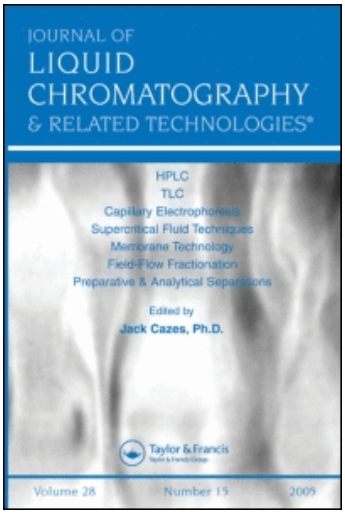


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# Liquid Chromatographic Analysis of Tryptophan and Serotonin Metabolites. Comparison of UV, Electrochemical and Spectrofluorimetric Detection

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# LIQUID CHROMATOGRAPHIC ANALYSIS OF TRYPTOPHAN AND SEROTONIN METABOLITES. COMPARISON OF UV, ELECTROCHEMICAL AND SPECTROFLUORIMETRIC DETECTION.

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## ABSTRACT

Tryptophan and five of its indolic metabolites have been separated by reversed-phase high performance liquid chromatography. The isocratic chromatographic system consisted of a column (20 x 0.46 cm i.d.) packed with RSil C18 HL5 and a methanol/$NaClO_4$ 0.2 M -$HClO_4$ (pH 1.4) mobile phase.

Three detection methods were tested : UV detection, fluorometric detection and electrochemical detection.

The limits of detection were found to be 4 picomoles (serotonin, 5 HTrp) and 20 pmoles (Trp, 5 HIAA, IAA , N-acetyl Trp) in the case of UV detection ; 1 pmole (serotonin), 4 pmoles (5H-Trp) and 10 pmoles (5 HIAA, Trp, IAA, N-acetyl Trp) for fluorometric detection ; and 1 pmole for electrochemical detection.

Analysis of human plasma were carried out using the above three detection methods to compare their relative specificity.

## INTRODUCTION

Tryptophan is metabolized via two major pathways, the kynurenine pathway and the indolic pathway. The second one, quantitatively

less important, presents however a great physiological importance, mainly because it is the precursor of serotonin (5-hydroxy tryptamin), fundamental brain neurotransmitter.

Serotonin is predominantly metabolized in 5-hydroxy indolacetic acid, an urinary metabolite, variations of which are dependent on those of serotonin.

Abnormal metabolism of tryptophan has been associated with a number of disease states in man, including Hartnup disease, Down's syndrome, schizophrenia, depression (1-6)... Aberrations of tryptophan metabolism are also associated with some cancerous tumors.



These metabolic disorders result in modification of normal concentrations of tryptophan metabolites in an organism ; therefore it seemed of interest to possess a sensitive, reliable, specific and rapid technique for measuring tryptophan (Trp), serotonin, 5-hydroxy indolacetic acid (5 HIAA), and other indol derivatives such as 5-hydroxy tryptophan (5-HTrp), indolacetic acid (IAA) and N-acetyl tryptophan (N-acetyl Trp).

The chromatographic technique generally used for aminoacid analysis is ion exchange (7-9), a time-consuming method requiring relatively large sample volumes.

HORVATH (10-12) has demonstrated the interest of aqueous eluents used simultaneously with nonpolar stationary phases, for the separation of small polar molecules. This method, previously used by different authors (14-17), was adapted and optimized for the separtion of Trp and its indolic metabolites.

Reported in this paper are the separation of these compounds using reversed-phase HPLC, and the comparative results of three detection methods : UV, fluorometric and electrochemical detection.

## EXPERIMENTAL

### . Apparatus

The chromatographic equipment consisted of a Chromatem 380 pump (Touzart & Matignon, France), a Rheodyne Model 71-20 sample injection valve, a Pye Unicam variable wavelength UV monitor (Philips, France), a JY 3 fluorimeter (Jobin Yvon, France), a DELC "Thin layer" electrochemical detector (Tacussel, Lyon France) and a PRG 5 polarograph (Tacussel) as potentiostat. The cell of the electrochemical detector, adjusted to a volume of 8 µl, was equiped with a glassy carbon working electrode, the reference potential being supplied by a silver-silver chloride electrode. The cell was used in the d.c. mode throughout.



Peak surfaces were integrated with an Autolab System I integrator (Spectra Physics, USA).

The signal output of the detectors was displayed on a 0470 L Linseis recorder and an EPL Tacussel recorder (in the case of electrochemical detection).

