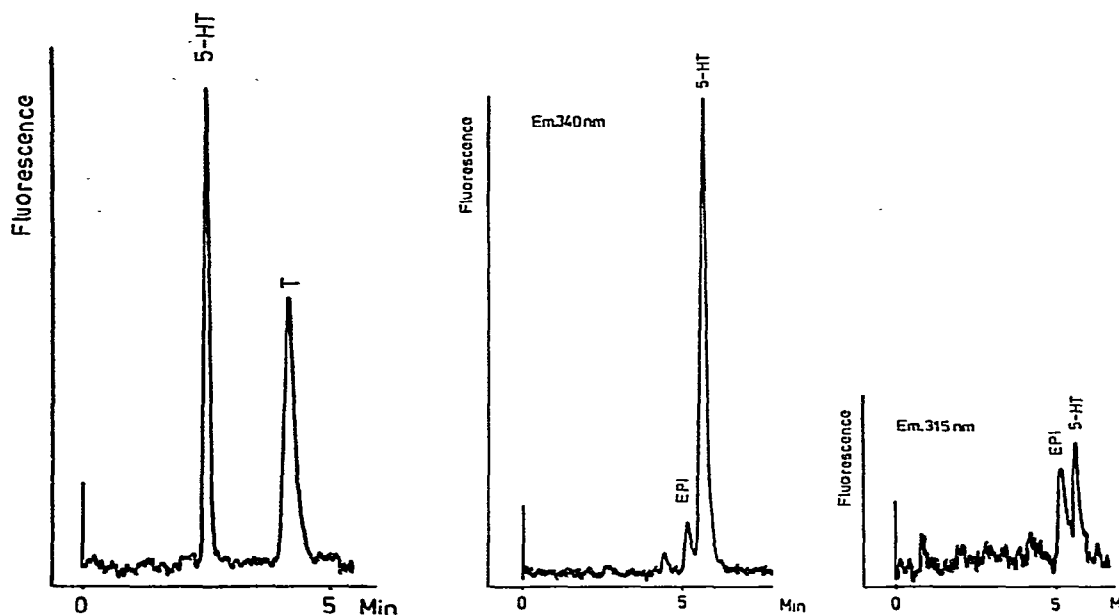
Fig. 6. Separation of serotonin and tryptamine (500 pg each) with 0.1 M HClO$_4$ on Spherisorb S5 silica. Excitation, 280 nm; emission, 340 nm.

Fig. 7. Suppression of serotonin compared with epinephrine (25-μl injection with 750 pg of each) by changing the emission from 340 to 315 nm; 0.1 M monochloroacetic acid on Spherisorb S5 silica.
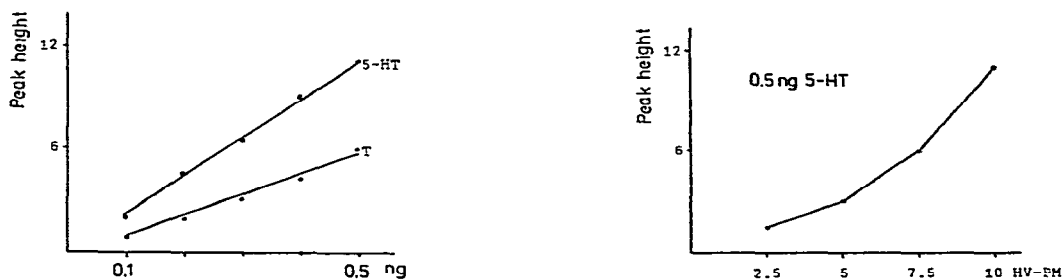
Fig. 8. Relationship between amount injected and peak height (cm) of serotonin and tryptamine. Excitation, 280 nm; emission, 340 nm.

Fig. 9. Variation of the photomultiplier potential, causing deviation from linearity. A linear relationship between the sensitivity control and the signal height was obtained at a constant photomultiplier potential.

