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ANALYSIS OF SEROTONIN AND DERIVATIVES BY REVERSED-PHASE ION-PAIR PARTITION CHROMATOGRAPHY WITH FLUOROMETRIC AND ELECTROCHEMICAL DETECTION

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SUMMARY

A flexible and efficient system is described for reversed-phase ion-pair partition-chromatographic analysis of serotonin, its precursors tryptophan and 5-hydroxytryptophan, its main metabolite 5-hydroxyindoleacetic acid, and tryptamine. The chromatographic system consists of tri-*n*-butylphosphate as stationary phase and buffered water–methanol mixtures, containing perchlorate, as mobile phases. Retention can be selectively influenced by means of the pH, the perchlorate concentration, and the methanol content of the mobile phase, as well as the temperature of the phase system. The compounds of interest can be separated within 10 min and no interference from catecholamines and derivatives was observed. Compared with electrochemical detection, fluorometric detection yielded more favourable detection limits and was more selective when supernatants of brain tissue homogenates were directly injected. Both detection systems showed inadequate selectivity if urine samples were directly injected, but 5-hydroxyindoleacetic acid could readily be assayed.

INTRODUCTION

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is present in distinct areas in the mammalian central nervous system. It has prominent effects on, *e.g.*, body temperature, sensory perception, and sleep[1], and enhancement of 5-HT transmission in depressed patients alleviates their symptoms[2]. Outside the central nervous system 5-HT is thought to play a role in migraine[3]. 5-HT is stored in blood platelets and in the enterochromaffin cells of the gastrointestinal tract. Tumours of the enterochromaffin cells are called "carcinoid". In patients with this "carcinoid syndrome" blood levels of 5-HT are significantly increased, as is the urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA)[4].

As to studies on 5-HT transmission, it has become obvious that for a better understanding of this process accurate analysis of 5-HT turnover and metabolism is

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necessary. 5-HT synthesis occurs in two steps: L-tryptophan (TRP) is hydroxylated to 5-hydroxytryptophan (5-HTP), which is decarboxylated to 5-HT (see Fig. 1). The catabolism results in the production of 5-HIAA, which is the end-product of 5-HT metabolism. However, TRP itself may be decarboxylated as well, leading to the formation of tryptamine (T). This step does not take place in 5-HT neurons[5], but tryptamine synthesis may be affected by drug treatment[6].

Various analytical methods have been employed to measure 5-HT and its main precursors and metabolites in biological tissues and fluids, including thin-layer chromatography[7], ultraviolet spectrophotometry[8], fluorometry[9,10], gas chromatography with electron-capture[11] or mass spectrometric[12] detection, radioenzymatic assay[13] and radioimmunoassay[14]. These methods either lack selectivity and sensitivity or they are too expensive or time-consuming for routine analysis. Recently, high-performance liquid chromatography (HPLC) with electrochemical detection (ED)[15–22], fluorometric detection (FMD)[23] or combined FMD–ED[24,25] has been applied to the analysis of the indoles. However, these methods have severe drawbacks, *viz.* extensive sample pretreatment is necessary, not all the compounds of interest can be assayed simultaneously, analysis times are rather long, and the compounds of interest are not completely separated from each other or from interfering compounds.

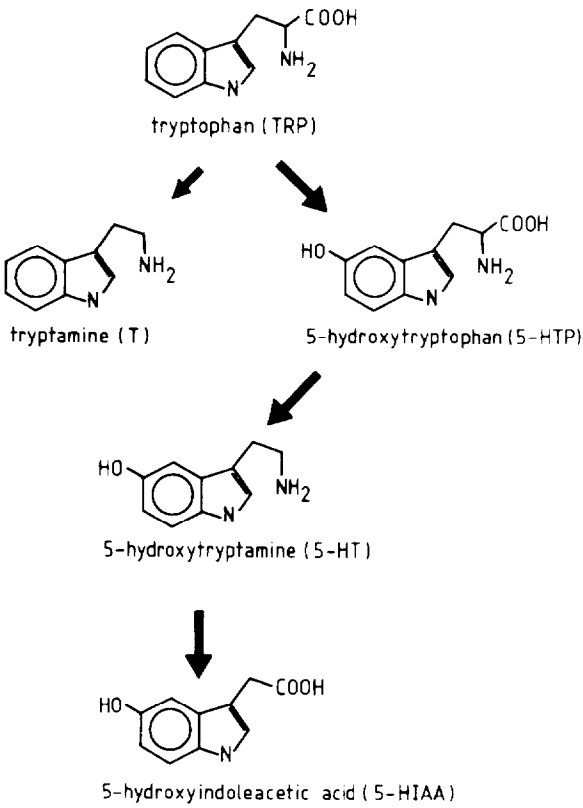


Fig. 1. Serotonin metabolism.