### . Chromatographic system

A 20 x 0.46 cm i.d. column was packed (13) with RSIL C18 HL5 (Interchim, Montluçon, France), particle size 5 µm).

The solvents used for the slurry and the packing procedure were respectively 1-butanol and methanol. The composition of the mo-

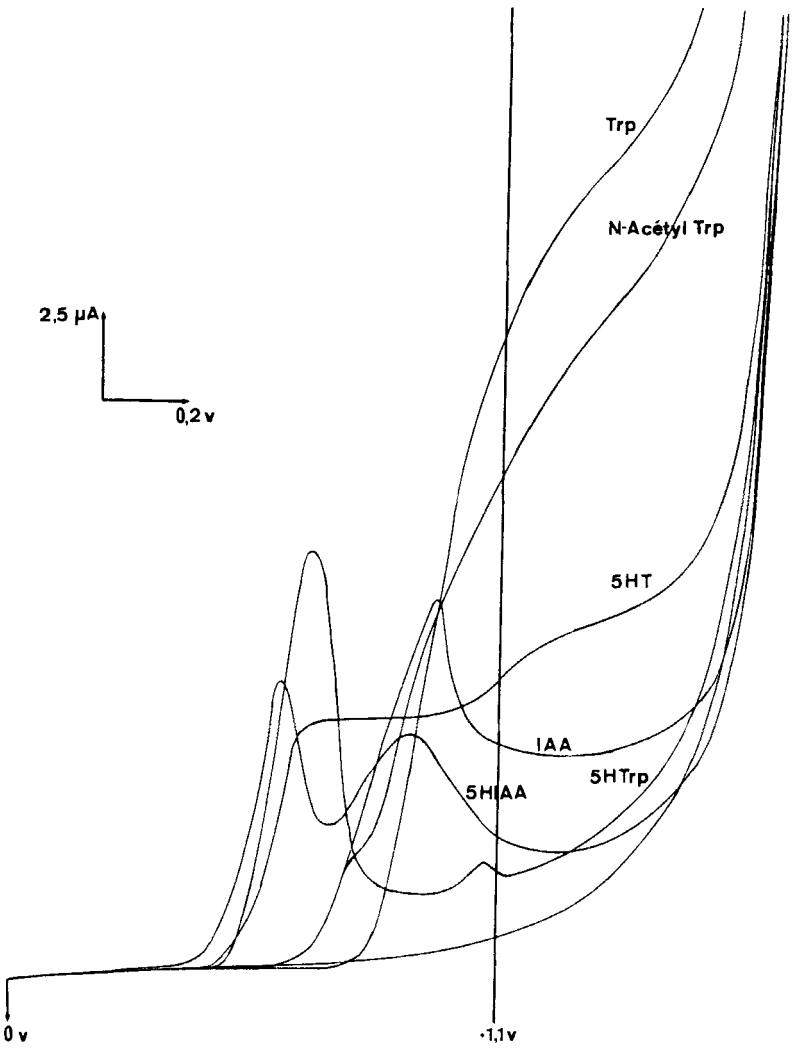


Figure 1 - Intensity-potential curves of tryptophan and indolic metabolites (concentrations $2.10^{-4}$M). Glassy carbon rotating disc electrode, 3 mm diameter 3000 r.p.m.

bile phase consisted of methanol and $NaClO_4$ 0.2 M - $HClO_4$ , pH 1.4 (30:70). The mixture was degassed for 5 minutes in an ultrasonic bath before use.

. Reagents

Standard solutions were prepared by dissolving tryptophan and metabolites (SERVA, Analytical grade) in the mobile phase.

Methanol was purchased from MERCK (Chromatographic grade), 1-butanol, $NaClO_4$ and $HClO_4$ were purchased from PROLABO (NORMAPUR guaranteed reagent).

Healthy human blood was collected in heparinized tubes and centrifugated. The plasma was defecated with a 100 g/l sulfosalycilic acid solution (4 vol. plasma/1 vol. acid), then stored at -25°C prior to analysis.

. Procedure

The compounds under study were detected at 280 nm, which corresponds to the maximum absorbance of tryptophan.

The excitation and fluorescence wavelength used in fluorometric detection were respectively 300 nm and 335 nm.

The oxidation potential used in electrochemical detection was +1.1 V. It was determined by plotting the current vs potential curves for each solute (fig.1).

The detection limits ( $\frac{\text{signal}}{\text{background noise}} = 5$) were determined using standard solutions of compounds.

