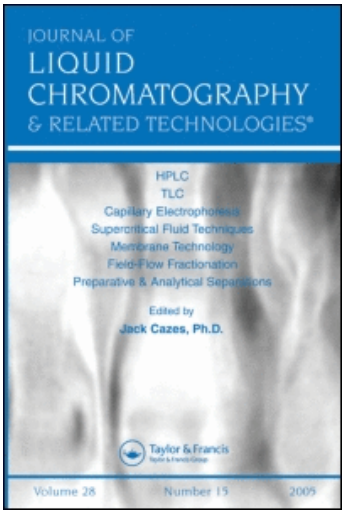

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RAPID AND SPECIFIC DETERMINATION OF SEROTONIN, ITS PRECURSORS AND METABOLITES IN RAT BRAIN TISSUE BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A liquid chromatographic analysis with electrochemical detection is proposed for simultaneous determination of four indols (i.e. tryptophan, 5-hydroxytryptophan, serotonin and 5-hydroxyindoleacetic acid) in various rat brain areas. Optimal elution conditions have been determined by studying chromatographic parameters : pH, buffer salt, counter-ion, organic modifier. Eventual interferences with the catecholaminergic compounds (i.e. norepinephrine, dopamine and their major metabolites) known to be detectable in the same conditions, have been eliminated. The specificity of the four indol detection was demonstrated by chromatographic, electrochemical and pharmacological proofs.

INTRODUCTION

Liquid chromatographic methods with electrochemical detection (LC-ECD) are now widely accessible for laboratories interested in

biogenic amine metabolism studies. Various analytical procedures have been described that permit simultaneous quantitation of catecholamines and indoleamines, their precursors and metabolites mainly in brain tissues (1, 2). Among the different methods proposed for the study of indols alone, few authors have reported on the specificity of their methods (3, 4). It is essential to verify this particular point [even though LC-ECD improves the specificity (5, 6) compared to U.V or fluorimetric detection] and thus to decrease the interference risk. As a matter of fact, some interferences have been described (1, 7), which might explain the discrepancies observed between the endogenous levels of biogenic amines reported by different authors for the same brain region.

Among the more obvious methods which have been used in judging the specificity of a new LC-ECD procedure one can propose :

* First, the chromatographic separation mechanism being one of the factors of specificity, the study of the chromatographic behaviour of a compound in various elution conditions will confirm its identity (4, 8, 9, 10). In the same manner the addition of small amounts of standard derivatives in a brain sample should not modify the peak shape but only increase the peak height (11).

* Second, evaluation of the electrochemical response : for the electrochemically active substances which could interfer, no problem occurs if their half-wave potential is higher than this of the studied compound. In the other case, a dual electrodes detector will be suitable (12, 13). For a given derivative, the peak height will be maximal in a very narrow potential range, and thus peak identity will be usefully confirmed by studying the electrochemical response versus applied potential (3, 14).

* Third, according to SALLER and SALAMA (14), a pharmacological proof of the identity of the studied peaks may be obtained by using some substances inhibiting various enzymatic steps of the metabolism pathway in central nervous system. The levels of the measured compounds vary in a predictable manner after various drug treatments

(e.g pargyline, a monoamine oxidase inhibitor, will elevate 5-HT levels and decrease the levels of its deaminated metabolite). The effects of these substances on the endogenous levels, evaluated by the new method, will have to be in agreement with those expected (15, 16, 17).

The interference risk may also be decreased by sample pretreatment using either alumina adsorption, or anion-exchange resin or sephadex (1, 18, 19), but this purification step is time-consuming thus some authors have proposed a single perchloric deproteinization before the injection onto the chromatographic column.

The present work was designed to propose a new LC-ECD procedure allowing the simultaneous determination of four indol derivatives : tryptophan (Trp), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (serotonin, 5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in cerebral areas. This method will have to be rapid without handling and time-consuming purification step, sensitive and specific enough to avoid predictible interferences, among 11 catechol derivatives well known to coexist with the indols and accuratelly detectable in rat brain areas, according to results so far published.