Reversed-phase ion-pair partition chromatography (RP-IPPC), based on the use of tri-*n*-butylphosphate (TBP) as stationary phase and of buffered aqueous media containing perchlorate as mobile phases, has proved to yield efficient systems, suitable for the analysis of catecholamines and their precursors and metabolites in brain tissues and urine[26–29]. This liquid–liquid system has also been adapted to the analysis of hydrophilic carboxylic acids, amino acids, and dipeptides[30], notably for the assay of 5-HIAA and indoleacetic acid (IAA) in urine[31,32]. In the latter systems[30–32] the mobile phases consisted of buffered water–methanol mixtures without perchlorate, which implies that amines (5-HT and T) and amino acids (TRP and 5-HTP) cannot be assayed because of their low retention.

In our paper a system is described which enables simultaneous analysis of serotonin, its precursors, and its main metabolite, next to tryptamine. The indoles can be detected by UV detection[31], ED[15–22,24,25] and FMD[23–25,33]. However, for trace analysis, only ED and FMD can be used. For both detection systems minimum detectable amounts in the low picogram range have been reported[16,33]. In our study, FMD and ED have been compared with respect to selectivity and sensitivity.

Although the method was primarily developed for brain tissue analysis, it has also been applied to the analysis of urinary 5-HIAA, which can be important in the clinical diagnosis of "carcinoid tumours".

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from custom-made and commercially available parts and consisted of a constant-flow pump (Constametric I, LDC, Riviera Beach, FL, U.S.A.), a thermostatted eluent reservoir, a Bourdon-type manometer, an injection system (Rheodyne 7120, Berkeley, CA, U.S.A.) with a 100-μl sample loop, a thermostatted stainless-steel column (10 cm × 3.0 mm I.D.) and several detection systems. The amperometric detector was a wall-jet type (PU 4022, Pye Unicam, Cambridge, U.K.) with a glassy carbon working electrode and a silver–silver chloride reference electrode. The fluorometric detector was a double-monochromator type with a 150-W xenon source and a 20-μl flow cell (SFM 23 LC, Kontron, Zurich, Switzerland). Columns were packed by means of a high-pressure air amplifier booster pump (DSHF-302, Haskell, Burbank, CA, U.S.A.).

Chemicals and reagents

Serotonin creatinine sulphate monohydrate and 5HIAA were purchased from Aldrich (Beerse, Belgium), tryptamine hydrochloride from EGA Chemie (Steinheim, F.R.G.), and TRP and 5-HTP from Sigma (St. Louis, MO, U.S.A.). All chemicals were of analytical or reagent grade and were used without further purification, except water, which was purified with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). TBP was obtained from Aldrich. The chromatographic support material Polygosil C_{18} (mean particle size, 5 μm) was obtained from Macherey & Nagel (Düren, F.R.G.).

Chromatography

The column-packing procedure was based on a slurry technique as described

elsewhere[34]. 1,1,1-Trichloroethane was used as dispersing solvent and methanol as displacing solvent. After the column was washed with *ca.* 100 ml of water, the mobile phase (saturated with TBP) was pumped through, and the column was loaded *in situ* by injection of aliquots of 20 μl of TBP until supersaturation of the effluent was observed. After loading, the pores of the column material are more or less completely filled with TBP and a stable phase system is obtained. For Polygosil C_{18} the porosity (ε_m) of the loaded column amounts to *ca.* 0.56. The porosity is hardly influenced by changes of temperature or methanol content of the mobile phase.

The capacity ratio, k'_i, of a compound i was determined from its retention time (t_{R_i}) and the retention time of an unretained compound (t_{R_0}) for which potassium iodide (injection of *ca.* 20 μl of a 1 m*M* solution) was used. t_{R_i} and t_{R_0} were corrected for extracolumn volumes between the injector and the detector by means of the following equation:

$$t_{R,\text{corr.}} = t_{R,\text{meas.}} - \frac{V_{\text{extracol.}}}{w} \tag{1}$$

where $t_{R,\text{corr.}}$ and $t_{R,\text{meas.}}$ are the corrected and measured retention times respectively, $V_{\text{extracol.}}$ is the extracolumn volume (determined experimentally) and w is the flow-rate.

Sample preparation

Brain tissue. Striata of female Wistar rats (180–200 g) were removed, homogenized in 0.5 ml of 0.2 *M* perchloric acid and centrifuged according to a method described elsewhere[28]. The supernatant was directly injected onto the column.

Urine samples. Urine was diluted 1:1 with 0.5 *M* perchloric acid and a 1-ml portion was filtered through a plug of Whatman GF/B filter by centrifugation as described elsewhere[28]. The filtrate was directly injected onto the column.

RESULTS AND DISCUSSION

Chromatography

In previous studies concerning RP-IPPC analysis of catecholamines and derivatives[26,28] or α-methyldopa and metabolites[27], the perchlorate concentration and the pH of the mobile phase were used as parameters for optimizing the separation.

