Journal of Chromatography, 223 (1981) 315—320
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 807

DETERMINATION OF INDOLES AND CATECHOLS IN RAT BRAIN AND PINEAL USING LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC AND AMPEROMETRIC DETECTION

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(Received October 14th, 1980)

SUMMARY

Tryptophan, serotonin, 5-hydroxyindoleacetic acid, and homovanillic acid were determined in rat brain by the direct injection of a centrifuged tissue homogenate into a liquid chromatographic—fluorometric/amperometric system. The above indoles, along with melatonin, were also determined in single rat pineal glands. The utility of the system in determining several additional catechols and indoles in brain was examined.

INTRODUCTION

The measurement of levels of neurotransmitters, their precursors, and metabolites, present in brain is of obvious importance in neurochemistry. Recently, high-performance liquid chromatography (HPLC) coupled with fluorometric [1—5] or amperometric [6—23] detectors has allowed many of the determinations to be made with greater simplicity and sensitivity. We have used a combined liquid chromatographic—fluorometric/amperometric system to determine several neurochemically important indoles and catechols in brain and pineal with a minimum of sample preparation.

MATERIALS AND METHODS

The liquid chromatograph and detection system employed was essentially that described previously [24]. It consisted of an Altex 110A pump (Altex Scientific, Berkeley, CA, U.S.A.), a Waters U6K injector, and a μBondapak C_{18} reversed-phase column (300 mm × 3.9 mm I.D., 10 μm average particle

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size) (Waters Assoc., Milford, MA, U.S.A.). A modified Aminco Fluoromonitor (American Instrument Co., Silver Spring, MD, U.S.A.) was used with a 254-nm low-pressure mercury lamp source and a 360-nm peak transmittance emission filter. The amperometric detector was composed of a Bioanalytical Systems electrochemical controller (LC-2A), a glassy carbon working electrode, a Ag/AgCl reference electrode and a Teflon thin-layer detector cell and reference electrode compartment (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A 51-μm spacer gasket was used, and the potential of the working electrode was set at +0.70 V versus the reference electrode. Background currents of 1—2 nA were observed. The detectors were connected in series, with the amperometric detector downstream. Standards were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions (10 mg/100 ml) were made up in distilled water with 0.1% ascorbate added. Diluted standards (0.1—5.0 ng/μl) were made up daily in distilled water. Solvent systems were prepared by mixing the proper proportion (v/v) of glass-distilled methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) with 0.01 *M* sodium acetate previously adjusted to the desired pH with glacial acetic acid.

Weighed whole rat brains (1—2 g) were placed in polycarbonate centrifuge tubes containing 4.0 ml of 0.1 *M* $HClO_4$ (400 μl of 1 *M* $NaHSO_3$ added per liter). The brain was sonicated at 0—4°C at a medium setting for two 15-sec periods using a Branson Polytron Sonicator (Branson Sonic Power Co., Danbury, CT, U.S.A.). After adding 0.5 ml of 4.0 *M* $HClO_4$ and vortex mixing, the samples were spun at 10,000 *g* for 10 min and a portion of the supernatant stored in a 1.5-ml polyethylene tube. When sonicating brain punches and areas weighing from 2 mg to ca. 1 g, a similar v/w ratio of 0.1 *M* $HClO_4$ was used down to a minimum of 200 μl.

Rat pineals were placed in 1.5-ml polyethylene tubes and sonicated in 200 μl of 0.1 *M* $HClO_4$ and centrifuged at 10,000 *g* without the addition of 4.0 *M* $HClO_4$.

TABLE I

CHROMATOGRAPHIC AND DETECTABILITY DATA

Compound	Chromatographic conditions*	Retention time (min)	Detection limit (pg)**	
			Fluorometric	Amperometric
Serotonin (5-HT)	1	3.2	7	10
	2	2.8		
Tryptophan (TRP)	1	4.7	20	400
	2	4.1		
5-Hydroxyindoleacetic acid (5-HIAA)	1	6.9	35	10
	2	5.1		
Homovanillic acid (HVA)	1	8.5	2000	25
	2	6.3		
5-Hydroxytryptophol (5-HTOL)	2	6.1	35	15
Melatonin (MEL)	3	5.4	25	50

*Chromatographic conditions: 1: 88% pH 4.25 0.01 *M* sodium acetate—12% methanol; 2: 85% pH 4.50 0.01 *M* sodium acetate—15% methanol; 3: 65% pH 4.25 0.01 *M* sodium acetate—35% methanol.

