Journal of Chromatography, 182 (1980) 277—284
Biomedical Applications
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 553

SIMPLE AND SENSITIVE PROCEDURE FOR THE ASSAY OF SEROTONIN AND CATECHOLAMINES IN BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTION

G. P. JACKMAN*, V.J. CARSON, A. BOBIK and H. SKEWS

Clinical Research Unit, Alfred Hospital and Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181 (Australia)

(First received October 23rd, 1979; revised manuscript received December 27th, 1979)

SUMMARY

A simple, rapid and specific method for the determination of serotonin and catecholamines in brain is described. After tissue homogenisation, catecholamines are isolated by adsorption onto alumina and elution with perchloric acid. Serotonin is isolated by extraction into *n*-heptanol and back-extraction into acid. High-performance liquid chromatography of the acid extracts is performed with a C_{18} reversed-phase column and simple mobile phases. Detection is by the intrinsic fluorescence of the amines on excitation at 200 nm. Detection limits are 100 pg for norepinephrine, 300 pg for dopamine and 20 pg for serotonin. The results are found to correlate well with a catechol O-methyl transferase radioenzymatic assay for catecholamines and a ninhydrin derivatisation procedure for serotonin.

INTRODUCTION

Catecholaminergic and serotoninergic neurones have been shown to be involved in the central nervous control of cardiovascular reflexes but their exact role in this and the pathogenesis of hypertension remains unclear [1, 2]. Studies in this Institute aimed at elucidating these pathways have involved the study of cardiovascular reflexes after the depletion of these neurotransmitters by 6-hydroxydopamine and 5,6-dihydroxytryptamine [3—5]. In order to assess the degree of depletion of these transmitters and their possible role in the observed cardiovascular responses, reliable specific and sensitive assays are required for routine use.

We have found that the combination of adsorption on alumina for catecholamines and a simple solvent extraction for serotonin as originally described by Shellenberger and Gordon [6] gives excellent recoveries for the amines and

*To whom correspondence should be addressed.

relatively pure extracts suitable for assay. The original procedure has been modified, first to incorporate a very sensitive radioenzymatic assay for catecholamines and now a high-performance liquid chromatographic (HPLC)—fluorimetric assay by which both catecholamines and serotonin may be determined simply and with excellent sensitivity.

EXPERIMENTAL

Reagents

Norepinephrine (*d,l*-7-^{3}H, 5—15 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and S-adenosylmethionine (tritiated S-methyl, 7—15 Ci/mmole) from the Radiochemical Centre (Amersham, Great Britain). Norepinephrine bitartrate, dopamine, normetanephrine and 3-methoxytyramine hydrochlorides were purchased from Sigma (St. Louis, MO, U.S.A.). Serotonin creatinine sulfate was obtained from Fluka (Buchs, Switzerland). N-Tris(hydroxymethyl)methylglycine (Tricine) and *n*-heptanol were purchased from Hopkin and Williams (Chadwell Heath, Great Britain). Heptanol was distilled before use and after use was washed with water and redistilled for re-use. Kieselgel 60 F254 thin-layer plates, 0.25 mm thick (Merck, Darmstadt, G.F.R.), were used in chromatography. Liquifluor (New England Nuclear) and Instagel (Packard Instrument, Downers Grove, IL, U.S.A.) were used in liquid scintillation counting. Alumina, activity grade I, 70—230 mesh (Merck) was pretreated according to the method of Anton and Sayre [7]. Catechol O-methyl transferase (COMT) was prepared by a modified method of Axelrod and Tomchick [8] involving the dialysis of the 30—50% ammonium sulfate precipitated fraction against 0.001 *M* phosphate buffer pH 7.0 for 16 h. The final protein concentration was 16 to 24 mg/ml and 1-ml aliquots were stored at −20°C for up to three months before use without loss of activity. Acetonitrile, 190 nm HPLC grade was purchased from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC was redistilled from alkaline permanganate before use. All other reagents were of A.C.S. certified grade.

Chromatographic system

A 5000 series liquid chromatograph fitted with a universal loop injector (Varian, Palo Alto, CA, U.S.A.) was used in conjunction with a Micropak MCH-10 octadecylsilane reversed-phase column (300 × 4 mm I.D.). A 40-mm guard column was packed with pellicular C_{18} material (Vydac SC reversed phase, Varian). The detector was a Schoeffel FS 970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. The amines were detected by excitation at 200 nm and their fluorescence emission was selected by a Corning 7-60 glass filter with an approximate bandpass of 320—400 nm.