MATERIALS AND METHODS

Chemicals

All chemicals used in the present study were of analytical grade : EDTA-disodium salt (Titriplex III), potassium dihydrogene phosphate, sodium acetate and citric acid were purchased from Merck. The mobile phases A, B and C were prepared with bidistilled desionized water. Methanol (PROLABO, Paris) and acetonitrile (OSI) were for liquid chromatography.

All the standard compounds : norepinephrine (NE), epinephrine (E), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3,4-dihydroxyphenylalanine (DOPA), normetanephrine (NMN), metanephrine (MN), 4-hydroxy, 3-methoxyphenylglycol (MHPG), 3-methoxytyramine (3-MT), L-tryptophan (Trp), 5-hydroxytryptophan

(5-HTP), 5-hydroxytryptamine (serotonin, 5-HT), 5-hydroxyindolacetic acid (5-HIAA) and 4-hydroxy, 3methoxymandelic acid (VMA) were obtained from SIGMA. L-cystein used as antioxydant was of cell-culture quality (SIGMA). Ion-pairing reagent was heptane sulfonic acid in acetic solution from WATERS (Pic B7). Pargyline hydrochloride used as a pharmacological tool was from SIGMA.

Apparatus

The LC-ECD apparatus consisted of a BECKMAN A-112 solvent delivery system, an automatic injector (ISS-100, Perkin-Elmer) with a refrigerated tray, a stainless-steel column (150x4.5 mm) packed with a 5µm regular particulate bonded-phase ODS (Ultrasphere, BECKMAN). The electrochemical detection was provided by a glassy carbon electrode versus an Ag/AgCl reference electrode (METROHM ED 666). The potential was set at +0.8 volt using a METROHM VA 135 potentiostat and the detector sensitivity at 1 nA full scale deflection.

Mobiles Phases

Throughout this study, various mobile phase compositions were used. The modifications included the following changes :

- presence or absence of counter-ion (Pic B7)
- variations of pH values in the range 3.5 - 5.4,
- nature and molarity of the buffer salt (i.e. phosphate, acetate or citrate).
- nature and proportion of the organic modifier (i.e. acetonitrile or methanol),

The mobile phases were filtered through a 0.45 µm filter (MILLIPORE) and degassed by vacuum aspiration.

phase A : this moderately fast elution solvent, used for the separation of the endogenous aminergic derivatives was an adaptation of a previously described method (2) : a binary mixture of KH_2PO_4 0.1M, containing EDTA 0.1mM and sodium heptane sulfonate 5mM (Pic B7), with methanol (92,5/7,5 V/V). pH was adjusted at 3.75 and the flow-rate maintained at 1ml/min.

phase B : this phase was a mixture of 90 volumes of KH_2PO_4 0.2M, containing EDTA 0.1mM and 10 volumes of methanol. pH was adjusted at 5.4 and the flow rate set at 1 ml/min.

phase C : this elution solvent was a mixture of CH_3COONa 0.1M and KH_2PO_4 0.1M containing EDTA 0.1mM (97 volumes) and acetonitrile (3 volumes). pH was adjusted at 5.4 and the flow rate was maintained at 1ml/min.

The retention time (RT) of each derivative injected alone or in a mixture solution was recorded. K' values (capacity factor)were calculated and expressed as : (RT-T0)/T0 with T0= void volume.

Application of the Method

Quantitation of catecholaminergic and indolaminergic compounds in rat cerebral areas were performed using a method previously described which involved a rapid preparation of the cerebral area dissected and frozen at -80°C until the assay. For the assay, the brain sample was homogenized in a perchloric medium ($HCLO_4$ 0.2N) containing EDTA 0.1%, $Na_2S_2O_5$ 0.1% and l-cystein 0.1% as antioxydant (20). A centrifugation allowed to obtain a limpid supernatant which was injected onto the chromatographic column with mobile phase C as eluent. Standard solutions were injected at constant interval for calibration of the system.

For the evaluation of the pharmacological effects of pargyline, rats were given 75 mg/kg in saline solution (i.p. route) and killed 2 hours after administration. Indol derivatives were assayed in hypothalamus, hippocampus, striatum and cortex.

