Journal of Chromatography, 225 (1981) 27—35
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 921

DETERMINATION OF SEROTONIN, ITS PRECURSORS, METABOLITES AND [^{3}H]SEROTONIN IN LUNG BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

DALE E. MAIS, PATRICIA D. LAHR and TALMAGE R. BOSIN*

Section of Pharmacology, Medical Sciences Program, Indiana University School of Medicine, Bloomington, IN 47405 (U.S.A.)

(First received January 15th, 1981; revised manuscript received March 17th, 1981)

SUMMARY

A rapid, simple, sensitive method for the determination of serotonin, its precursors, metabolites and [^{3}H]serotonin in lung is described. Tissue preparation requires only homogenization in dilute perchloric acid and centrifugation prior to separation by high-performance liquid chromatography using a reversed-phase column. Detection is based on the native fluorescence of indole compounds and detection limits ranged between 30—90 pg injected. The method has been used to determine these compounds in mouse lung and plasma.

INTRODUCTION

The importance of the lungs in the control of circulating levels of vasoactive substances in health and disease has been established [1, 2]. Recent investigations [3—9] have demonstrated the pulmonary vasculature to be the major site controlling the arterial concentration of the vasoactive amine, serotonin (5-hydroxytryptamine, 5-HT). This control is accomplished by 5-HT uptake, storage, and metabolism and may be important in maintaining the fluidity of the pulmonary circulation. Drugs, and dietary and environmental toxicants which interfere with this pulmonary function may produce severe adverse effects. Furthermore, the control of circulating 5-HT levels by the lung and its function therein have been the subject of considerable speculation which has included such pathological states as anaphylaxis [10, 11], delayed-type hypersensitivity [12] and pulmonary hypertension [13].

Various analytical methods have been employed to measure 5-HT and its precursors and metabolites in tissues and fluids, including thin-layer chromato-

0378-4347/81/0000—0000/$02.50

graphy [14], ultraviolet spectrometry [15], fluorescence spectrometry [16], gas chromatography [17], gas chromatography—mass spectrometry (GC—MS) [18] and radioimmunoassay (RIA) [19]. All of these analytical methods suffer from limitations of simplicity, selectivity and/or sensitivity. Recently these compounds have been analyzed in brain, cerebrospinal fluid, serum and urine by high-performance liquid chromatography (HPLC) with fluorescence [20—25] and electrochemical (EC) [26—31] detection.

In light of the importance of lung—serotonin interactions, we have developed a simple, sensitive method for the determination of tryptophan (TRP), 5-hydroxytryptophan (5-HTP), 5-HT, exogenous [^{3}H] 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxyindole-3-acetic acid O-sulfate (5-HIAA-O-sulfate), 5-hydroxytryptophol (5-HTOL), tryptamine, N-acetylserotonin and indole-3-acetic acid (IAA) in a single injection. This method has been used in the determination of these compounds in mouse lung and plasma.

EXPERIMENTAL

Reagents

Tryptophan, tryptamine hydrochloride, indole-3-acetic acid, 5-hydroxytryptophan, serotonin hydrogen oxalate, N-methylserotonin hydrogen oxalate, N-acetylserotonin, 5-hydroxyindole-3-acetic acid, and 5-hydroxytryptophol were purchased from Regis (Morton Grove, IL, U.S.A.). 5-Hydroxytryptamine binoxalate [1,2-^{3}H(N), specific activity 27.0 Ci/mmol] and 5-hydroxytryptamine binoxalate [2-^{14}C, specific activity 51.5 mCi/mmol] were purchased from New England Nuclear (Boston, MA, U.S.A.). The purity of all labeled compounds was determined by thin-layer chromatography on 250-μm silica gel G plates purchased from Analtech (Newark, DE, U.S.A.), using a solvent system composed of acetone—2-propanol—water—ammonium hydroxide (50:40:7:3). The thin-layer plates were scanned on a Packard 7200 radiochromatogram scanner (Packard Instrument Co., Downers Grove, IL, U.S.A.). Liquid scintillation fluid was prepared by dissolving 5 g of PPO—POPOP (98:2) purchased from Research Products International (Elk Grove Village, IL, U.S.A.) and 100 g of naphthalene in 1 l of *p*-dioxane. Type VII purified bacterial β-glucuronidase and Type H-1 partially purified β-glucuronidase—sulfatase from *Helix pomatia* were purchased from Sigma (St. Louis, MO, U.S.A.). Glass distilled methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and 0.01 *M* sodium acetate buffer, pH 4.7, was prepared from reagent grade sodium acetate. Solvents were filtered through a 0.2-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and vacuum deaereated. Standard solutions (1 m*M*) were prepared in 0.1 *M* perchloric acid and diluted to the desired concentration.