. Results and discussion

The reversed-phase HPLC separation of a synthetic mixture of tryptophan and its metabolites is shown in figure 2.

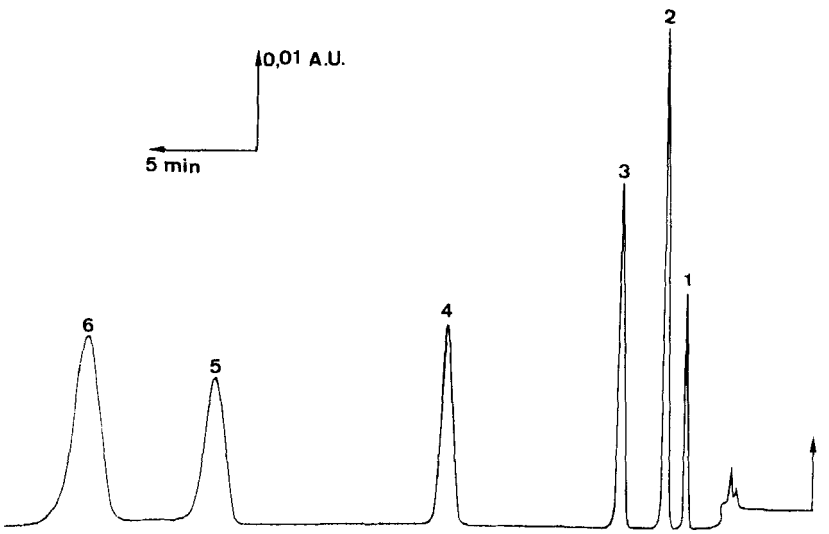


Figure 2 - Chromatographic separation of tryptophan and its indolic metabolites. Column : RSil C18 HL5 (20 x 0.46 cm i.d.). Mobile phase : methanol/$NaClO_4$ 0.2 M-$HClO_4$ pH 1.4 (30:70). UV detection 280 nm. Flow rate 0.6 ml/min. Peaks : (1) serotonin, (2) 5 HTrp ; (3) 5 HIAA , (4) Trp ; (5) N-acetyl Trp ; (6) IAA.

The phenolic compounds (serotonin, 5-HTrp, 5 HIAA) are eluted more rapidly than tryptophan and other metabolites, a fact which shows an important decrease in the stationary phase affinity due to phenolic ring.

The linearity of detectors was demonstrated in the 5-100 µM concentration range, by measurement of the peak surface.

Figures 3, 4 and 5 show the injection of standard solutions of 4 and 10 pmoles in the three detection modes, making it possi-

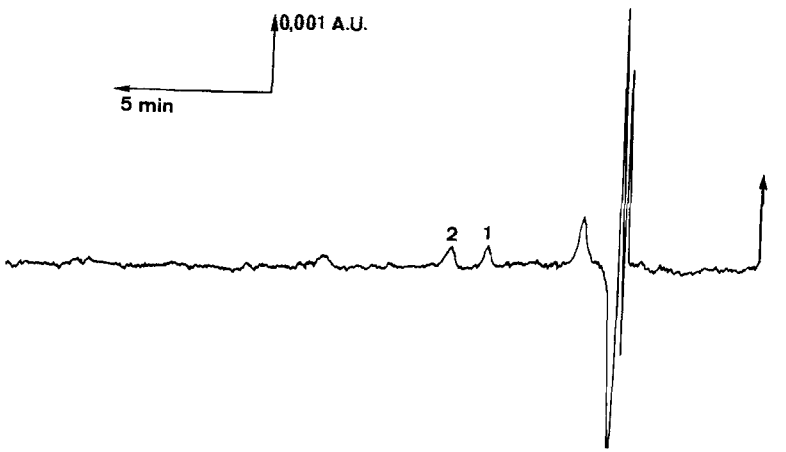


Figure 3 - UV detection wavelength 280 nm. Injection of 4 pmoles. column : Rsil C18 HL 5 (20 x 0.46 cm i.d.) , mobile phase : methanol/$NaClO_4$ 0.2 M - $HClO_4$ pH 1.4 (20:80). Flow rate : 0.6 ml/mn. Peaks : (1) serotonin ; (2) 5-HTrp.



ble to demonstrate their relative sensitivity. Due to a non reproducibility in the different batches of RSil C 18, the mobile phase composition had to be modified from 20 to 30 % methanol.