RESULTS AND DISCUSSION

Chromatographic Conditions

Mobile phase A separated a mixture of 15 compounds. As showed in figure 1, two compounds were eluted in the solvent front and 13 derivatives were separated within 30 minutes. This mobile phase is very

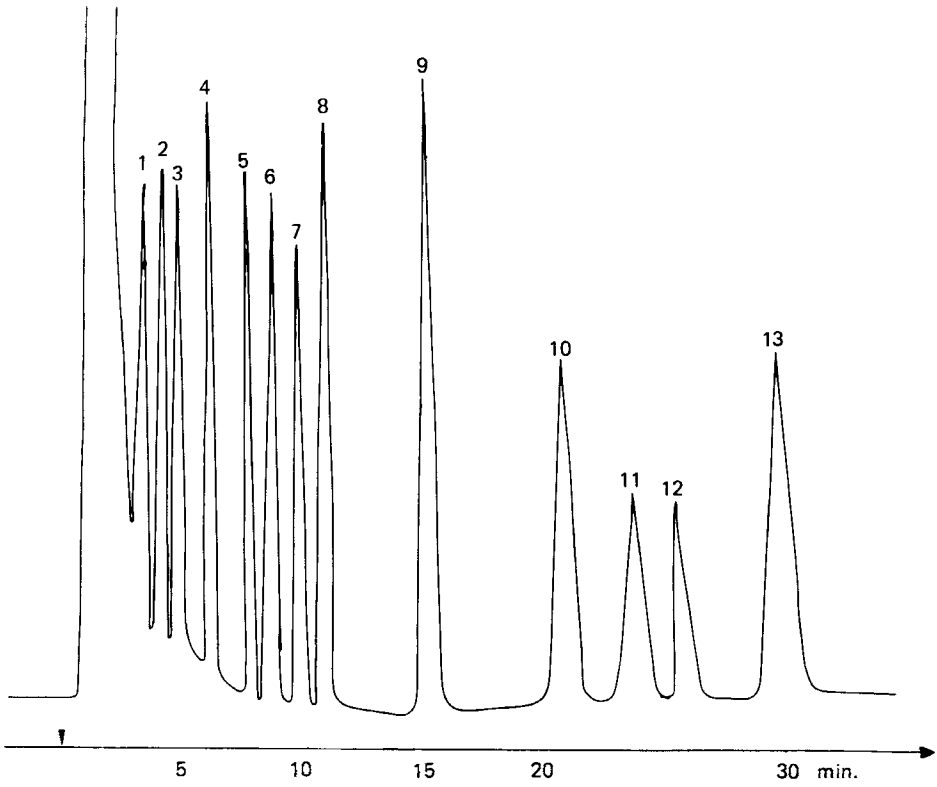

FIGURE 1 : Chromatogram of a standard mixture containing catechol and indol derivatives.

Chromatographic conditions : mobile phase A, 1ml/min.
Detection 0.85v, 1nA full scale.
0.4 ng of each compound were injected, except 8 ng for Trp.
1= MHPG., 2= NE., 3= E., 4= 5-HTP., 5= NMN., 6= DOPAC., 7=MN., 8= DA., 9= 5-HIAA., 10= HVA., 11= Trp., 12= 3-MT., 13 = 5-HT.
L-DOPA and VMA were in solvent front.

efficient, however, it could be interesting to separate only the indols, without interference with the catechols present and detectable in the sample. Thus modifications of mobile phase A have been performed.

* Effect of PIC B7

The influence of PIC B7 was displayed by the RT modifications of each studied compound. Absence of PIC B7 increased the retention times of the acidic metabolites (DOPAC, 5-HIAA and HVA) whereas it decreased those of the aminated derivates (NE, DA and 5-HT). 5-HTP and Trp had specific behaviour, probably due to the amino-acid structure of these compounds (table I). With mobile phase A, containing PIC B7, two indols (5-HT and Trp) are the latest eluted compounds. Thus, mobile phases without PIC B7 were used to shorten the total analysis time of these four indols without modifying the elution order.

