Journal of Chromatography, 275 (1983) 1—9
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1659

SIMULTANEOUS DETERMINATION OF 5-HYDROXYTRYPTAMINE, ITS AMINO ACID PRECURSORS AND ACID METABOLITE IN DISCRETE BRAIN REGIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

WILLIAM A. WOLF and DONALD M. KUHN*

Section of Biochemical Pharmacology, Hypertension Endocrine Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20205 (U.S.A.) and Department of Pharmacology, The George Washington University Medical Center, Washington, DC 20037 (U.S.A.)

(First received December 14th, 1982; revised manuscript received January 26th, 1983)

SUMMARY

L-Tryptophan, 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid (5-HIAA) from discrete regions of brain can be resolved by isocratic elution on a C-18 reversed-phase column and quantitated by native fluorescence measurement. This method is rapid and simple and can reproduceably detect 5-hydroxytryptamine, tryptophan and 5-HIAA with picogram sensitivity. The detection of 5-HTP in brain extracts from untreated animals is difficult because of the extremely low endogenous levels of 5-HTP. Quantitation of 5-HTP presents no problem in animals injected with 5-HTP or with an inhibitor of L-aromatic amino acid decarboxylase. Brain tissue requires minimal treatment for assay and the hydroxyindoles are stable enough to allow automated processing.

INTRODUCTION

The neurotransmitter 5-hydroxytryptamine (5-HT) has been implicated as a modulator of a variety of physiological processes, not the least of which is blood pressure and its regulation [1]. The amine itself does not apparently cross the blood brain barrier [2] and, as a result, the amino acid precursors of 5-HT, tryptophan and 5-hydroxytryptophan (5-HTP), are frequently injected systemically to increase the brain concentrations of 5-HT. However, it is becoming apparent that tryptophan and 5-HTP can exert physiological and behavioral effects which are not entirely mediated by 5-HT (synthesized from tryptophan or 5-HTP) but possibly by the amino acids themselves [3—6] or by the nonspecific influence of the amino acids on other neurotransmitter systems [7—9]. For these reasons, it is quite important that the concentrations of tryptophan and 5-HTP, in addition to those of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) be determined in discrete brain regions before and

after 5-HT percursor loading.

A large number of methods are currently available for measuring 5-HT and its precursors and metabolites in biological tissue [10]. However, even recent methods often require derivatization or fluorescence enhancement prior to high-performance liquid chromatography (HPLC) [11] or extraction of the tissue into organic solvents in preparation for HPLC [12], both of which restrict the number of substances in a particular biosynthetic pathway which can be subsequently analyzed. Furthermore, some HPLC methods are applied to whole brain [8, 12–14] which does not completely allow for the discrete analysis of neurotransmitter levels in the dynamic fashion in which they certainly function.

In the present report, we describe a method which allows the resolution and detection of tryptophan, 5-HTP, 5-HT, and 5-HIAA in small brain areas from rats. The method requires no pretreatment of the samples besides deproteination and the use of isocratic elution simplifies the instrumentation. Finally, this procedure takes advantage of the native fluorescence properties of tryptophan, 5-HT, and 5-HIAA for detection and quantitation. Determination of endogenous 5-HTP levels is difficult but estimates can be made by difference.

EXPERIMENTAL

Apparatus

The HPLC apparatus consists of a Dupont Chromatographic Pump Module (Model 870), a Perkin-Elmer Fluorescence Spectrophotometer (Model 650-10LC) and an LKB Potentiometric Recorder (Model 2210). Automated sample injections were performed by a Waters Intelligent Sample Processor (WISP Model 710B).

The chromatographic separation was achieved by reversed-phase using an octadecyl polymer-coated pellicular silica column (Spherisorb ODS, particle size 5 μm; 250 × 4.6 mm I.D. column prepacked from Chromanetics Corp.). A short precolumn (60 × 5 mm I.D.) packed with an octadecyl-coated pellicular support (CO:Pell ODS, Whatman, Clifton, NJ, U.S.A.) was used as a guard column.

The mobile phase consists of 0.01 *M* sodium acetate containing 15% methanol brought to pH 4.85 with acetic acid. This was degassed under vacuum prior to use. Solvent flow-rate was set at 1.4 ml/min and separation was performed at ambient temperature (23°C).