Figure 5 shows the improvement in electrochemical detection sensitivity which became 10 times greater due to a decrease in the cell joint thickness from 500 µm to 200 µm.

200 µm thickness (8 µl) is, in this case, the best compromise between sensitivity and baseline stability.

Detection limits in UV detection are 4 pmoles for serotonin and 5-HTrp ; and 20 pmoles for Trp, 5HIAA, IAA and N-acteyl Trp.

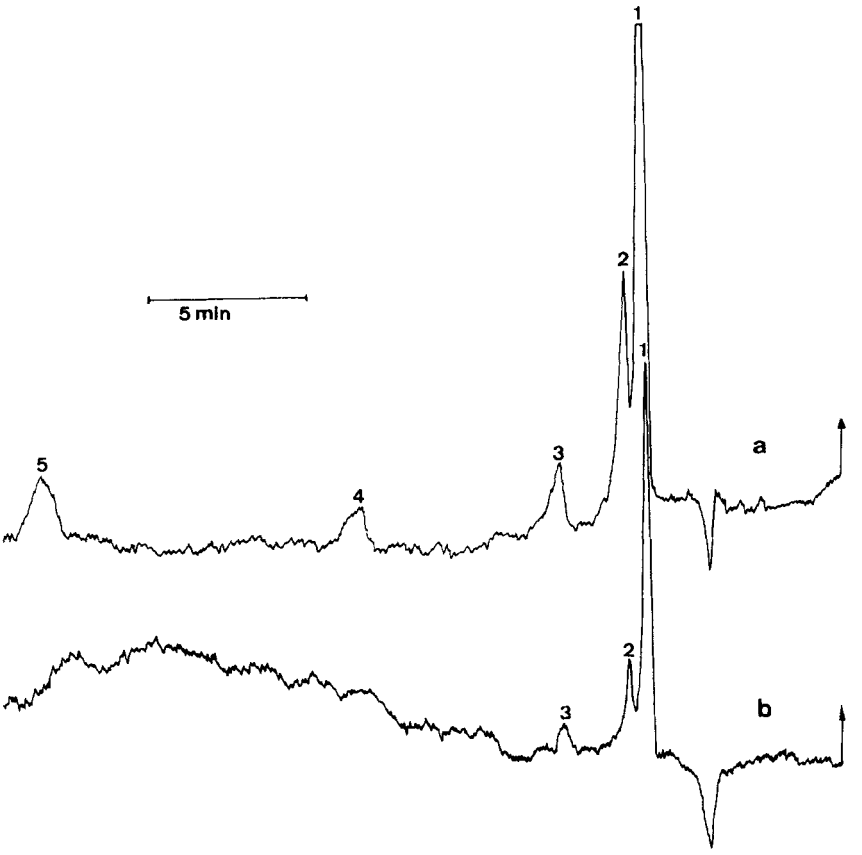


Figure 4 - Fluorometric detection, $\lambda_{exc}$ = 300 nm ; $\lambda_{fluo}$ = 335 nm (gain x 1000). Injection of (a) 10 pmoles , (b) 4 pmoles. Column : RSil C18 HL5 (20 x 0.46 cm i.d.) ; mobile phase : méthanol/$NaClO_4$ 0.2 M - $HClO_4$ pH 1.4 (30 : 70). Flow rate : 0.6 ml/min. Peaks : (1) serotonin ; (2) 5 HTrp ; (3) 5 HIAA ; (4) Trp ; (5) N-acetylTrp.