TABLE I

Effect of PIC B7 on Capacity Factor (K') Values of Catechols and Indols

	PIC B7 5 mM	Without PIC B7
NE	1.9	1.3
5-HTP	3.0	3.6
DOPAC	4.1	10.5
DA	5.7	1.2
5-HIAA	7.5	16.5
HVA	11.0	25.5
5-HT	14.2	5.2
Trp	16.0	10.1

K' = RT-T0/T0 with T0 = void volume RT = retention time. Chromatographic conditions : KH_2PO_4 O.1M + CH_3OH 7.5%, pH 3.75

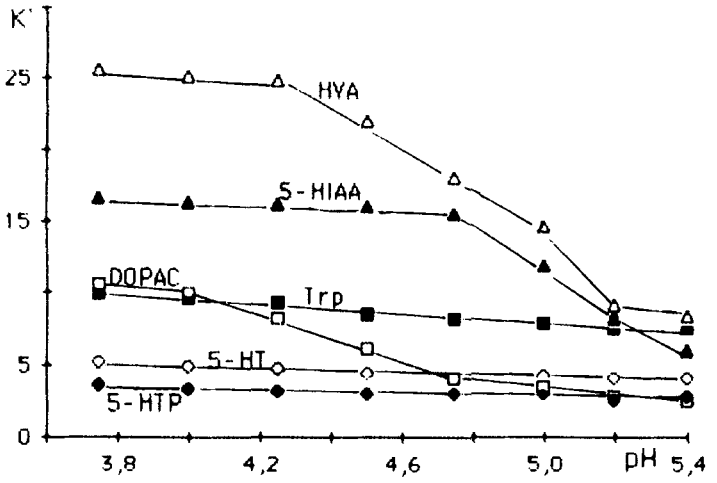

FIGURE 2 : Effect of the mobile phase pH on capacity factor (K') of four indol derivatives and two interferent catechols.

Chromatographic conditions : KH2P04 0.1M 92.5% + Methanol 7.5%, flow rate = 1ml/min.

* pH effects

pH effects were investigated without counter-ion in the mobile phase. pH greatly affects the K' values and the elution order : in the pH range 3.75 - 5.4, according to the pK_a values of the carboxylic groups, the retention times of the acidic metabolites (5-HIAA, DOPAC and HVA) decreased with increasing pH. Moreover, we observed that the K' values of the hydroxylated compounds as well as the aminated (5-HT, DA, NMN, and MN) were slightly affected by pH variations. For the indols, pH effects were obvious for 5-HIAA (fig. 2) and with phase B, at pH 5.4 the separation of the indols was achieved in the following order : DOPAC/5-HTP, 3-MT, 5-HT, 5-HIAA and Trp/HVA. But even though this phase B allowed rapid separation, the specificity was not achieved since DOPAC was still coeluted with 5-HTP and HVA with Trp.

* Effect of buffer salts

The separation of indolic compounds on the C_{18} bonded-phase column depended on the ionic strength and on the nature of the buffer salt in the medium. Moreover, salt is necessary to allow electrochemical response. In order to modify the molarity of the mobile phase B, we acted on the amount of KH_2PO_4. Increasing the mobile phase ionic strength in the range 0.1-0.2 M did not affect the elution order but slightly accelerated the elution of 5-HT and improved the resolution for 5-HT and 5-HIAA. When we modified the nature of the buffer salt (table II), with sodium acetate a faster separation and a modification in the elution order of 5-HT and 5-HIAA were obtained. Thus this salt, combined with potassium phosphate was chosen for the next analysis. But, even with this new combination DOPAC/5-HTP still coeluted.

* Effect of organic modifier

As indols are relatively non-polar compounds, the addition of various amount of organic modifier was able to modify RT. The indol capacity factors decreased as the concentration of methanol or acetonitrile increased. However, the methanol proportions affect the retention of all

TABLE II

Effect of Buffer Salt Nature on K' of Indols and Catechols.