Brain samples

Rabbits were obtained from an inbred colony established since 1961 (Commonwealth Serum Labs., Melbourne, Australia) and were killed with an overdose of sodium pentobarbitone. The brain and spinal cord were rapidly removed and dissected over ice into cerebral hemispheres, thalamus plus limbic (thalamus, basal ganglia and hippocampus) hypothalamus, midbrain,

pons plus medulla, cerebellum, cervical cord, thoracic cord and lumbar cord. These were then frozen in liquid nitrogen, weighed and stored in liquid nitrogen for up to four weeks.

Sample preparation

The sections of brain were homogenised with a Polytron homogeniser (Kinematica, Luzern, Switzerland) at setting 5 for 10 sec in 0.4 *M* perchloric acid containing 0.1% sodium metabisulfite and 0.05% Na_2EDTA. The ratio of acid to tissue was 6:1, v/w. The homogeniser was washed with 1 ml of acid and the washings added to the homogenate. The homogenates were centrifuged at 30,000 *g* for 10 min and the supernatants collected and stored over ice. The pellets were resuspended in 2 ml of 0.4 *M* perchloric acid, centrifuged and the two supernatants pooled. The total volume was then recorded.

Extraction of catecholamines and HPLC estimation

Tritiated norepinephrine (200,000 dpm) was added to 6 ml of the supernatant and buffered to pH 7.5 to 8.0 with 5 to 6 ml of Tricine buffer (Tricine 100 m*M* and Na_2EDTA 68 m*M* in 0.525 *M* sodium hydroxide) as described by Shellenberger and Gordon [6]. Alumina (300 mg) was added and the samples shaken gently for 15—20 min. After centrifugation at 1000 *g* for 5 min, the supernatant was removed for the serotonin determination. The alumina was washed three times with 20 ml of water by gentle inversion and centrifuged for 5 min at 1000 *g*. All water was carefully removed by aspiration. The catecholamines were eluted by the addition of 0.1 *M* perchloric acid (5 ml for radioenzymatic estimation and 1 ml for HPLC) with gentle shaking for 15 min. After centrifugation the acid eluate was transferred to clean tubes and stored at 4° C. Aliquots (50 µl) of the eluates were added to 10 ml of Instagel for scintillation counting to determine the recovery of catecholamines. The eluates are stable for at least one week at 4° C.

The eluates were assayed by injection of 50—100 µl directly onto the HPLC column. The mobile phase was 0.01 *M* perchloric acid—acetonitrile (99:1) and the flow-rate was 2 ml/min. Detection was by fluorescence (see Chromatographic system). Calibration was performed by the injection of standard solutions freshly prepared from stock solutions (1 mg/ml in 0.01 *M* hydrochloric acid).

Radioenzymatic estimation of catecholamines

Norepinephrine and dopamine are estimated in the alumina eluates by enzymatic conversion to tritiated normetanephrine and 3-methoxytyramine respectively. Enzyme inhibition [8, 9] by aluminium ions was effectively overcome by 1:1 dilution of the eluates with 0.1 *M* perchloric acid.

Duplicate aliquots (50 µl) of the diluted eluates were added to 50 µl of 0.2 *M* perchloric acid in 15-ml stoppered glass tubes. Blank samples were prepared with 100 µl of 0.15 *M* perchloric acid and external standards (100, 250 and 500 pg) in 50 µl of 0.1 *M* perchloric acid.

The reaction was started by the addition of 100 µl of an enzyme mixture containing 12.5 m*M* $MgCl_2$, 600 m*M* Tris HCl pH 9.6, [^{3}H]S-adenosylmethionine (2.5 µCi) and COMT (44%, v/v), prepared in ice immediately before

use. The samples were then incubated at 37°C for 45 min. The reaction was stopped by the addition of 100 µl of 1 *M* sodium hydroxide containing 0.8 *M* boric acid, 0.08 *M* Na_2EDTA and 3 m*M* normetanephrine and methoxytyramine. The O-methylated derivatives were extracted with 4 ml of a mixture of toluene—isoamylalcohol (3:2) by shaking for 2 min and the organic phase transferred to clean tubes containing 100 µl of 0.1 *M* acetic acid. Here and in all subsequent steps phase separation was simplified by freezing the aqueous phase in dry ice—ethanol. The tritiated derivatives were extracted into the acid phase by shaking for 2 min, the phases separated by centrifugation and the acid phase was washed with a further 2 ml of toluene—isoamyl alcohol mixture. The acid extracts were dried in vacuo at 50°C and taken up in 100 µl of 0.001 *M* methanolic hydrochloric acid which was quantitatively transferred to silica gel thin-layer plates. The plates were developed in a solvent system consisting of chloroform—ethanol—ethylamine (80:15:7, water saturated). After development the plates were air dried, the bands corresponding to the derivatives located under UV illumination (254 nm), and scraped into scintillation vials. To each vial was added 1 ml of 0.05 *M* ammonia.