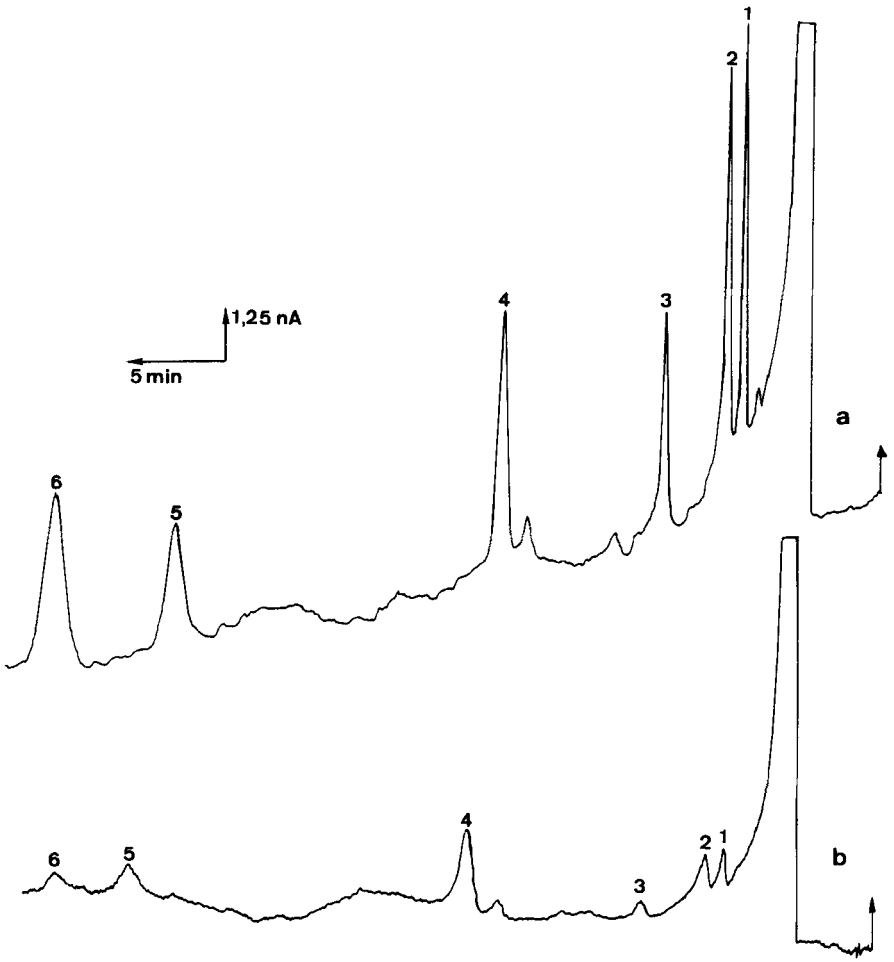


Figure 5 - Electrochemical detection, detector operating potential + 1,1 V.Electrochemical cell thickness : (a) 200 µm ; (b) 500 µm. Injection of 4 pmoles. Column : RSil C 18 HL 5 (20 x 0.46 cm i.d.); mobile phase : methanol/ $NaClO_4$ 0.2 M - $HClO_4$ pH 1.4 (25:75). Flow rate : 0.6 ml/min. Peaks : (1) serotonin ; (2) 5 HTrp ; (3) 5 HIAA ; (4) Trp ; (5) N-acetylTrp ; (6) IAA.

In fluorometric measurements the detection limits have been determined to be 1 pmole for serotonin ; 4 pmoles for 5-HTrp ; and 10 pmoles for 5 HIAA, Trp, IAA and N-acetyl Trp.

Electrochemical detection appears to be the most sensitive detection mode, the detection limits being 1 pmole for all the six compounds studied.

Fluorometric and electrochemical detection modes are particularly specific in the cases mentioned above, because most biological compounds, like other aminoacids, are not naturally fluorescent nor electrochemically active, and therefore will not interfere with either of these detection modes.



It has been possible to compare the relative importance of interferences in each mode of detection when analyzing tryptophan and its metabolites in biological fluids.

Figure 6 shows the chromatogram obtained after injection of 4 µl of blood plasma. The three detectors were monitored at their maximum sensitivity.

The UV and fluorometric detection sensitivity are comparable for the determination of tryptophan (concentration 40 µM). Interferences are less important in fluorometric detection. Nevertheless the peaks corresponding to hydroxylated indolic metabolites are not separated from the interference peak.

The major advantage of UV detection is its relatively easy use. Its use is particularly indicated in determining tryptophan and compounds in the case of higher concentrated samples.

Figure 6 c confirms that electrochemical detection provides the best sensitivity and specificity among the three methods studied.