Indoles in serum

Normal blood levels of catecholamines are too low to permit direct measurements without a pre-concentration step with any of the detection methods discussed. The serum levels of many indoles should, however, permit the use of fluorescence for direct measurements of deproteinized samples. Fig. 10 shows the chromatograms obtained with 10-μl injections of serum deproteinized with perchloric acid; 0.1 M acetic acid was used as the solvent in order to obtain sufficient resolution of the early
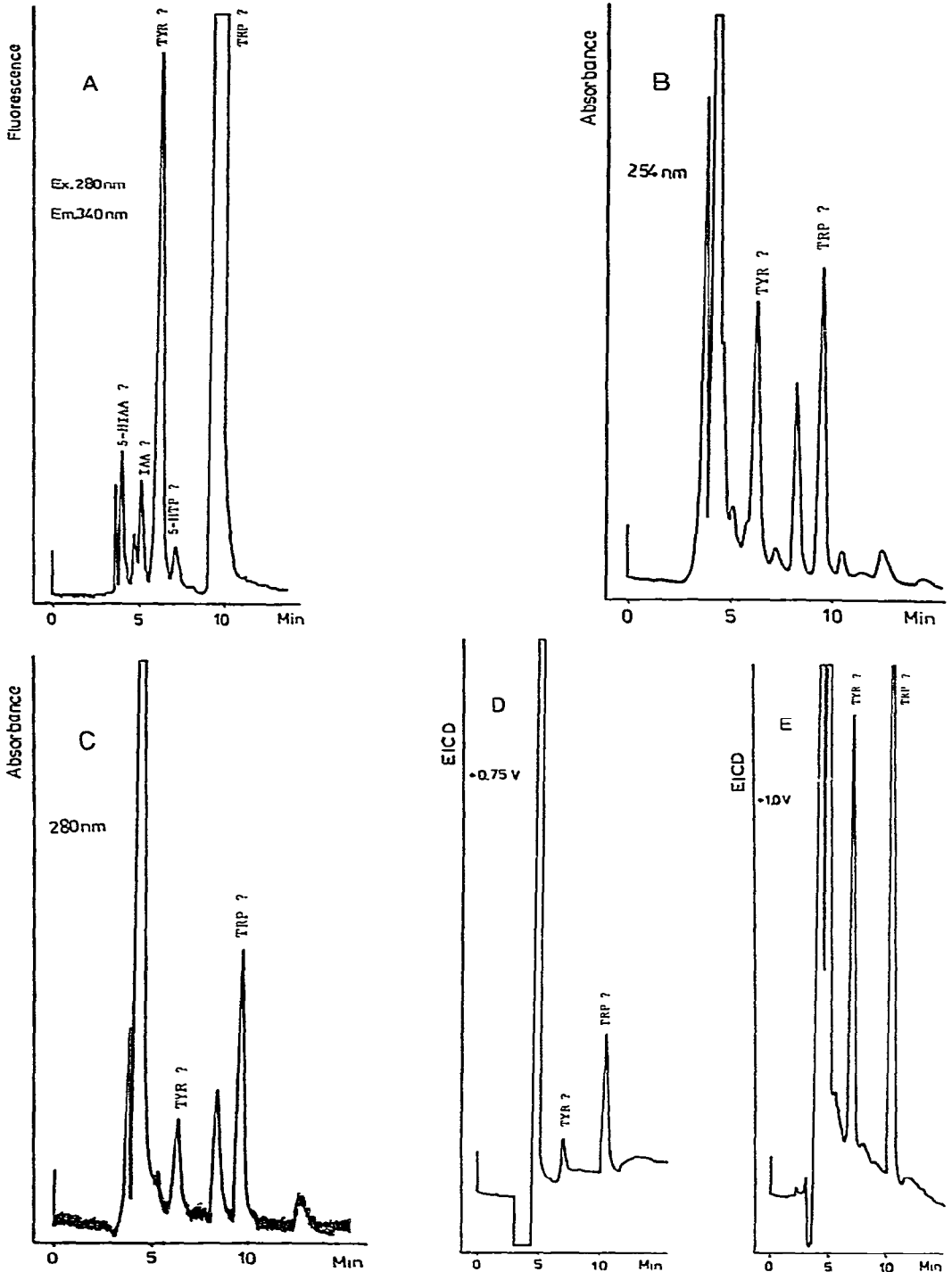
Fig. 10. Deproteinized serum (10 μl) injected on Spherisorb silica, with 0.1 M acetic acid as mobile phase, with (A) fluorescence detection (sensitivity, 10.0; photomultiplier potential adjustment, 6.0); (B, C) UV detection (0.01 a.u., 254 nm; 0.2 a.u., 280 nm); (D, E) electrochemical detection (1000 nA full-scale). The question marks do not query the identity of the components, but merely serve as a reminder that the impact of possible interferences may vary with the mobile phase chosen to suit the biogenic amines in question.