Apparatus

Liquid chromatography was performed using a Varian 5020 liquid chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with a universal loop injector, a 5-cm column guard packed with Vydac reversed-phase hydrocarbon (Separations Group, Hesperia, CA, U.S.A.) and a 30 cm × 3.9 mm I.D. μBondapak C_{18} reversed-phase column, particle size 10 μm (Waters Assoc.,

Milford, MA, U.S.A.). The mobile phase consisted of 12% methanol—0.01 *M* sodium acetate buffer (pH 4.7) and the flow-rate was 0.8 ml/min. Fluorescence detection was achieved using a Fluorichrom detector (Varian Assoc.) equipped with a deuterium arc source and using a 200 I excitation filter and a Corning 7-60 band filter (360 nm) for emission. Radioactive samples were counted in a Beckman LS-8000 liquid scintillation counter (Beckman Instruments, Fullterton, CA, U.S.A.) and programmed to count dual-labeled ($^3H/^{14}C$) samples.

Sample preparation

Male, Swiss-Webster mice weighing 20—25 g were purchased from Laboratory Supply Co., (Indianapolis, IN, U.S.A.). All animals were fed food and water ad libitum and were maintained on a 12:12 light/dark cycle for five days prior to use. 5-Hydroxytryptamine binoxalate [1,2-^{3}H(N)] was injected into the tail vein of mice at a dose of 0.2 μCi and 20 ng 5-HT binoxalate per g. The mice were loosely restrained under an inverted 100-ml blackened beaker with the tail protruding from the lip of the beaker. Fifteen min after administration of [^{3}H] 5-HT the animals were killed by ether asphyxiation/decapitation which involved placing the animal in a warmed (37°C), ether-saturated desiccator until all breathing ceased (1.1 min). The lungs were quickly removed, rinsed in ice-cold 0.9% saline, blotted dry and frozen at —80°C for analysis. Blood was collected from the decapitation site in glass tubes previously rinsed with EDTA anticoagulant solution [27]. Hematocrits were determined and compared to previously reported values [32]. Plasma samples were prepared by centrifuging the sample for 30 min at 600 *g* in a Beckman J-6B centrifuge cooled to 4°C. Aliquots of up to 300 μl of each plasma sample were transferred to 125 × 15 mm glass tubes and stored at —80°C for analysis.

Lungs were weighed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, U.S.A.) at a setting of 6 for 0.5 min in 2.0 ml of ice-cold 0.1 *M* perchloric acid containing 100 μl of EDTA—ascorbic acid solution (200 mg ascorbic acid per ml of 10% EDTA solution, prepared fresh daily), 50 μl (0.4 μg) N-methylserotonin (as internal standard for endogenous indole determinations) and 50 μl (10 nCi) of [^{14}C] 5-HT as internal standard for [^{3}H] 5-HT determinations. Plasma samples were handled in the same manner. The homogenates were centrifuged for 10 min at 4°C and 2500 *g* and the supernatants transferred to screw-capped glass centrifuge tubes and 100-μl aliquots were injected onto the column. Samples were stored at —80°C for longer than two weeks without evidence of degradation.

Quantitation

The quantitation of the endogenous indoles was based on the calculated fluorescence intensity ratio of each indole to N-methylserotonin (internal standard), and the use of a calibration curve that was prepared by adding differing amounts of each indole compound to 0.4 μg of N-methylserotonin and analyzing the samples (see above).