Vials containing normetanephrine were treated with 100 µl of 3% sodium periodate for 5 min. The reaction was stopped with 100 µl of 10% glycerol and acidified with 1 ml of 0.1 *M* acetic acid. The vanillin thus produced was extracted by brief shaking into 10 ml of toluene—Liquifluor scintillant for ^3H determination.

Methoxytyramine was extracted directly into 10 ml of toluene—isoamyl alcohol which was added to 10 ml of Instagel for ^3H determination.

The assay is linear to at least 500 pg of catecholamine. Blank values are typically 20—50 cpm for norepinephrine and 100—200 cpm for dopamine while the slopes of the standard curves are 25—35 cpm per pg of catecholamine.

Estimation of serotonin by the HPLC method

Aliquots of the buffered perchloric acid homogenates (2 ml) were taken from the alumina adsorption step, saturated with 1 g of sodium chloride and adjusted to pH 9.8 ± 0.4 with 80—120 mg of potassium carbonate. Standards were made up in 1 ml of 0.4 *M* perchloric acid and 1 ml of Tricine buffer. Then *n*-heptanol (4 ml) was added to each sample and shaken for 2 min, followed by centrifugation to separate the phases. A 3-ml aliquot of the heptanol extract is transferred to clean tubes and serotonin is back-extracted into 500 µl of 0.5 *M* NaH_2PO_4. The heptanol was carefully aspirated and the extract washed with 1 ml of *n*-heptane to remove residual heptanol.

The extract was then assayed by injection of 50 µl directly onto the HPLC column. The mobile phase was 0.01 *M* perchloric acid—acetonitrile (85:15) at a flow-rate of 2 ml/min. Calibration was by means of a curve prepared concurrently with the samples from external standards. Fluorescence detection was used (see Chromatographic system). Results with this method were compared with those obtained by using the method of Shellenberger and Gordon [6] without modification.

RESULTS AND DISCUSSION

For each sample the recovery of catecholamines was estimated by the recovery of tritiated norepinephrine. Use of this method to estimate dopamine recoveries is justified by the observation that the recoveries of several catecholamines are very similar [6, 10]. Recoveries were found to vary between 50 and 80% and were lower (50—70%) for the 1-ml eluates used in the HPLC assay. The unnatural catecholamine 3,4-dihydroxybenzylamine could be used with the HPLC assay — it elutes between epinephrine and dopamine — as an internal standard to avoid the necessity for recovery measurements, but the large variation in catecholamine levels between samples would require adjustment of the amount of internal standard for each sample.

The HPLC assay for catecholamines uses the intrinsic fluorescence of catechols upon excitation at 200 nm. In the mobile phases used for chromatography, catecholamines absorb maximally at 200, 220 and 280 nm (uncorrected) and serotonin at 205, 220, 275 and 295 nm. It was found experimentally that the signal-to-noise ratio for fluorescence detection was optimum at an excitation wavelength of 200 nm. This method is sensitive to 100 pg of norepinephrine and 300 pg of dopamine (Fig. 1), but relatively unspecific since the same conditions are used to detect serotonin. Specificity is achieved with the combination of adsorption onto alumina and reversed-phase HPLC. The chromatographic system is stable, highly reproducible and linear to at least 20 ng (the response is offscale for 60 ng of norepinephrine). Calibration is by the injection of known standards, since no difference is found between internal and external standards. The coefficient of variation of the absolute fluorescence response to the injection of 2 ng of catecholamine is less

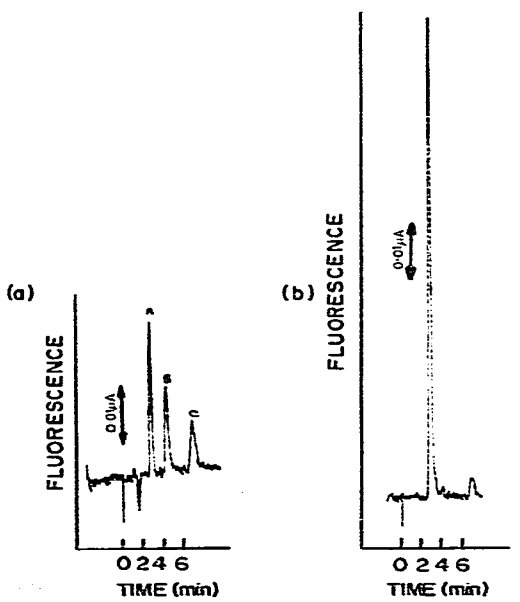

Fig. 1. Chromatograms of (a) 2 ng of norepinephrine (A), epinephrine (B) and dopamine (C) and (b) 100 μl of the eluate from a control hypothalamus weighing 40 mg.