The fluorescence spectrophotometer was set at an emission wavelength of 345 nm while the excitation wavelength was 285 nm. The spectral bandpass was set at 10 nm.

Chemicals and reagents

Tryptophan, 5-HTP, 5-HT (creatinine sulfate), N-acetylserotonin (NAS), and 5-HIAA standards were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol was HPLC grade from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other chemicals and reagents were of the highest quality commercially available. Stock solutions of tryptophan, 5-HTP, 5-HT, 5-HIAA and NAS were made to a concentration of 1.0 mg/ml in distilled, deionized water containing

0.1% ascorbic acid. Stock solutions were frozen and stored at −20°C, protected from light, and were diluted to the appropriate concentration prior to HPLC analysis with the same buffer used to dilute tissue samples (see below).

Tissue preparation

Male Wistar rats were obtained from the NIH animal colony and used when 10—12 weeks of age. Rats were housed in group cages under constant humidity and ambient temperature. The lighting schedule consisted of a 12-h light—dark cycle (7 a.m.—7 p.m.). Food and water were available ad libitum. Animals were sacrificed and the following brain regions were immediately dissected out and frozen on dry ice: anterior hypothalamus, midbrain tegmentum, nucleus tractus solitarius (NTS) and striatum. All samples were stored in liquid nitrogen until assay. Samples were rapidly thawed and prepared for analysis by addition of 100 ng of NAS as internal standard followed by homogenization in 4 volumes (or at least 100 μl) of ice cold 0.16 *N* perchloric acid containing 0.1% EDTA and 0.1% ascorbate. Tissue was homogenized in 1.5-ml polypropylene test tubes with a motor-driven PTFE pestle which had been cut and shaped to fit the test tubes. The homogenate was centrifuged at 40,000 *g* for 20 min at 4°C. The supernatant was then diluted 1:6 with ice cold 0.5 *M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) buffer, pH 6.0 and immediately frozen on dry ice until analysis. We have found that the 5-hydroxyindoles and 5-HIAA in particular are much more stable when diluted into Hepes pH 6.0 than when diluted into $NaPO_4$ buffer, pH 7.9 or Tris·HCl pH 7.4. In some cases rats were injected intraperitoneally with L-tryptophan (methyl ester hydrochloride salt) or 5-HTP dissolved in 0.9% saline. The injection volume was 2 ml/kg and doses are based on the weight of the free amino acid.

RESULTS

A chromatogram demonstrating the resolution of tryptophan, 5-HTP, 5-HT, 5-HIAA, and NAS standards is shown in Fig. 1. It can be seen from Fig. 1 that all substances are sufficiently resolved to allow accurate quantitation. All standards including the internal standard are chromatographed within 18 min. Standards and tissue are stable for at least 7 h when prepared by the protocol described above, allowing automatic sample processing.

These same substances can be detected in brain tissue as shown in Fig. 2A. Identification of the peaks found in the chromatogram of brain tissue was achieved by matching the retention times of peaks from tissue with those of the standard compounds. Further confirmation was achieved by addition of a known quantity of each standard to a duplicate sample of brain extract followed by chromatography of both samples. As can be seen in Fig. 2B the characteristics of the identified peaks remained unchanged, although they were appropriately taller with the added standard, and no new peaks emerged. The first two peaks, eluting at 3 min and 3.7 min, are as yet unidentified, but they are neither tryptamine nor indoleacetic acid. In order to enhance quantitation of the 5-HTP peak in brain tissue from untreated rats it was necessary to add 100 pg of 5-HTP standard to each sample before analysis.

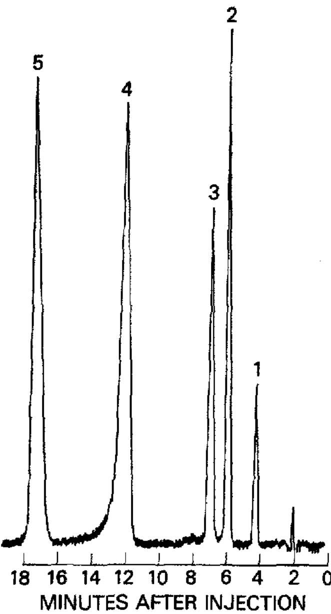

Fig. 1. Chromatogram of standards prepared as described in Methods. Injection volume is 40 μl. Peaks: 1, 5-HTP (200 pg); 2, 5-HIAA (1.20 ng); 3, tryptophan (2 ng); 4, 5-HT (2 ng); and 5, NAS (2 ng).