The quantitation of [^{3}H] 5-HT was based on the [^{3}H] 5-HT to [^{14}C] 5-HT ratio determined by collecting the detector effluent associated with the 5-HT peak in 10 ml of liquid scintillation fluid and counting. A calibration curve was

prepared by adding known amounts of [^{3}H] 5-HT and 10 nCi of [^{14}C] 5-HT to blank lung tissue and analyzing the samples (see above).

Identification and quantitation of 5-HIAA-O-sulfate

The substance to be identified (Fig. 1A, peak 2) was repetitively collected from lung chromatographic analysis and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 2.0 ml of water and divided equally. The divided samples were adjusted to pH 5.9 and pH 5.0, and treated

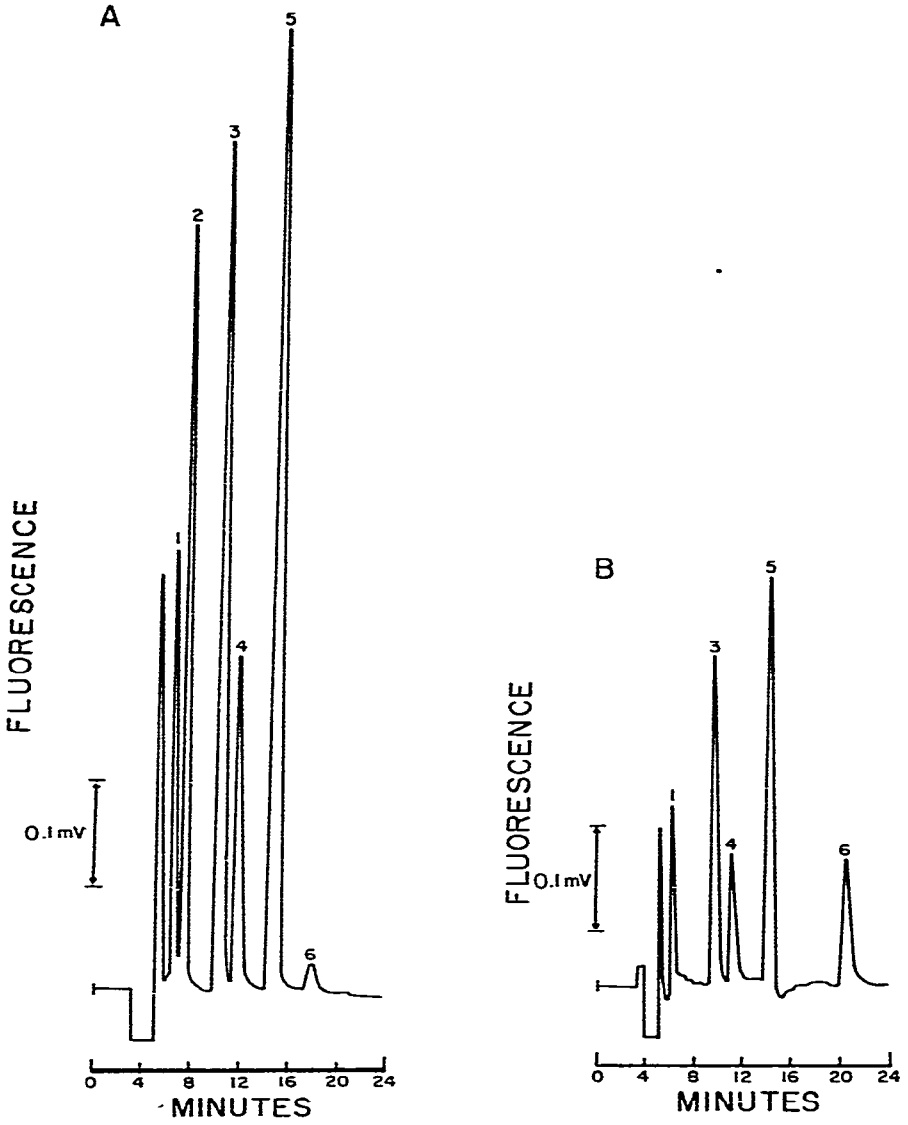

Fig. 1. Chromatograms of mouse lung sample before (A) and after (B) acid hydrolysis. Peaks: 1 = 5-HTP; 2 = 5-HIAA-O-sulfate; 3 = 5-HT; 4 = N-methylserotonin; 5 = TRP; 6 = 5-HIAA. See Experimental for chromatographic conditions. Samples were eluted with 12% methanol in 0.01 *M* sodium acetate buffer, pH 4.7 and a flow-rate of 0.8 ml/min.