If the mobile phase does not contain a modifier, a linear relationship is found between k' and perchlorate concentration for compounds with an amine function[26–28]. This indicates that amine–perchlorate or amino acid–perchlorate ion pairs exist only in the organic phase[26,27]. In mobile phases containing 20% (v/v) methanol this linear relationship is not found anymore (Fig. 2). The convex curves can be explained by assuming that ion pairs exist in the organic phase as well as in the aqueous phase. Using amine extraction as an example, the following new equilibria must be taken into account:

$$X^-_{\text{aq.}} + BH^+_{\text{aq.}} \overset{K_{\text{i.p.},1}}{\rightleftharpoons} BHX_{\text{aq.}} \tag{2}$$

$$BHX_{\text{aq.}} + nS_{\text{org.}} \overset{K_{\text{ex.}}}{\rightleftharpoons} BHXS_{n,\text{org.}} \tag{3}$$

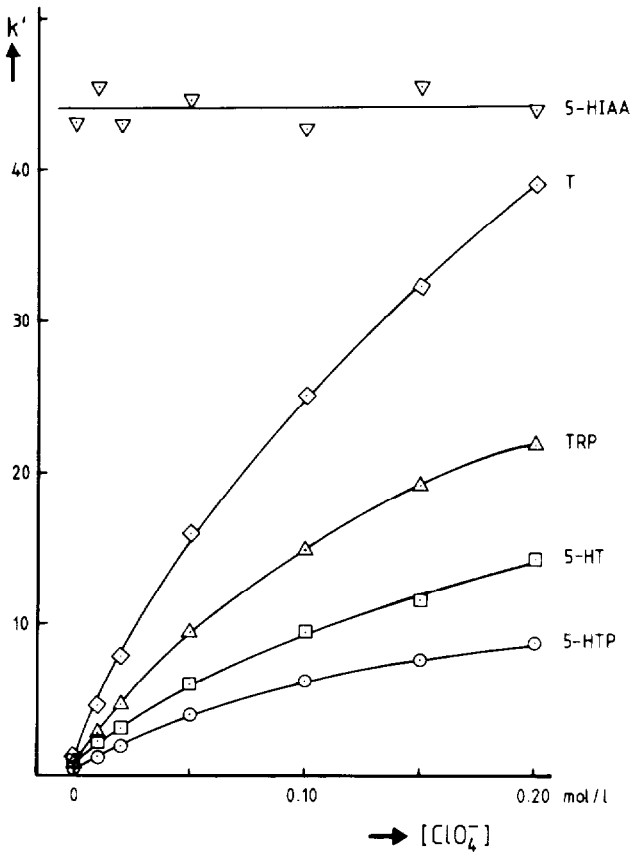


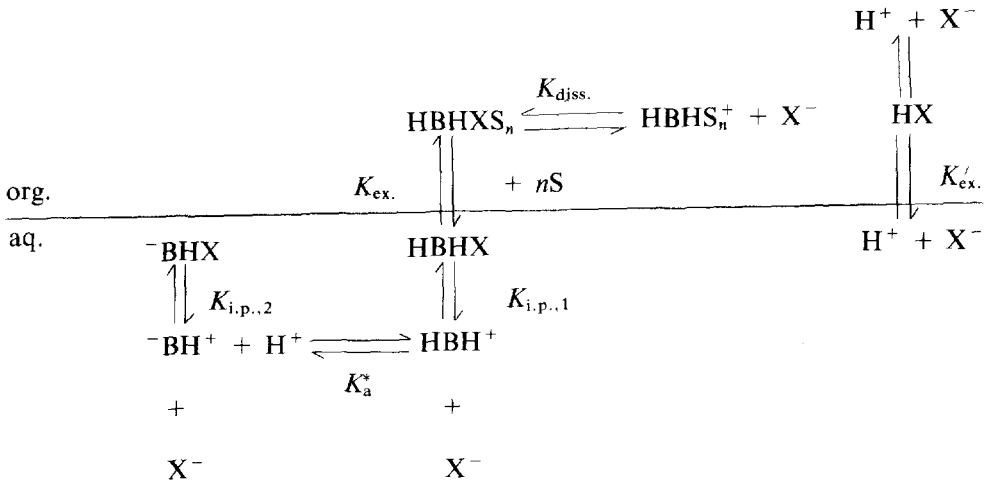
Fig. 2. Dependence of capacity ratios on the total counter-ion concentration. Mobile phase, phosphate buffer (pH 2.40) methanol (8:2); total buffer concentration, 0.05 *M*; temperature, 298°K.

where X^- is the perchlorate ion, BH^+ is the protonated amine, $K_{i.p.,1}$ is the ion-pair formation constant in the aqueous phase, S is tri-*n*-butylphosphate, which forms adducts with the ion pairs[26,27], and $K_{ex.}$ is the ion-pair extraction coefficient. Now an equation can be derived (see also refs. 26 and 27) for the distribution of an amine

$$D_{amine} = K_{ex.} \cdot [S]^n_{org.} \left(1 + \frac{K_{diss}}{[X^-]_{org.}}\right)\left(1 + \frac{K_{i.p.,1}}{[X^-]_{aq.}}\right)^{-1} \quad (4)$$

predicting a convex dependence of k' on the perchlorate concentration of the mobile phase ($K_{diss.}$ is the dissociation constant of the ion pair in the organic phase).