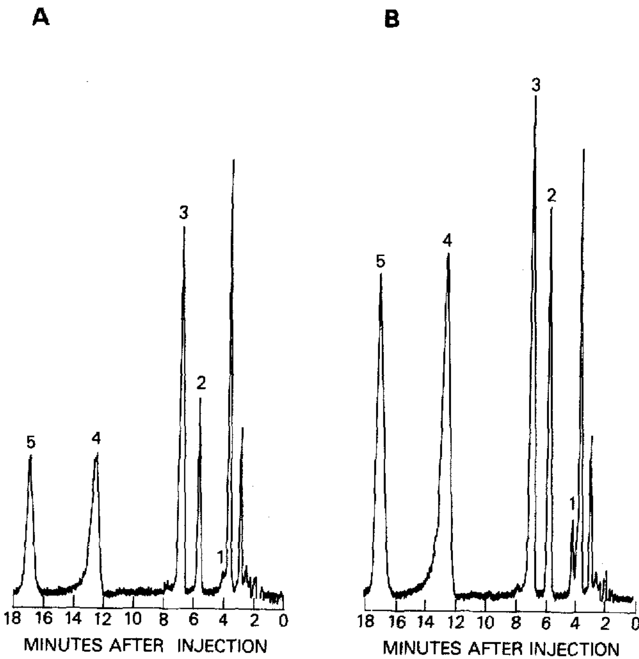

Fig. 2.