with 9.3 mg (12,000 units) of Type VII purified β-glucuronidase and 10 mg (3000 units and 300 units) of partially purified Type H-1 β-glucuronidase—sulfatase, respectively. Each sample was incubated for 18 h at 37°C, after which it was adjusted to pH 1.0 with dilute perchloric acid, centrifuged, and analyzed.

A 0.5-ml aliquot of the lung supernate was treated with 100 μl of EDTA—ascorbic acid solution and then adjusted to a final concentration of 1.0 *M* perchloric acid by the addition of 90 μl of concentrated perchloric acid. The solution was heated for 10 min at 100°C, cooled, and neutralized with 180 μl of 2 *M* potassium acetate solution. The sample was centrifuged to remove the potassium perchlorate precipitate and a 100-μl aliquot was analyzed.

The quantitation of 5-HIAA-O-sulfate was based on the quantitative acid hydrolysis of the lung supernate, as described above. From the increased level of 5-HIAA following hydrolysis, the initial concentration of 5-HIAA-O-sulfate was readily calculated. Under the hydrolysis conditions 5-HTOL was not oxidized to 5-HIAA, the other indole derivatives were not converted to 5-HIAA, and both 5-HIAA and N-methylserotonin (internal standard) were stable. The peak height differences observed between Fig. 1A and B are due to dilution of the sample during the hydrolysis of the sample. All peak height ratios remained the same.

RESULTS AND DISCUSSION

The chromatographic charactersistics of the authentic indole compounds are depicted in Fig. 2 and the chromatographic and detectability data are presented in Table I. Under the experimental conditions, all of the indole compounds are readily separated and fluorescence detection provides detection limits which are comparable to GC—MS [18], RIA [19] and HPLC—EC [26—31].

The analysis of mouse lung indoles is presented in Fig. 1A. All of the compounds, except the one producing peak 2, were confirmed by both spiking the sample with authentic material and chromatographing the samples with differing percentages (5—15%) of methanol. In each instance the compound coeluted with the appropriate standard. The identification of 5-HIAA-O-sulfate as the compound chromatographing as peak 2 was based on its rapid hydrolysis to 5-HIAA, as shown by the marked increase in 5-HIAA in the hydrolyzed chromatogram (Fig. 1B). Under the hydrolysis conditions neither 5-HTOL nor the other indole compounds present in the lung sample were converted to 5-HIAA. In addition, both 5-HIAA and N-methylserotonin, the internal standard, were stable under these conditions, thereby providing an easy means of quantitating this metabolite. The identification of the metabolite as a sulfate conjugate was based on the facile hydrolysis of the concentrated metabolite by partially purified Type H-1 β-glucuronidase—sulfatase and the absence of hydrolysis with Type VII purified β-glucuronidase. Based on this differential hydrolysis the conjugate was identified as a sulfate.

The chromatographic analysis of mouse plasma is presented in Fig. 3A. The identification and quantitation of 5-HIAA-O-sulfate as the metabolite responsible for peak 2 in the chromatogram was based on the same criteria as