	Potassium phosphate 0.2 M	Sodium acetate 0.2 M	Sodium citrate 0.2 M
DOPAC	2.0	1.0	1.9
5-HTP	2.2	1.8	2.0
5-HT	3.0	3.9	2.7
5-HIAA	5.3	3.2	4.7
HVA	6.8	3.5	5.8
Trp	6.8	5.7	6.2

Chromatographic conditions : buffer salt 91%, CH3OH 9%, pH 5.4

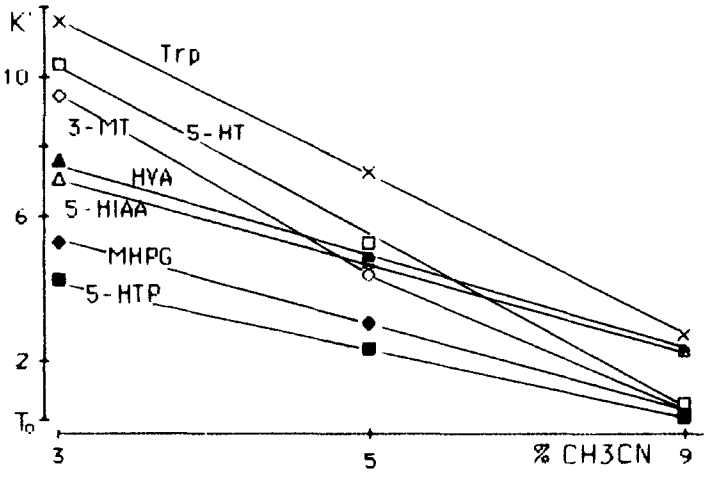

FIGURE 3 : Effect of acetonitrile proportions in the mobile phase, on the capacity factors (K') of four indol derivatives.

Chromatographic conditions : (KH2PO4 0.1M/acetate 0.1M) x% + acetonitrile, pH= 5.4, flow rate = 1 ml/min.
To = solvent front.

the compounds in the same direction without modifying the elution order. To improve the separation, we changed the nature of the organic modifier and worked with acetonitrile. First, we have compared the effect of acetonitrile and methanol in the same proportion (9%) and investigate the effects of various proportions of acetonitrile (fig. 3). Like FEENSTRA et al. (21), we have noted with acetonitrile a decrease by 50% of all RT and a modification in the elution order. In the optimized conditions (phase C), the acetonitrile proportion was reduced till 3% (fig. 4).

By modifying the different parameters of the mobile phase we obtained an elution solvent which insure the separation of the four indols in less than 14 minutes, with a good specificity. In fact, most of the catechol derivatives presents in the test-mixture are eluted in the

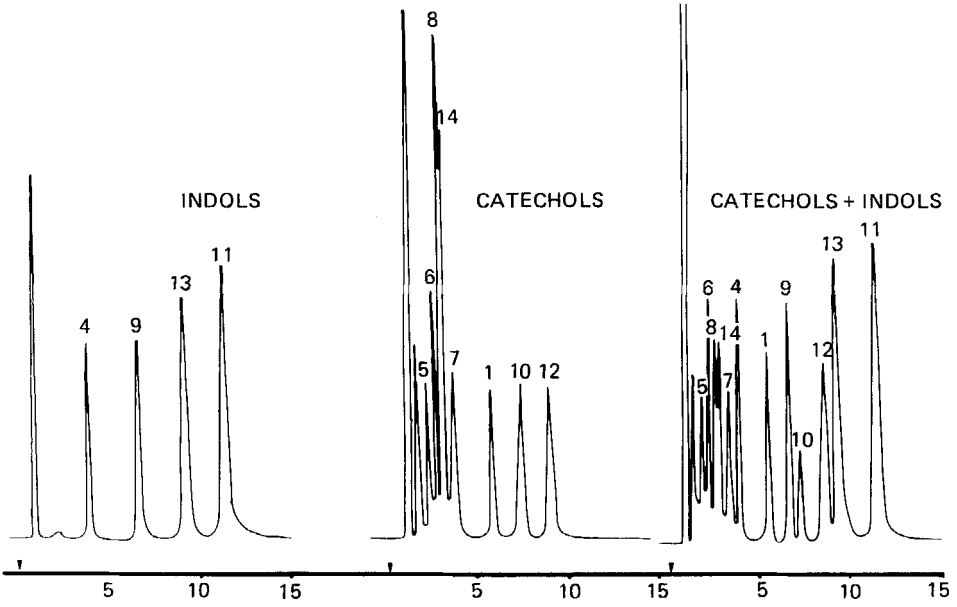

FIGURE 4 : Chromatograms of standard mixtures containing either indols or catechols or both.
Chromatographic conditions : mobile phase C, flow rate = 1ml/min. Detection set at 0.8v, 1nA full scale.
Quantities injected and number meaning are given in Fig.1. 14 = 3-O-methylDOPA (0.4 ng).