than 10% for any one assay or for all assays over a two-month period. This is comparable with procedures using electrochemical detection [11, 12] or fluorescence after *o*-phthalaldehyde derivatisation [13]. For comparison, samples were assayed by radioenzymatic method using catechol O-methyl transferase. The procedure combines the method of Peuler and Johnson [14] and DaPrada and Zürcher [15] for the differential assay of catecholamines. The inhibitory effects on the enzyme of aluminium ions [8, 9] in the eluates are effectively overcome by dilution of the eluates so that an external standard curve may be used. However, since only 25 µl of the 5 ml of eluate are assayed, the basic sensitivity of the assay, 1—2 pg for norepinephrine and 4—8 pg for dopamine, corresponds to 200—400 pg for norepinephrine and 800—1600 pg dopamine per eluate.

The results of the comparison are shown in Fig. 2. The slopes of the regression lines are 0.961 for norepinephrine and 0.954 for dopamine or a difference of only 4%. While similar results can be obtained with either method, the speed and simplicity of the HPLC method make it preferable.

HPLC with electrochemical detection has been used to assay alumina eluates [10—12, 16] and can sometimes offer an absolute sensitivity comparable with the radioenzymatic assay [10, 12]. However, electrode life is often limited and sensitivity variable [10], while we find that fluorescence detection is very stable without any special precautions. Sasa and Blank [17, 18] have used electrochemical detection to assay butanol extracts of tissue homogenates, but not only are recoveries low and different for internal and external standards, but the chromatographic system must resolve catecholamines, serotonin and metabolites such as the metanephrines, which are also extracted. This leads to long chromatography times, which are avoided in our procedure by assaying catecholamines and serotonin independently. An HPLC assay for urinary catecholamines after alumina adsorption uses *o*-phthalaldehyde derivatisation and fluorescence detection [13], but only offers a two-fold

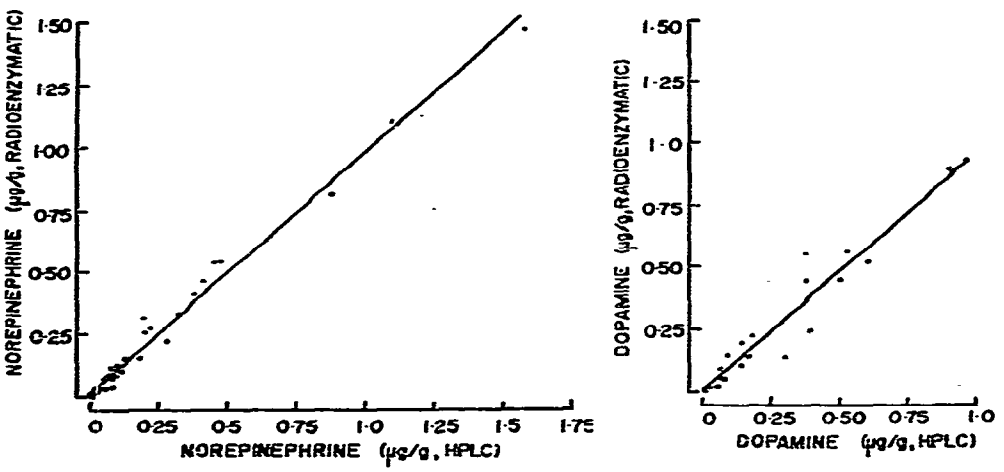

Fig. 2. Comparison between the radioenzymatic and HPLC assays for catecholamines. Samples were dilute eluates of the radioenzymatic assay. Slopes of the regression lines are 0.961 for norepinephrine and 0.954 for dopamine. Correlation coefficients are 0.993 and 0.986, respectively.

increase in sensitivity compared with our procedure which involves no derivatisation.

The extraction procedure for serotonin is taken directly from the method of Shellenberger and Gordon [6]. The high intrinsic fluorescence of serotonin when excited at low wavelengths permits the use of small samples. Some specificity is also achieved by the fact that another HPLC—fluorescence assay has shown the relative fluorescence of serotonin to be at least 40 times greater than that of its common precursors and metabolites [19]. The mild extraction procedure minimises interferences while giving recoveries of about 80%. No difference was found between internal and external standards. The calibration graph is linear within the range 0—100 ng. The reagent blank shown in Fig. 3 indicates the possibility of using much smaller samples, particularly since only 10% of the final extract is actually assayed, corresponding to about 15 mg of tissue.