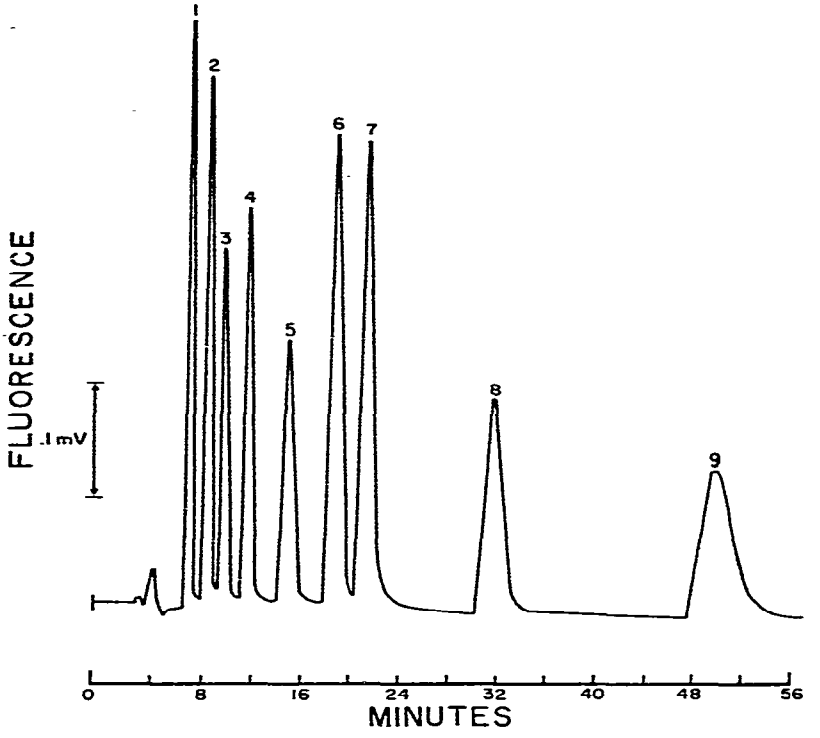

Fig. 2. Chromatogram of standard indole compounds. Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = N-methylserotonin; 4 = TRP; 5 = 5-HIAA; 6 = 5-HTOL; 7 = tryptamine; 8 = N-acetylserotonin; 9 = IAA. See Experimental for chromatographic conditions. Elution conditions were the same as for Fig. 1.

TABLE I

CHROMATOGRAPHIC AND DETECTABILITY DATA

For chromatographic conditions see Experimental.

Compound	Retention time (min)	Detection limit (pg)*
5-Hydroxytryptophan	7.6	45
Serotonin	9.1	30
N-Methylserotonin	10.4	50
Tryptophan	12.4	50
5-Hydroxyindole-3-acetic acid	15.7	90
5-Hydroxytryptophol	19.6	70
Tryptamine	21.6	100
N-Acetylserotonin	31.9	300
Indole-3-acetic acid	50.0	210

*Injected quantity of authentic material giving a signal-to-noise ratio of 2.0.