solvent front and solely 3 minor catechol metabolites (MHPG, HVA and 3-MT) are still present in the chromatogram. However, these three compounds did not disturb the analysis since they usually are detected as traces in few brain areas (7). The 5-HTP peak is clearly distinguishable from the solvent front and 5-HT appears to be free from 3-MT interfering peak.

Electrochemical Detection Study

The response of the electrochemical detector depends on the applied voltage and on the nature of the compound being oxidized. The

catechols (NE, E, DA, DOPAC) and 5-hydroxylated indols (5-HTP, 5-HT and 5-HIAA) are readily oxidized at 0.65 volt. At this voltage, the 3-Omethoxylated metabolites (MHPG, NMN, MN, 3-MT and HVA) do not appear but show maximum response at 0.85 volt (7). The hydroxyindols give a highest response at 0.85 volt whereas the catechols reach a plateau in their voltammograms (7). Tryptophan, compound of interest, gives no response at potential less than 0.85 volt and has no limit current at potential up to 1,1 volts (5). Then to choose the right potential, one must consider the detection potential of the studied compounds. If the chosen potential is low enough, few interfering substances will be co-detected, but enhancing potential decreases the specificity and increases interference risk.

By setting the detector potential at different voltages and studying the electrochemical detection response, additionnal degrees of specificity are obtained. We have measured the peak height of the indol derivatives in a standard mixture and in brain areas (hypothalamus, hippocampus, striatum and cortex) at two different potential levels (0.65 and 0.85 volt). The current (peak height h) obtained at the most positive voltage is divided by the response (h') obtained at the lower voltage, yielding to the current ratio h/h'. This ratio must be identic for each indol measured in the biological samples, comparatively with the reference compound. The results are presented in table III.

The electrochemical detection showed maximum response at the same oxidative potentials in the samples and standard mixture. Thus, the h/h' ratios print out the specificity of the described method by confirming the identity of the compound. As expected from few authors (3, 4, 14), Trp was weakly detectable at the usual working potential(0.85 volt) and should required higher applied voltage for a better detection response. But such a potential may increase the extent of interfering compounds in the chromatograms. Moreover, in brain areas, Trp levels are 10 times higher than the other indols and thus, even with a weak response, this precursor remains sufficently detectable.

TABLE III

Peak Current Ratios (0.85v/0.65v) of Hydroxyindols Measured in Standard Solution and Various Rat Brain areas

	5-HTP	5-HIAA	5-HT
Standard	1.84 ± 0.3	1.13 ± 0.1	1.30 ± 0.2
Hypothalamus	2.20 ± 0.7	1.10 ± 0.1	1.08 ± 0.1
Hippocampus	1.60 ± 0.3	1.50 ± 0.2	1.26 ± 0.1
Striatum	N.D.	1.23 ± 0.2	1.38 ± 0.1
Cortex	1.60 ± 0.4	1.14 ± 0.1	1.15 ± 0.1

Chromatographic conditions : mobile phase C, sensitivity = 1nA. Values are means ± Sd of 3 determinations. N.D. = no detectable.

Application of the method : validation by pharmacological proof

In table IV are summarized endogenous levels of 5-HT and 5-HIAA in various rat brain areas, as can be seen, our values are in the same range than those reported in the literature for the same areas. The identity of the chromatographic peaks was further confirmed by adding small amounts of standard into brain samples. Chromatograms obtained were no modified. This new evidence confirms the absence of factor inactivating these standard or interfering with the chromatographic separation and/or the electrochemical detection. In figure 5 are given examples of tracings obtained after injection of supernatants from homogenized hypothalamus, striatum and hippocampus. As can been seen, in striatum extract, HVA and 3-MT do not interfere with chromatographic separation of 5-HIAA and 5-HT.