eluting acidic indoles. The silica separation column was protected with a 5-cm silica pre-column. As a serum blank without indoles was not available, conclusions regarding the amount of the different indoles present should be drawn with great care, but the advantages of the fluorescence detection for this purpose are obvious.

Another example of the potential usefulness of this procedure for a component with higher retention (tryptamine) is shown in Fig. 11. An aqueous solution of a stronger acid (monochloroacetic acid) was used as the eluent. Serum, without and with tryptamine added (0.1 μg/ml), was deproteinized and 15-μl samples were injected. No major interferences were found. The tryptamine levels in serum may be far too low to be detectable, but the procedure could prove useful for the determination of tryptamine in cerebrospinal fluid or in brain tissue extracts. A more detailed examination of the analytical aspects of fluorescence detection of several indoles in biological fluids is in progress.
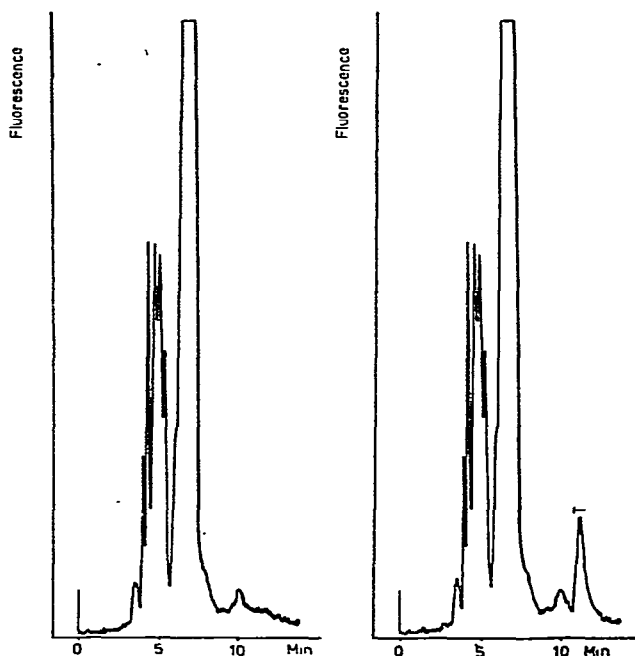

Fig. 11. Deproteinized serum (15 μl), with and without tryptamine added, injected on Spherisorb S5 with a 5-cm silica guard column. Mobile phase: 0.1 M monochloroacetic acid. Excitation, 280 nm: emission, 340 nm.

CONCLUSION

At present no HPLC detector has the ability to detect smaller amounts of catecholamines than the EICD. This does not necessarily mean that the EICD is superior in the analysis of catecholamines. Based on the usual requirements of stability and reproducibility, detection by native fluorescence may compete favourably with the EICD.

In the analysis of indoles, detection by native fluorescence has proved superior

to the ElCD with regard to sensitivity, stability and reproducibility.

If no other differences were apparent, the spectrofluorimeter was preferred to the carbon paste ElCD owing to the simplicity of handling and maintenance.

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