For amino acids, owing to dissociation of the carboxylic group in the mobile phase, the mechanism is more complex:



K_a^* is the conditional acid dissociation constant, $K_{i.p.,2}$ is the ion-pair formation constant of the zwitterion form ($^-BH^+$) of the amino acid and $K'_{ex.}$ is the perchloric acid extraction coefficient. It is assumed that the negatively charged perchlorate ion pair of the zwitterion (^-BHX) cannot be extracted into the organic phase.

From the equilibria described above, an equation can be derived for the distribution coefficient of an amino acid:

$$D = \frac{K_{ex.} \cdot [S]^n_{org.} \cdot K_{i.p.,1} \left(1 + \frac{K_{diss.}}{[X^-]_{org.}}\right) [X^-]_{aq.}}{\left(K_{i.p.,1} + \frac{K_{i.p.,2} \cdot K_a^*}{[H^+]_{aq.}}\right) [X^-]_{aq.} + 1 + \frac{K_a^*}{[H^+]_{aq.}}} \tag{5}$$

Although this equation is much more complex than eqn. 4, a convex dependence of k' on the perchlorate concentration is still predicted, provided that the pH of the mobile phase is kept constant ($K_{diss.} << [X^-]_{org.}$, see refs. 26 and 27).

Fig. 3 shows the effect of the pH of the mobile phase on the retention. As expected, the amine retention is not affected by the pH. Distribution of acids can be described as follows[30]:

$$D_{acid} = \frac{K_{HA}}{1 + (K_a^*/[H^+]_{aq.})} \tag{6}$$

where K_{HA} is the partition coefficient of the undissociated acid. At low and high pH values (pH $<<$ pK_a^* and pH $>>$ pK_a^*), eqn. 6 can be simplified to eqns. 7 and 8, respectively.

$$\log D = \log K_{HA} \tag{7}$$

$$\log D = \log K_{HA} + pK_a^* - pH \tag{8}$$

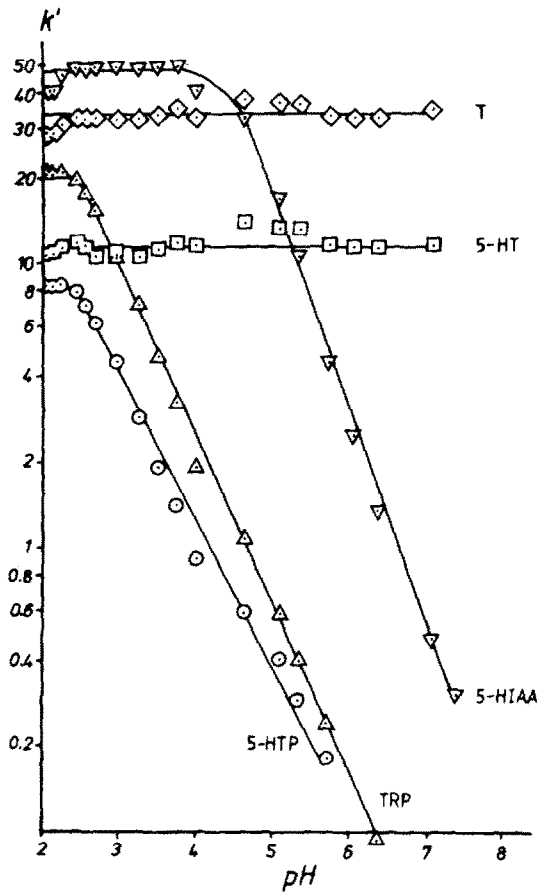


Fig. 3. Influence of pH of the mobile phase on the capacity ratios. Mobile phase, phosphate buffer–methanol (8:2); total perchlorate and buffer concentrations, 0.15 *M* and 0.05 *M*; temperature, 298°K.

According to these equations, plots of log k' *vs.* pH are linear at low and high pH values, and at the point of intersection of these linear parts of the curve pH = pK_a^*.