We also tested by pharmacological verification, the authenticity of the indol peaks detected. After pargyline administration (a monoamine-oxidase inhibitor), we observed an increase of 5-HT levels by 100-300% depending on the area, whereas 5-HIAA levels were decreased by

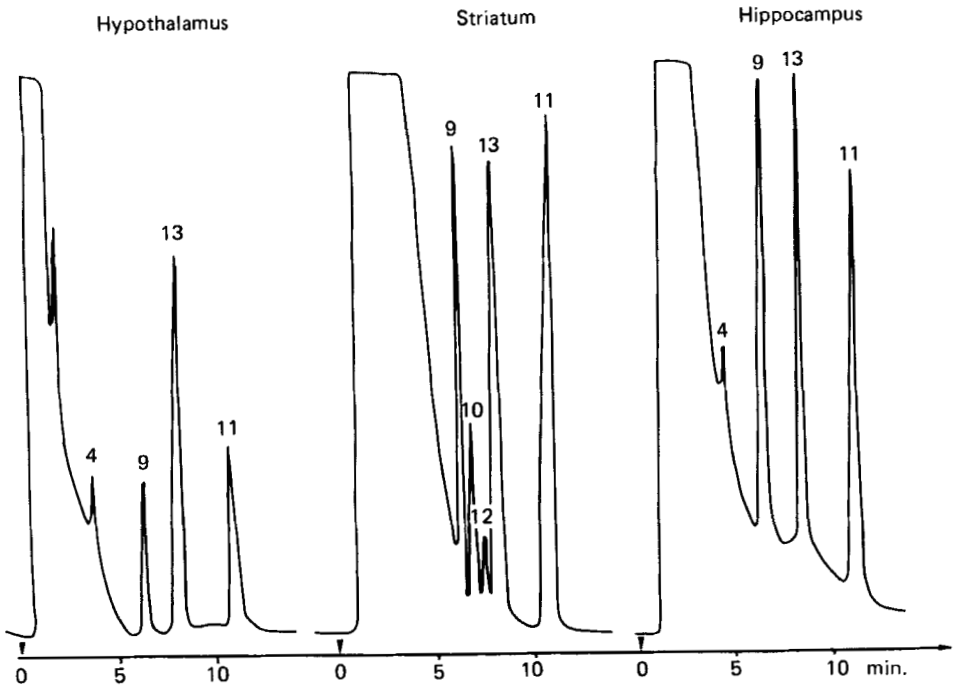

FIGURE 5 : Chromatograms from hypothalamus, striatum and hippocampus of control rats.

Chromatographic conditions : mobile phase C, 1ml/min.
Detection set at 0.8v, 1nA full scale.
Number meaning are given in Fig.1.

TABLE IV

Various Endogenous Levels of 5-HT and 5-HIAA in Rat Brain Areas, Reported by Authors Using LC-ECD Procedure.

		Our values	(1)	(16)	(18)	(25)	(26)	(27)
5-HT	Hypothalamus	650	-	-	1100	350	1800	980
	Hippocampus	260	-	-	-	170	-	570
	Striatum	500	590	140	-	280	-	-
5-HIAA	Hypothalamus	500	470	-	460	850	410	1060
	Hippocampus	240	200	-	270	400	-	720
	Striatum	500	470	140	290	590	-	-

Values are expressed as ng/g of wet tissues.

50-70%. These drug effects were in agreement with those reported in previous studies either with fluorimetric or LC-ECD assays (9, 14, 16, 18, 19, 22, 23, 24).

In summary, for achieving the optimal separation, all mobile phase components have been systematically examined. The identity of chromatographic peaks obtained in various rat brain extracts were demonstrated in several ways, because the use of one of them is likely insufficient. The present method, after both chromatographic and biological validations, permits to measure 5-HTP, 5-HIAA, 5-HT and Trp within 15 minutes. Catecholamines and other very polar compounds are eluted very quickly and do not interfere in the determination. These results have been verified for 11 catecholaminergic compounds likely detectable in many rat brain regions. This method sensitive, reliable and requiring minimal sample handling and preparation appears very efficient for rapid and specific brain indoleamine determination.