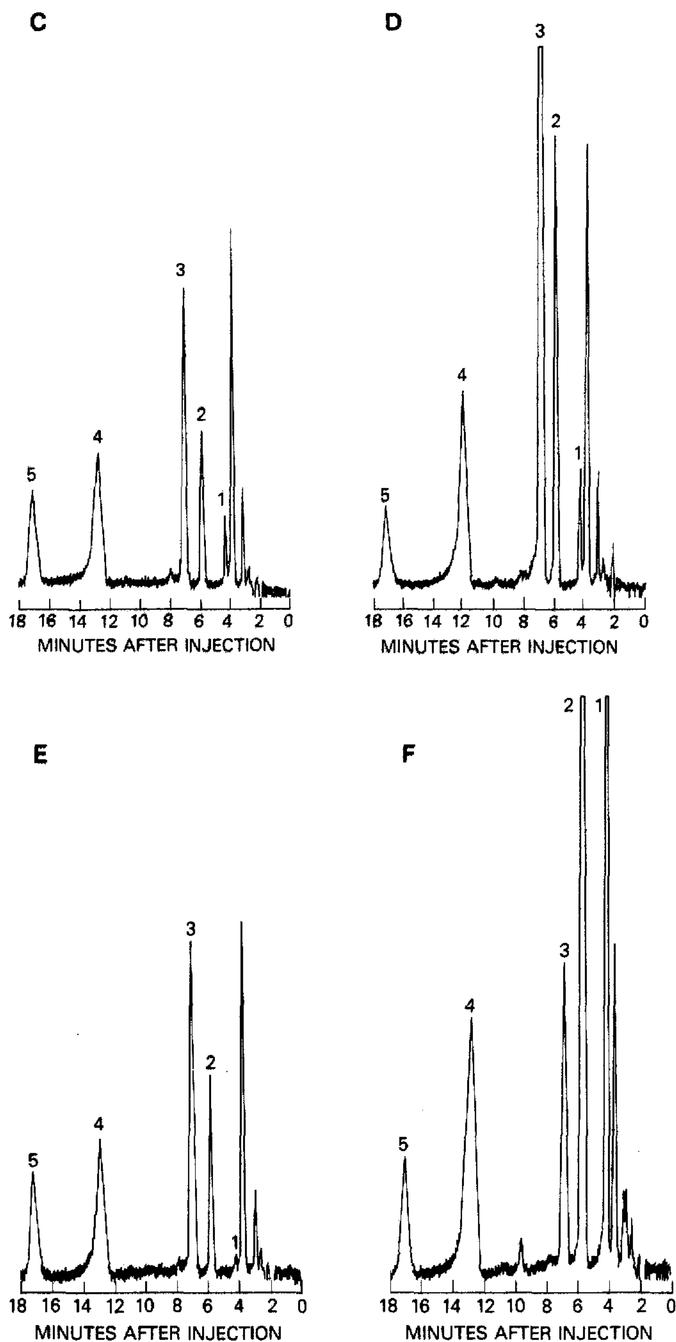

Fig. 2. Chromatogram of brain tissue extracts. (A) Midbrain tegmentum from an untreated rat, (B) same as A but with addition of 100 pg of 5-HTP, 0.60 ng of 5-HIAA, 1 ng of tryptophan, 1 ng of 5-HT, and 1 ng of NAS, (C) extract from midbrain tegmentum with 100 pg of 5-HTP added, (D) midbrain tegmentum from an animal injected with 100 mg/kg of tryptophan 2 h prior to sacrifice, (E) midbrain tegmentum from untreated rat without exogenous 5-HTP, and (F) midbrain tegmentum from an animal injected with 50 mg/kg of 5-HTP 1 h prior to sacrifice. Peaks: 1, 5-HTP; 2, 5-HIAA; 3, tryptophan; 4, 5-HT; and 5, NAS. All injections were made in a volume of 40 μl of which 20 μl was tissue extract.

The endogenous amount of 5-HTP was then calculated as the difference between the 5-HTP standard peak and the 5-HTP peak in the spiked tissue sample. This method of determination should be viewed with some caution, however.

The effects of tryptophan and 5-HTP loading can also be seen in Fig. 2D and F, respectively. An injection of 100 mg/kg of tryptophan 2 h prior to sacrifice substantially increased the brain levels of tryptophan, and moderately increased 5-HT and 5-HIAA while the levels of 5-HTP were not significantly changed. An injection of 50 mg/kg of 5-HTP 1 h prior to sacrifice did not change brain tryptophan levels but increased the levels of 5-HTP, 5-HT, and 5-HIAA. Note the extremely large amounts of tryptophan and 5-HTP remaining in brain after their respective injections (Fig. 2D and F).

The assay is linear over a wide range of standard concentrations as shown in Fig. 3. The chromatographic and detectability data for tryptophan, 5-HTP, 5-HT, 5-HIAA, and NAS are summarized in Table I. Although more sensitive methods are available (see ref. 10), the present method is sensitive enough to allow measurement of the 5-hydroxyindoles from brain tissue. Omission of the tissue dilution step (above) correspondingly increases the peak height of each compound in the brain extracts (data not shown). It should be pointed out too that the present detectability values were determined with a 5:1 signal-to-noise ratio. The coefficient of variation of the absolute fluorescence of 1.0 ng of 5-HT was 5.8% for inter-assay variability and 1.3% for intra-assay variability for all assays over a 2—3 month period. The coefficients of variation for the other indoles were similar to that of 5-HT.

Finally, this procedure has been used to analyze tryptophan, 5-HTP, 5-HT, and 5-HIAA in numerous rat brain areas in untreated animals and in animals injected with tryptophan and the results are summarized in Table II. Recoveries varied between 50—80%. In general, recovery of all compounds was higher with larger homogenization volumes.

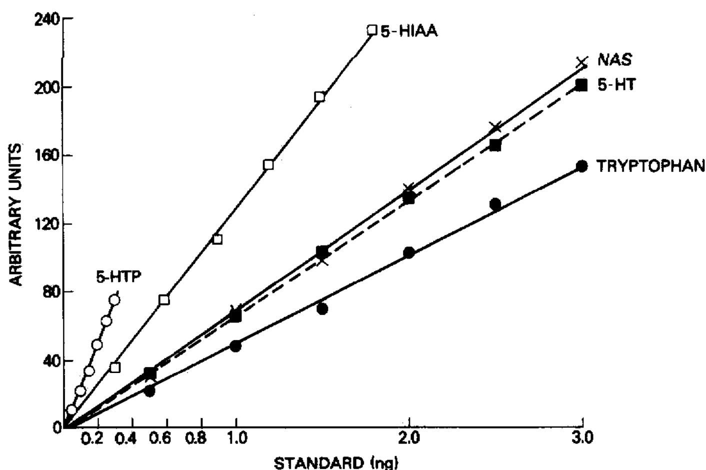

Fig. 3. Linearity of fluorescence response (in arbitrary units) with increasing quantities of 5-HTP, 5-HIAA, tryptophan, 5-HT, and NAS. Each data point represents the average of at least four determinations.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF 5-HTP, 5-HIAA, TRYPTOPHAN, 5-HT, AND NAS

The conditions are described in Methods; λ_{ex} = 285 nm, λ_{em} = 345 nm.