For amino acids at low and high pH values eqn. 5 can be simplified to eqns. 9 and 10, respectively.

$$\log D = \log (\text{numerator eqn. 5}) - \log (K_{i.p.,1} \cdot [X^-]_{aq.} + 1) \quad (9)$$

$$\log D = \log (\text{numerator eqn. 5}) - \log (K_{i.p.,2} \cdot [X^-]_{aq.} + 1) + pK_a^* - pH \quad (10)$$

According to eqns. 9 and 10 for amino acids the two linear parts of the log k' *vs.* pH curves intersect at $pH = pK_a^*$ only if $K_{i.p.,1} = K_{i.p.,2}$.

In Fig. 3, two linear parts can indeed be distinguished in the curves for the acids and amino acids. At their point of intersection, pH equals 4.55 (5-HIAA), 2.50 (TRP) and 2.40 (5-HTP). These values agree well with those reported in literature:

4.65 for 5-HIAA[35] and 2.38 for TRP[36]. For the acid (5-HIAA) this result is as expected, for the amino acid (TRP) it seems to indicate (see eqns. 9 and 10) that $K_{i.p.,1} \approx K_{i.p.,2}$.

According to theory (see eqns. 8 and 10) for acids and amino acids the slope of the log k' *vs.* pH curves in the pH-dependent range should equal -1. However, the slopes, as calculated from Fig. 3, range from -0.5 to -0.8. Although as yet a full explanation of this phenomenon cannot be given, the deviation from theory is at least partly caused by the presence of methanol in the mobile phase: if the mobile phase does not contain a modifier, slopes of TRP, 5-HTP and 5-HT range[37] from -0.8 to -1.0. It should be noted that in the present study the pH of the mobile phase was measured after addition of methanol to the mobile phase.

Perchlorate concentration and pH are not sufficient as parameters for optimization of the separation of the five compounds of interest. At lower pH values (below 3), required for sufficient retention of the amino acids, the capacity ratio of 5-HIAA is unacceptably high (*ca.* 200 at 298°K and pH 2–3), even though Polygosil C_{18}, selected as a support material, has a relatively small pore volume corresponding to a small phase ratio.

Generally, temperature can also be used as a parameter to influence retention. Depending on the compound, the capacity ratio is halved at each 10–15° increase of temperature and, in agreement with theory, plots of log k' *vs.* the reciprocal of absolute temperature (Van't Hoff plots) show straight lines (see Fig. 4). Although a higher temperature can be used to shorten the total analysis time, the selectivity coefficient $r_{5\text{-HIAA,T}}$, which is already unfavourably high at 298°K, becomes even more

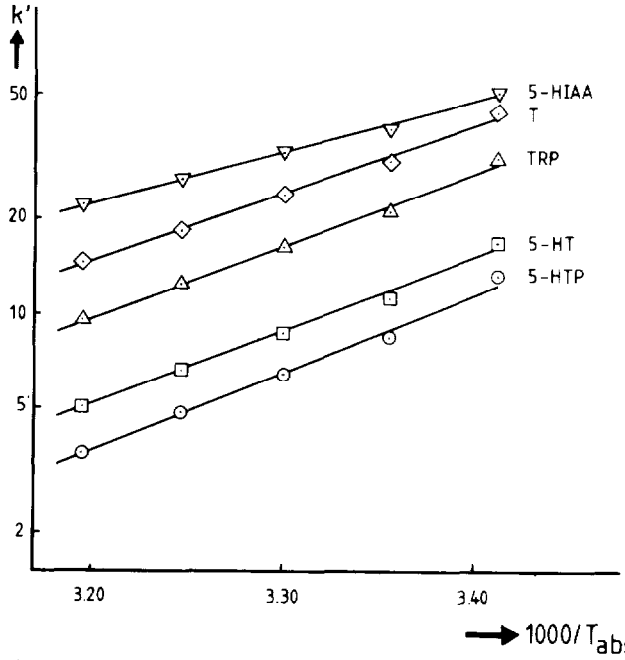


Fig. 4. Temperature dependence of the capacity ratios. Mobile phase, phosphate buffer (pH 1.95)–methanol (8:2); total perchlorate and buffer concentrations, 0.14 *M* and 0.05 *M*.

unfavourable at higher temperatures (see Fig. 4). Obviously, this phenomenon lengthens the analysis time.

Addition of methanol to the mobile phase results in a decrease of retention for all compounds, especially for 5-HIAA (see Fig. 5), and thus is an excellent parameter for decreasing the analysis time. In our experience, methanol contents above 30% should be avoided because this could possibly strip off the stationary phase.

Fig. 6 shows the separation of the five indoles under optimized conditions. Retention can be influenced selectively by one of the mobile-phase parameters: pH (5-HTP and TRP), perchlorate concentration (5-HTP, 5-HT, TRP and T), and methanol content (5-HIAA). In the optimized phase system, catecholamines and their derivatives do not interfere in the analysis: all of these compounds are eluted before 5-HTP, except HVA and DOPAC, which are eluted between 5-HT and TRP.