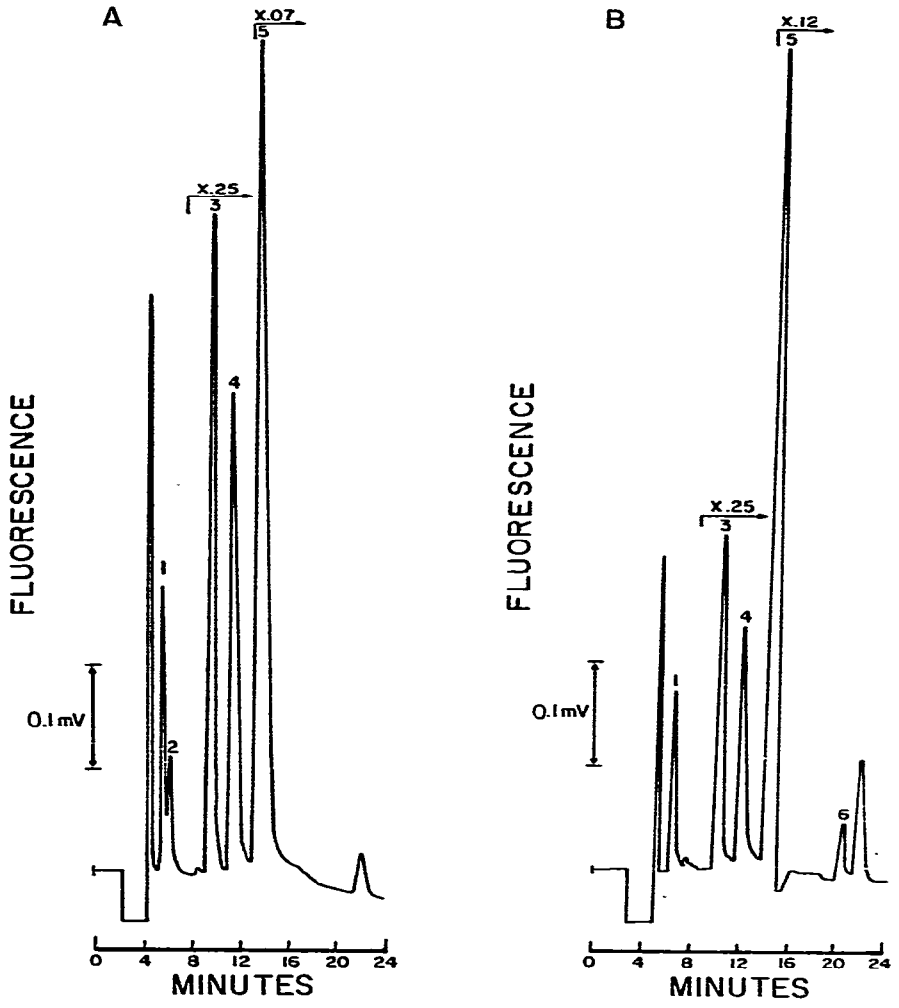

Fig. 3. Chromatogram of mouse plasma sample before (A) and after (B) acid hydrolysis. Peaks: 1 = 5-HTP; 2 = 5-HIAA-O-sulfate; 3 = 5-HT; 4 = N-methylserotonin; 5 = TRP; 6 = 5-HIAA. See Experimental for chromatographic conditions. Elution conditions were the same as for Fig. 1.

described above for lung samples and the corresponding hydrolyzed plasma sample is shown in Fig. 3B.

The determinations of TRP, 5-HTP, 5-HT, [^{3}H] 5-HT, 5-HIAA, and 5-HIAA-O-sulfate levels in mouse lung and plasma are shown in Table II. The lung 5-HT and 5-HIAA values are similar to those previously reported [33, 34], while the remaining indoles have not been previously reported in mouse lung. The plasma 5-HT levels are considerably lower than those reported [33] and may reflect differences in analytical method or differences in the method of sacrifice. The latter have been shown [35, 36] to markedly affect the lung and circulating levels of 5-HT.

In summary, we have described a simple, sensitive procedure for the rapid

TABLE II

LEVELS OF SEROTONIN, ITS PRECURSORS, METABOLITES AND [^{3}H]SEROTONIN IN MOUSE LUNG AND PLASMA

Values are expressed as μg/g or μg/ml and radioactivity is expressed as nCi/g or nCi/ml. All values are mean ± S.E.M.

Sample	TRP	5-HTP	5-HT	[^{3}H] 5-HT	5-HIAA	5-HIAA-O-sulfate
Lung	10.98 ± 0.89	0.45 ± 0.05	2.89 ± 0.26	1544 ± 138	0.12 ± 0.04	9.96 ± 0.39
Plasma	12.04 ± 1.34	0.15 ± 0.03	1.17 ± 0.16	586 ± 72	ND*	0.27 ± 0.01

*Not routinely detected.

determination of exogenous [^{3}H] 5-HT and endogenous 5-HT, its precursors and metabolites in lung. With a method capable of simultaneously measuring both endogenous 5-HT as well as exogenous [^{3}H] 5-HT, the dynamics of lung—serotonin interactions and their alteration by drugs, environmental toxicants, and disease can be more readily studied.

ACKNOWLEDGEMENTS

This work was carried out under a Grant-in-aid from the American Heart Association, Indiana Affiliate, and a Young Investigator Pulmonary Research Grant (HL 19573) from the National Institute of Heart, Lung and Blood.