Compound	Retention time (min)	Limit of sensitivity* (pg)
5-HTP	4.2	40
5-HIAA	5.8	75
Tryptophan	6.8	190
5-HT	12.2	145
NAS	17.4	130

*Determined at a signal-to-noise ratio of 5:1.

TABLE II

LEVELS OF 5-HTP, 5-HIAA, TRYPTOPHAN, AND 5-HT IN VARIOUS BRAIN REGIONS FROM UNTREATED ANIMALS OR FROM ANIMALS INJECTED WITH 100 mg/kg TRYPTOPHAN 2 h PRIOR TO SACRIFICE

Values are expressed as ng/mg wet weight, n = 3.

	Tryptophan	5-HTP	5-HT	5-HIAA
Tegmentum	4.38 ± 0.36*	0.04 ± 0.01	1.24 ± 0.01	0.93 ± 0.07
	17.78 ± 4.4**	0.07 ± 0.01	1.92 ± 0.36	2.42 ± 0.29
Anterior	4.92 ± 0.37	0.04 ± 0.01	1.02 ± 0.04	0.52 ± 0.03
hypothalamus	14.03 ± 4.3	0.06 ± 0.01	1.49 ± 0.06	1.04 ± 0.13
NTS	5.18 ± 0.34	0.05 ± 0.01	1.08 ± 0.13	0.51 ± 0.03
	21.20 ± 0.47	0.12 ± 0.01	2.01 ± 0.10	1.61 ± 0.05
Striatum	5.52 ± 0.25	0.04 ± 0.01	0.63 ± 0.02	0.56 ± 0.01
	15.38 ± 4.6	0.04 ± 0.02	1.11 ± 0.15	0.85 ± 0.06

*Top row of numbers for each brain area represents values for untreated rats.
**Bottom row refers to values from tryptophan-treated rats.

DISCUSSION

Chromatography of deproteinized, buffered brain extracts on a reversed-phase column at pH 4.85 allows the resolution of tryptophan, 5-HTP, 5-HT, and 5-HIAA in discrete brain areas. These substances can then be easily quantitated by native fluorescence measurement. Although the present method lacks the absolute sensitivity of electrochemical detection [15—17], it is sensitive enough to allow the measurements of 5-HT, its amino acid precursors, and the major 5-HT metabolite 5-HIAA. The present method is also quite useful for experiments where animals have been treated with tryptophan or

5-HTP in order to increase brain 5-HT concentrations since these 5-HT precursors can be measured simultaneously.

Apart from quantitation of 5-HT levels in discrete brain regions the present method can be used to study the in vivo rates of 5-HT synthesis and turnover in animals treated with L-aromatic amino acid decarboxylase inhibitors (5-HTP accumulates) and with monoamine oxidase inhibitors (5-HT accumulates) or acid transport inhibitors (e.g., probenecid, 5-HIAA accumulates), respectively. This particular application was demonstrated recently by Reinhard et al. [16] using HPLC with electrochemical detection. Similarly, the in vitro assay of tryptophan hydroxylase, the initial and rate limiting enzyme in the biosynthesis of 5-HT [18], could be improved and extended from the presently employed methods [19—21] by virtue of the increased sensitivity and the ease of measurement of the substrate tryptophan offered by this HPLC method. Meek and co-workers [22, 23] were the first to apply 5-HTP detection by HPLC to the measurement of tryptophan hydroxylase activity. However, the concurrent measurement of tryptophan and 5-HTP levels will allow more careful kinetic analysis of tryptophan hydroxylase, including substrate utilization and reaction stoichiometry. Finally, the present method can be simply adapted to measure L-aromatic amino acid decarboxylase activity in vitro.

One drawback of the current procedure is the detection and quantitation of 5-HTP. Endogenous brain 5-HTP levels are normally quite low and detection and measurement by HPLC with electrochemical detection [15] offers no advantages over the present method. Although the 5-HTP peak is observable (see Fig. 2), it is juxtaposed to an unidentified fluorescent peak in tissue. The endogenous levels of 5-HTP can be estimated by difference without interference from the unidentified peaks, as described above, but quantitation presents no difficulty in rats injected with 5-HTP (Fig. 2F) or with an inhibitor of L-aromatic amino acid decarboxylase (unpublished observations).