REFERENCES

1) Wagner, J., Vitali, P., Palfreyman, M.G., Zraika, M. and Huot, S., **J. Neurochem.**, 38, 1241, 1982.

2) Morier, E. and Rips, R., Chromatography in biochemitsry, medecin and environmental research, Frigerio A., ed., Elsevier, Amsterdam, 1983, p.217.

3) Laasko, J.T., Koskiniemi, M.L., Wahlroos, O. and Harkonen, M., **Scand. J. Clin. Lab. Invest.**, 43, 463, 1983.

4) Semerdjian-Rouquier, L., Bossi, L. and Scatton, B. **J. Chromatogr.**, 218, 663, 1981

5) Richards, D.A., **J. Chromatogr.**, 175, 293, 1979.

6) Hashimoto, H. and Maruyama, Y., Methods in Biogenic Amine Research, Parvez, S., Nagatsu, T., Nagatsu, I. and Parvez, H., eds, Elsevier, Amsterdam, 1983, p.35.

7) Lasley, S.M., Michaelson, L.A., Greenland, R.D. and McGinnis, P.M., **J. Chromatogr.**, 305, 27, 1984.

8) Koch, D.D. and Kissinger, P.T., **J. Chromatogr.**, 164, 441, 1979.

9) Nielsen, J.A. and Johnston, C.A., **Life Sci.**, 31, 2847, 1982.

10) Hunt, W.A. and Dalton, J.K., **Anal. Biochem.**, 135, 269, 1983.

11) Reinhard, J.F., Moskowitz, M.A., Sved, A.F. and Fernstrom, J.D., **Life Sci.**, 27, 905, 1980.

12) Blank, C.L., **J. Chromatogr.**, 117, 35, 1976.

13) Mayer, G.S. and Shoup, R.E., **J. Chromatogr.**, 255, 533, 1983.

14) Saller, C.F. and Salama, A.I., **J. Chromatogr.**, 309, 287, 1984.

15) Arneric, S.P., Goodale, D.B., Flynn, J.R. and Long, J.P. **Brain Res. Bull.**, 6, 407, 1981.

16) Lackovic, Z., Parenti, M. and Neff, N.H.
Eur. J. Pharmacol., 69, 347, 1981.

17) Diggory, G.L. and Buckett, W.R.
J. Pharmacol. Meth., 11, 207, 1984.

18) Westerink, B.H.C., **J. Liq. Chromatogr.**, 6, 2337, 1983.

19) Oka, K., Kojima, K., Togari, A., Nagatsu, N. and Kiss, B. **J. Chromatogr.**, 308, 43, 1984.

20) Trouvin, J.H., Prioux-Guyonneau, M., Cohen, Y. and Jacquot, C. **Gen. Pharmacol.**, 17, 69, 1986.

21) Feenstra, M.G.P., Homad, J.W., Dijkstra, D., Mulder, T.B.A., Rollema, H., Westerink, B.H.C. and Horn, A.S.
J. Chromatogr., 230, 271, 1982.

22) Kempf, E. and Mandel, P., **Anal. Biochem.**, 112, 223, 1981.

23) Tozer, T.N., Neff, N.H. and Brodie, B.B.
J. Pharmacol. Exp. Ther., 153, 177, 1966.

24) Peuler, J.J. and Passon, P.G., **Anal. Biochem.**, 52, 574, 1973.

25) Aymard, N., Belkahla, F., Curet, O. and Boulu, R.G.
Act. Pharmacol. Biol. Clin., 2, 175, 1983.

26) Taylor, R.B., Reid, R., Kendle, K.E., Geddes, C. and Curle, P.F. **J. Chromatogr.**, 277, 101, 1983.

27) Lister, R.G. and File, S.E., **J. Pharm. Pharmacol.**, 35, 601, 1983.