REFERENCES

1 A.P. Fishman and G.G. Pietra, N. Engl. J. Med., 291 (1974) 884, 953.
2 H. Steinberg and A. Fisher, in W.B. Essman (Editor), Serotonin in Health and Disease, Vol. V, Spectrum Publications, New York, London, 1978, p. 69.
3 V.A. Alabaster and Y.S. Bakhle, Brit. J. Pharmacol., 40 (1970) 468.
4 A.F. Junod, J. Pharmacol. Exp. Ther., 183 (1972) 341.
5 Y. Iwasawa, C.N. Gillis and G. Aghajanian, J. Pharmacol. Exp. Ther., 186 (1973) 498.
6 Y. Iwasawa and C.N. Gillis, J. Pharmacol. Exp. Ther., 188 (1974) 386.
7 C.N. Gillis and J.A. Roth, Brit. J. Pharmacol., 59 (1977) 585.
8 Y.S. Bakhle and M.B.H. Yoridim, Brit. J. Pharmacol., 65 (1979) 147.
9 J.D. Catrovas and C.N. Gillis, J. Pharmacol. Exp. Ther., 213 (1980) 120.
10 T.R. Waalkes, H. Weissbach, J. Bozicevich and S. Udenfriend, J. Clin. Invest., 36 (1957) 1115.
11 M.D. Gershon and L.L. Ross, Proc. Soc. Exp. Biol. Med., 115 (1961) 367.
12 R.K. Gershon, P.W. Askenase and M.D. Gershon, J. Exp. Med., 142 (1975) 732.
13 A.P. Fishman, Circ. Res., 35 (1974) 657.
14 H.E. Geissler and E. Mutschler, Z. Klin. Chem. Klin. Biochem., 12 (1974) 151.
15 S.S. Hassan, Anal. Chem., 47 (1975) 1429.
16 F.P. Miller, R.H. Cox, W.R. Snodgrass and R.P. Maickel, Biochem. Pharmacol., 19 (1970) 435.
17 P.H. Degen, J.R. DoAmarol and J.D. Barchas, Anal. Biochem., 45 (1972) 634.
18 O. Beck, F.-A. Wiesel and G. Sedvall, J. Chromatogr., 134 (1977) 407.
19 B. Peskar and S. Spector, Science, 179 (1973) 1340.
20 A.P. Graffeo and B.K. Karger, Clin. Chem., 22 (1976) 184.
21 A.M. Krstulovic, P.B. Brown, D.M. Rosic and P.B. Champlin, Clin. Chem., 23 (1977) 1984.
22 G.M. Anderson and W.C. Purdy, Anal. Chem., 51 (1979) 283.
23 A.M. Krstulovic and C. Matzura, J. Chromatogr., 163 (1979) 72.

24 G.M. Anderson, J.G. Young and D.J. Cohen, J. Chromatogr., 164 (1979) 501.
25 G.P. Jackman, V.J. Carson, A. Bobik and H. Skews, J. Chromatogr., 182 (1980) 277.
26 R.M. Wightman, P.M. Plotsky, E. Strope, R.J. Delcore and R.N. Adams, Brain Res., 131 (1977) 345.
27 S. Sasa, C.L. Blank, D.C. Wenke and C.A. Sczupak, Clin. Chem., 24 (1978) 1509.
28 D.D. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
29 W.H. Lyness, N.M. Friedle and K.E. Moore, Life Sci., 26 (1980) 1109.
30 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
31 T. Flatmark, S. Wahlstrom and J. Haavik, Anal. Biochem., 107 (1980) 71.
32 J.J. Friedman, Amer. J. Physiol., 196 (1959) 420.
33 W.B. Essman, in W.B. Essman (Editor), Serotonin in Health and Disease, Vol. I, Spectrum Publications, New York, London, 1978, p. 125.
34 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 179 (1971) 207.
35 A.R. Buckpitt, R.P. Maickel and T.R. Bosin, Biochem. Pharmacol., 27 (1978) 569.
36 T.R. Bosin and P.D. Lahr, Biochem. Pharmacol., in press.