Detection

Electrochemical detection. Fig. 7 shows the current–voltage (*I E*) plots for the five indoles. The three compounds with a 5-hydroxy group are relatively easily oxi-

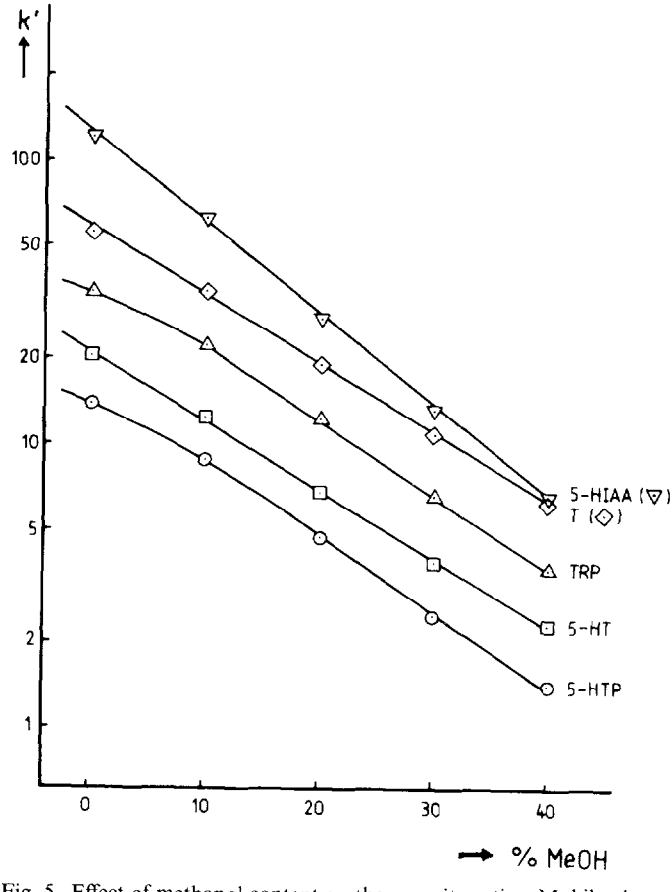


Fig. 5. Effect of methanol content on the capacity ratios. Mobile phase, phosphate buffer (pH 2.00) with various percentages of methanol; total concentrations of perchlorate and buffer, 0.15 *M* and 0.05 *M*; temperature, 308°K.

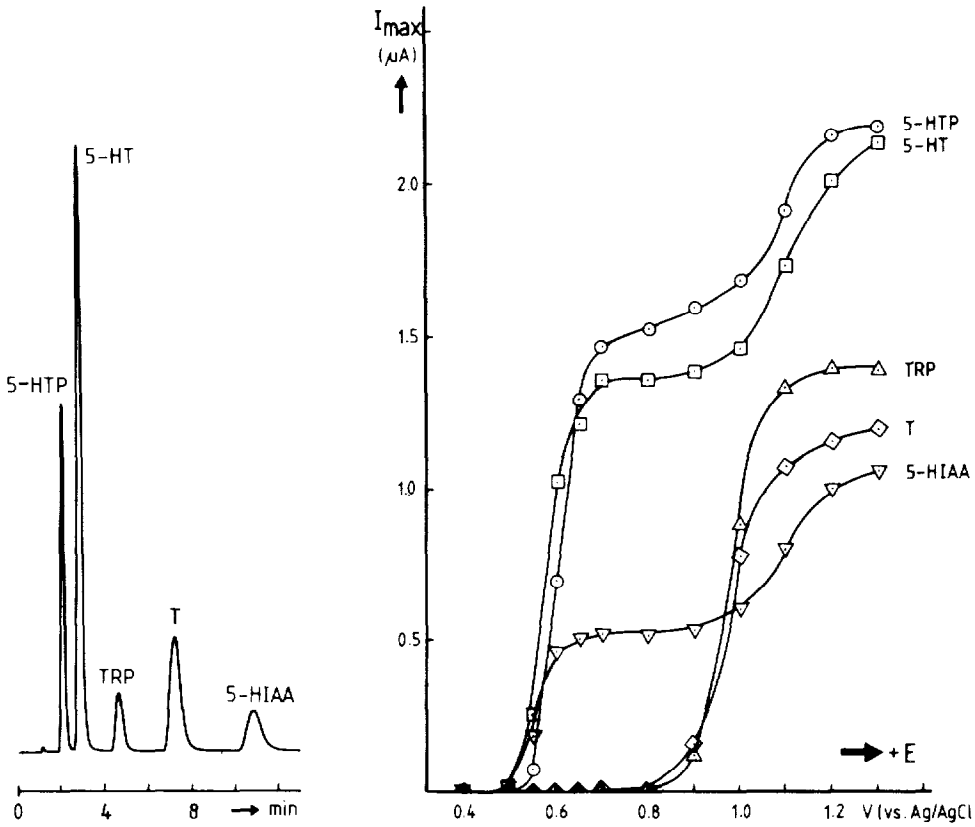


Fig. 6. Separation of the five indoles under optimized conditions. Mobile phase, phosphate buffer (pH 1.95)–methanol (8:2); total perchlorate and buffer concentrations, 0.14 *M* and 0.05 *M*; temperature, 308°K; flow-rate, 1.0 ml/min; detection, fluorometric, λ_{ex} = 282 nm, λ_{em} = 333 nm; amounts injected, 2–3 nmol per compound.