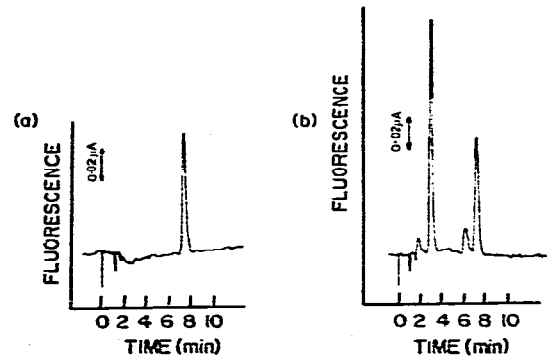

Fig. 3. Typical chromatograms for serotonin. (a) Reagent blank. The peak eluting at 8 min originates with the reagents and is present in all chromatograms. (b) Tissue sample containing serotonin which elutes at 3.2 min. The peak corresponds to approximately 3 ng of serotonin injected.

A direct comparison between the HPLC assay and the fluorimetric ninhydrin derivative assay [6] gave an excellent correlation ($r = 0.999$). The regression line was not significantly different from the line of identity.

Alternative serotonin assays have involved derivatisation with *o*-phthaldehyde [20] which is sensitive to about 5 ng. The ninhydrin assay is only sensitive to 30 ng but is more reliable [6]. A double enzymatic procedure has been described, but is time consuming and only sensitive to 1 ng [21]. The enzymes are unstable and must be prepared frequently. Radioimmunoassay has been found to be sensitive to 100 pg, but cross-reacts with 5-methoxytryptamine [22]. The antibody is also unstable and requires careful calibration. Our procedure involves no derivatisation, is extremely stable and can detect 20 pg of serotonin routinely.

In summary, the procedure we have described offers the advantage of simple extraction methods, rapid sensitive chromatography without derivatisation and the ability to assay catecholamines or serotonin independently if required.

REFERENCES

1 J. Axelrod, Clin. Sci. Mol. Med., 51 (1976) 415—421.
2 J.P. Chalmers, Clin. Sci. Mol. Med., 55 (1978) 45s—56s.
3 P.I. Korner, J.R. Oliver, J.A. Reynoldson, G.A. Head, V.J. Carson and M.M. Walker, Eur. J. Pharmacol., 53 (1978) 83—93.
4 J.A. Reynoldson, G.A. Head and P.I. Korner, Eur. J. Pharmacol., 55 (1979) 257—262.
5 P.I. Korner, J.A. Reynoldson, G.A. Head, J.R. Oliver and V.J. Carson, J. Cardiovascular Pharmacol., 1 (1979) 311—328.
6 M.K. Shellenberger and J.H. Gordon, Anal. Biochem., 39 (1971) 356—372.
7 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360—375.
8 J. Axelrod and R. Tomchick, J. Biol. Chem., 233 (1958) 702—705.
9 V.K. Weise and I.J. Kopin, Life Sci., 19 (1976) 625—636.
10 P. Hjemdahl, M. Daleskog and T. Kahan, Life Sci., 25 (1979) 131—138.
11 C. Refshauge, P.T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R.N. Adams, Life Sci., 14 (1974) 311—322.
12 R. Keller, A. Oke, I. Mefford and R.N. Adams, Life Sci., 19 (1976) 995—1004.
13 L.D. Mell, Jr., A.R. Dasler and A.B. Gustafson, J. Liquid Chromatogr., 1 (1978) 261—277.
14 J.D. Peuler and G.A. Johnson, Life Sci., 21 (1977) 625—636.
15 M. DaPrada and G. Zürcher, Life Sci., 19 (1976) 1161—1174.
16 H. Hashimoto and Y. Maruyama, J. Chromatogr., 152 (1978) 387—393.
17 S. Sasa and C.L. Blank, Anal. Chem., 49 (1977) 354—359.
18 S. Sasa and C.L. Blank, Anal. Chim. Acta, 104 (1979) 29—45.
19 A.M. Krstulovic and C. Matzura, J. Chromatogr., 163 (1979) 72—76.
20 R.P. Maickel, R.H. Cox, Jr., J. Saillant and F.P. Miller, Int. J. Neuropharmacol., 7 (1968) 275.
21 J.M. Saavedra, M. Brownstein and J. Axelrod, J. Pharmacol. Exp. Ther., 186 (1973) 508—515.
22 B.M. Jaffe, in B.M. Jaffe and H.R. Behrman (Editors), Methods of Hormone Radioimmunoassay, 2nd ed., Academic Press, London, 1978, pp. 527—539.