In summary, the method described in this report has the advantages of allowing the simultaneous measurement of tryptophan, 5-HTP, 5-HT, and 5-HIAA in discrete brain areas with minimal tissue preparation. Most other HPLC methods for measuring the hydroxyindoles with fluorometric detection require various extraction or ion-exchange steps and these are often coupled with pre- or postcolumn (HPLC) fluorescence enhancement (see ref. 10 for review). Furthermore, fluorescence detection generally involves less technical maintenance than electrochemical detection and provides a more stable baseline. Finally, the present method is more versatile and is far easier to perform than the commonly used radioenzymatic procedures [24, 25] which only measure 5-HT, or the recently described radioimmunoassays for 5-HT and 5-HIAA [26].

ACKNOWLEDGEMENTS

We thank Dr. Walter Lovenberg for his comments on the manuscript and for his advice and encouragement. W.A. Wolf was supported by a National Science Foundation pre-doctoral fellowship.

REFERENCES

1 D.M. Kuhn, W. Wolf and W. Lovenberg, Hypertension, 2 (1980) 243.
2 J.L. Lexchin, K.D. Simpson and H.D. Stancer, Neurochem. Res., 2 (1977) 39.
3 E.D. Hall, Neuropharmacology, 20 (1981) 109.
4 M.E. Safdy, E. Kurchacova, R.N. Schut, H. Vidrio and E. Hong, J. Med. Chem., 25 (1982) 723.
5 H. Zimmerman, S.L. Kaplan and W.F. Ganong, Neuroendocrinology, 34 (1982) 27.
6 G.A. Wilson and B.L. Furman, Eur. J. Pharmacol., 78 (1982) 263.
7 M. Sallanon, C. Buda, M. Janin and M. Jouvet, Eur. J. Pharmacol., 82 (1982) 29.
8 L.M. Yunger and J.A. Harvey, J. Pharmacol. Exp. Ther., 196 (1976) 307.
9 H. Echizen and C.R. Freed, J. Pharmacol. Exp. Ther., 220 (1982) 579.
10 D.M. Kuhn and W. Lovenberg, in H. Parvez, S. Parvez, I. Nagatsu and T. Nagatsu (Editors), Methods in Biogenic Amine Research, Elsevier, New York, 1982, in press.
11 G. Curzon, B.D. Kantamaneni and M.D. Tricklebank, Brit. J. Pharmacol., 73 (1981) 555.
12 C.A. Marsden, J. Neurochem., 36 (1981) 1621.
13 K. Ishikawa and J.L. McGaugh, J. Chromatogr., 229 (1982) 35.
14 A.J. Falkowski and R. Wei, Anal. Biochem., 115 (1981) 311.
15 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
16 J.F. Reinhard, M.A. Moskowitz, A.F. Sved and J.D. Fernstrom, Life Sci., 27 (1980) 905.
17 Z. Lackovic, M. Parenti and N.H. Neff, Eur. J. Pharmacol., 69 (1981) 347.
18 E. Jequier, W. Lovenberg and A. Sjoerdsma, Mol. Pharmacol., 3 (1967) 274.
19 P.A. Friedman, A.H. Kappelman and S. Kaufman, J. Biol. Chem., 247 (1972) 4165.
20 H.G. Baumgarten, S.J. Victor and W. Lovenberg, J. Neurochem., 21 (1973) 251.
21 A. Ichiyama, S. Nakamura, Y. Nishijuka and O. Hayaishi, J. Biol. Chem., 245 (1970) 1699.
22 J.L. Meek and L.M. Neckers, Brain Res., 91 (1975) 336.
23 J.L. Meek and S. Lofstrandh, Eur. J. Pharmacol., 37 (1976) 377.
24 J.M. Saavedra, M. Brownstein and J. Axelrod, J. Pharmacol. Exp. Ther., 186 (1973) 508.
25 I. Hammel, Y. Naot, E. Ben-David and H. Ginsburg, Anal. Biochem., 90 (1978) 840.
26 M.R. Geffard, J.J. Prizillout and M.A. Delaage, J. Neurochem., 39 (1982) 1271.