Fig. 7. Hydrodynamic voltamograms for the five indoles. Mobile phase, phosphate buffer (pH 2.2)–methanol (8:2); total concentrations of perchlorate and buffer, 0.15 *M* and 0.05 *M*; temperature, 298°K.

dized; oxidation of TRP and T is more difficult, and a plateau value in the *I–E* relationship is obtained only at $E \geqslant 1.1$ V *vs.* Ag AgCl. As to the compounds with a 5-hydroxy function, there is a second oxidation reaction at approximately the same potential where TRP and T are oxidized. Compared with data reported in the literature[16,21], the *I–E* curves are shifted to higher potentials owing to the relatively low pH (2.2) used in our experiments. Based on a signal-to-noise ratio of 3, detection limits range from 60 pg (5-HT, 5-HTP) to 150 pg (5-HIAA). These figures are not as favourable as those reported in ref. 16, but it should be emphasized that in this study little attention has been paid to optimization of detection limits.

In our experience, there is a gradual decrease (in terms of days to weeks) of detector response, presumably caused by contamination of the glassy carbon working electrode. Proper function can be restored by cleaning the working electrode with methanol, but best results were obtained when the surface was polished with 0.3-μm and 0.05-μm alumina, respectively. Electrochemical pretreatment of the working elec-

TABLE I

OPTIMAL WAVELENGTHS FOR FLUOROMETRIC DETECTION

Mobile phase, phosphate buffer (pH 1.95) methanol (8:2); total perchlorate and buffer concentration, 0.14 *M* and 0.05 *M*; temperature, 308°K.

Compound	λ_{ex} *(nm)*	λ_{em} *(nm)*
5-HTP, 5-HT, 5-HIAA	296	333
TRP, T	282	354

trode, *e.g.*, by applying a potential of +1.4 V for 10–30 min., only adversely affected detector function.

Fluorometric detection. Table I shows the optimal wavelengths for FMD of the five indoles. Analogously to ED, FMD distinguishes two groups of compounds, *viz.* compounds with and without the 5-hydroxy function. If all five compounds have to be assayed with maximum sensitivity, excitation and emission wavelengths of 282

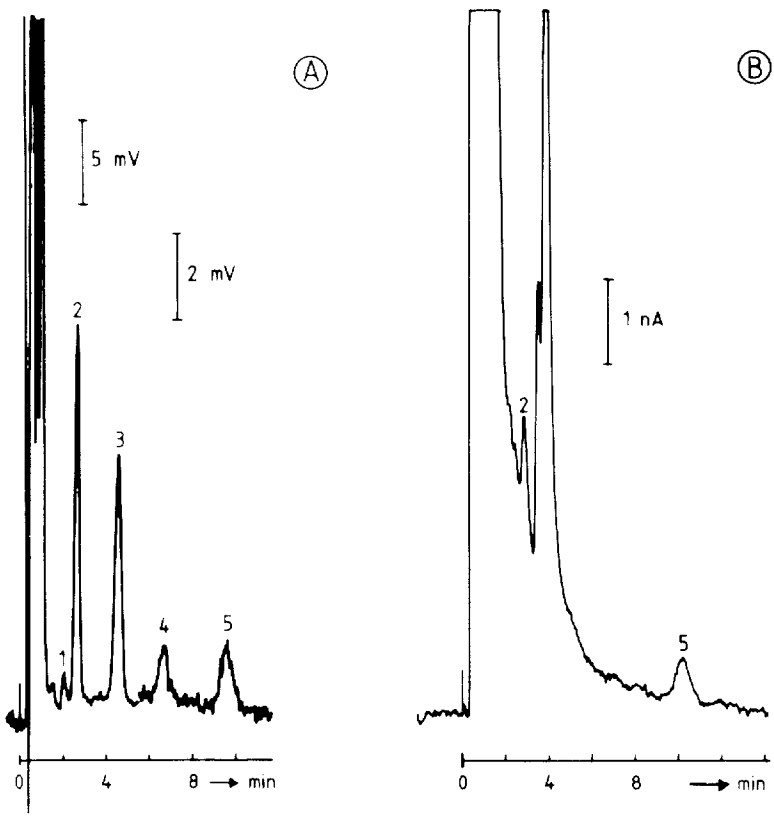


Fig. 8. Chromatograms obtained after injection of 100 µl of supernatant corresponding to 16 mg of striatal tissue. (A) Fluorometric detection, λ_{ex} = 296 nm, λ_{em} = 333 nm. (B) Electrochemical detection, E = +0.80 V *vs.* Ag/AgCl. Chromatographic conditions as in Fig. 6, except for flow-rate = 1.1 ml/min. Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = TRP; 4 = T; 5 = 5-HIAA.

and 333 nm, respectively, are a good compromise. Based on a signal-to-noise ratio of 3, the detection limits under optimized conditions range from 15 pg (5-HT) to 150 pg (5-HIAA). These values are comparable with those reported in ref. 33.