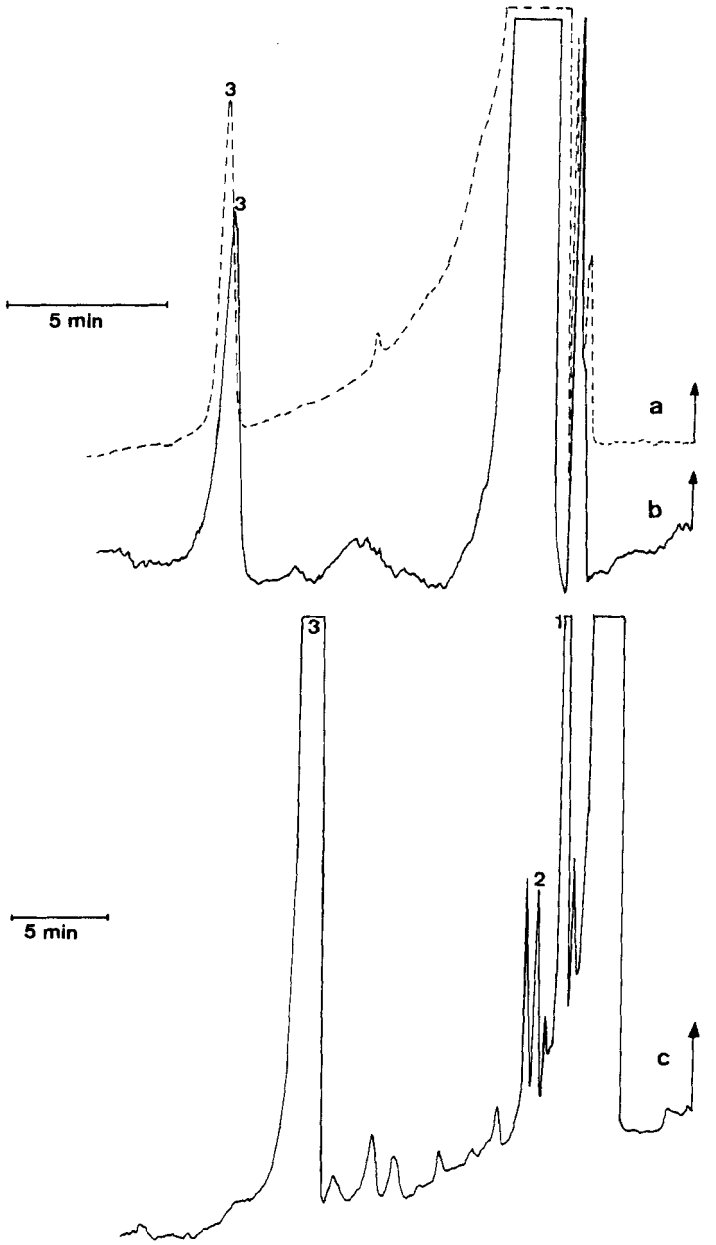


Figure 6 - Chromatogram of a 4 µl serum using (a) UV detection , (b) fluorometric detection , (c) electrochemical detection. Peaks : (1) serotonin ; (2) 5-HTrp ; (3) Trp.

With this technique the peak of interferences is comparatively the lest important. Electrochemical detection is, among the three methods tested ,the only one allowing the detection of the peaks of serotonin and 5-HTrp , which are not overlapped by the interference peak.

Therefore, electrochemical detection is,in the present case, the most interesting detection method, mainly because of its detection limit (1 pmole) and its specificity.

## BIBLIOGRAPHY


1. p. POITOU, R. BOULU - Path. Biol. 25, N°8, 565.
2. J.P. GARNIER, B. BOUSQUET, C. DREUX - J. of Liquid Chromatogr. 2(4), (1979), 539.
3. J.B. JEPSON - Advan. Pharmac. 6B (1968), 171.
4. D. O'BRIEN, A. GROSHEK - Arch. Dis. Child 37, (1962), 17.
5. J.A. OATES, A. SJOERDSMA - Neurology, 10 (1960), 1076.
6. A.J. COPPEN - J. Psychiat. Res. 9 (1972), 163.
7. J.C. DICKINSON, P.B. HAMILTON - J. Neurochem. 13, (1966), 1179.
8. C.M. PLUM - J. Neurochem. 23, (1974), 595.
9. T.L. PERRY, S. HANSEN, J. KENNEDY - J. Neurochem. 24 (1975),587.
10. C. HORVATH, W. MELANDER, I. MOLNAR - J. Chromatogr. 125 (1976), p.129.
11. C. HORVATH, W. MELANDER, I. MOLNAR - Anal. Chem. 49 (1977),142.
12. I. MOLNAR, C. HORVATH - J. Chromatogr.142 (1977), 623.
13. B. COQ, C. GONNET, J.L. ROCCA - J. Chromatogr. 106, (1975),249.
14. S. YONG, S. LAU - J. Chromatogr. 175 (1979), 343.
15. D. A. RICHARDS - J. Chromatogr. 175 (1979) , 293.
16. D. D. KOCH, P. T. KISSINGER - J. Chromatog. Biomed. Appl. 164 (1979), p.441.
17. A.M. KRSTULOVIC, C.MATZURA - J. Chromatogr. Biomed. Appl. 163 (1979), p.72