APPLICATIONS

The method was applied to brain tissue and urine analysis. FMD and ED were performed in series.

Striatum

Fig. 8 shows chromatograms obtained after injection of 100 μl of supernatant, corresponding to 16 mg of striatal tissue. Using FMD (Fig. 8a), all five compounds can be readily quantified. It is obviously advantageous that the sensitivity of FMD for catecholamines and metabolites is an order of magnitude lower than for the indoles. This is clearly demonstrated by comparison with ED (Fig. 8b). The two compounds which are eluted after 5-HT are HVA and DOPAC. These compounds are hardly detected by FMD. Besides, compared with FMD, the front peak observed by ED is more pronounced, and again this is due, at least partly, to the catecholam-

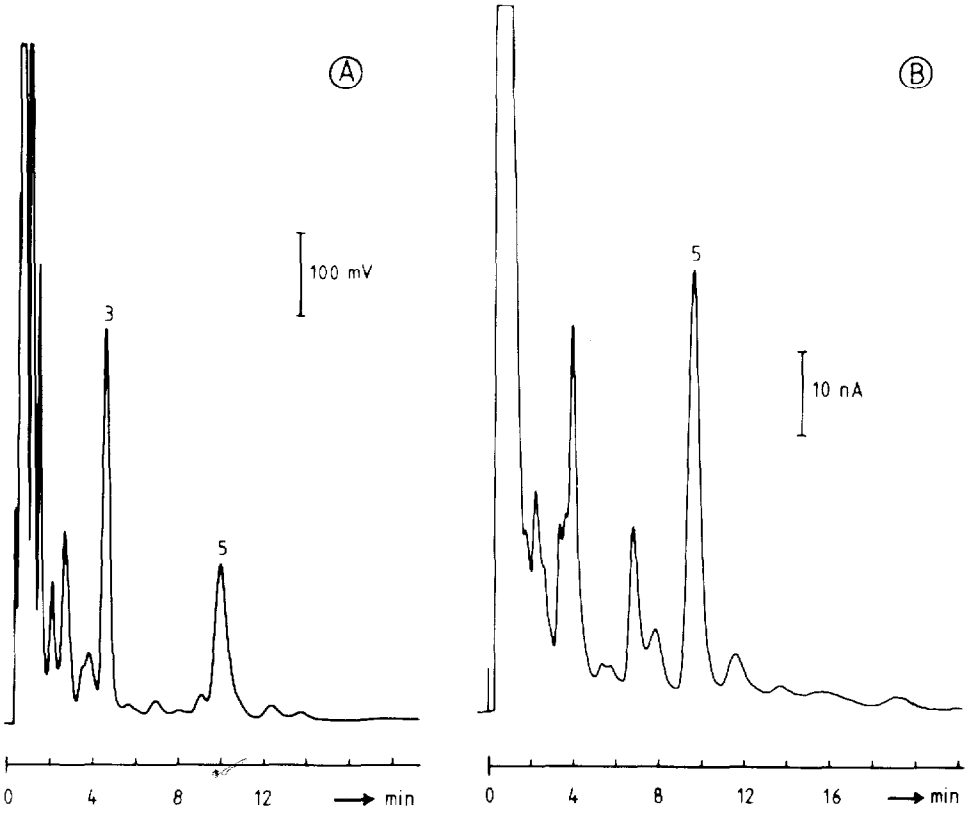


Fig. 9. Chromatograms obtained from a urine sample. Injection volume, 100 μl. Conditions as in Fig. 8, except for E_{ED} = +0.70 V *vs.* Ag/AgCl.

ines (*i.e.* dopamine, vanillylmandelic acid and noradrenaline), which are eluted faster than 5-HTP. The potential applied to the working electrode was set at +0.8 V, at which potential TRP and T are not oxidized. Although these compounds can also be detected at higher potentials (above 1.0 V), the selectivity and noise of the detection system will be adversely affected.

Urine

Urine samples contain more interfering compounds than brain tissue samples. When no sample clean-up is performed, neither FMD nor ED have sufficient selectivity for the assay of all five indoles. However, it is possible to determine TRP (FMD) and 5-HIAA (ED and FMD). Particularly if "dirty" samples, like urine, are assayed, the use of both detectors in series is strongly recommended for peak identity confirmation.

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