**Injected amount giving a signal twice the peak-to-peak baseline fluctuation.

The compounds were determined in the brain and pineal homogenates by directly injecting 10—100 μl of the supernatants into the chromatograph. The chromatographic conditions are given in Table I. The compounds were quantitated by peak height measurments; single point standards were used as a linear response was observed over the working range.

RESULTS AND DISCUSSION

The chromatographic conditions, retention times, and detection limits for the compounds determined are given in Table I. A chromatogram of serotonin (5-HT), tryptophan (TRP), 5-hydroxyindoleacetic acid (5-HIAA), and homovanillic acid (HVA) standards is shown in Fig. 1. Standards were determined

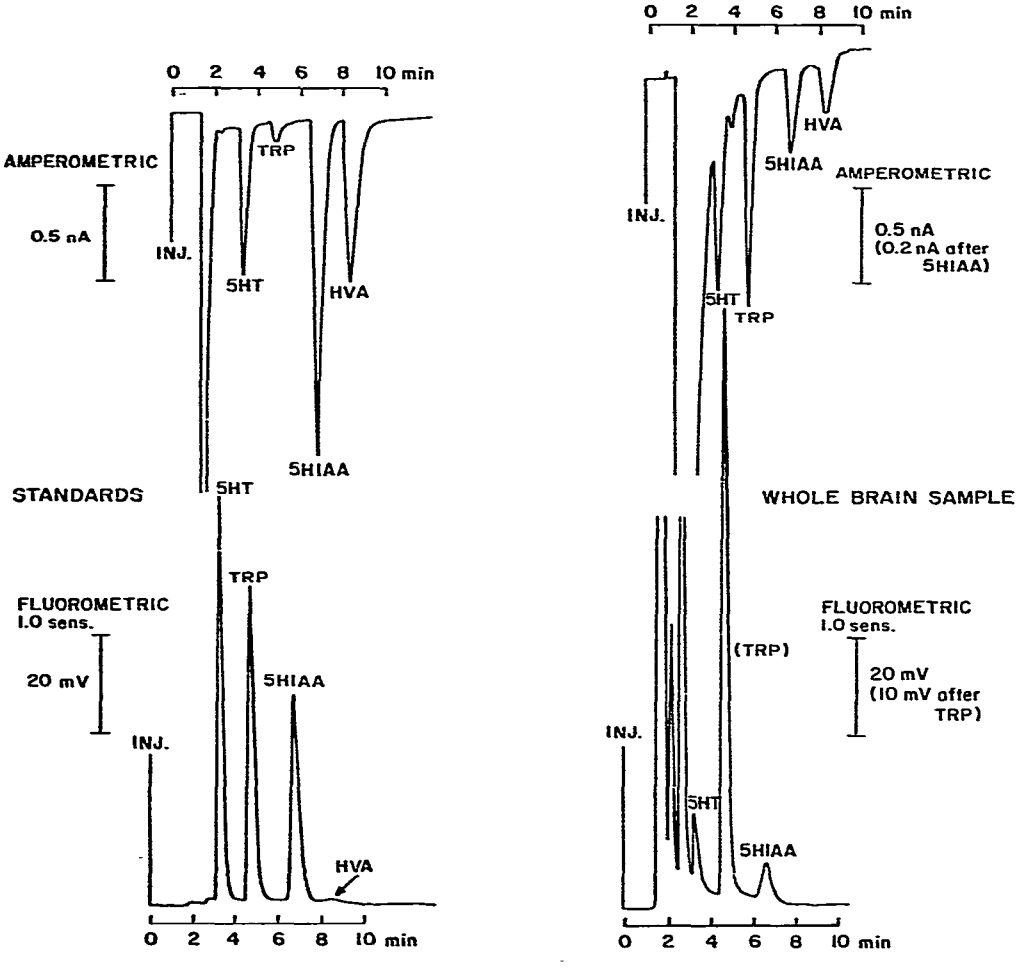

Fig. 1. Chromatogram of standards (5-HT 5 ng, TRP 10 ng, 5-HIAA 10 ng, HVA 10 ng) with fluorometric and amperometric detection. Chromatographic condition 1 (see Table I).

Fig. 2. Determination of 5-HT (130 ng/g), TRP (2500 ng/g), 5-HIAA (110 ng/g), and HVA (53 ng/g) in whole rat brain (1.8 g). Injection volume 20 μl. Chromatographic condition 1 (see Table I).

with typical coefficients of variation of 3—7%. The chromatogram in Fig. 2 depicts the determination of the compounds in whole rat brain. All of the compounds can be determined in brain both fluorometrically and amperometrically (except HVA fluorometrically). The amperometric and fluorometric values agreed well; usually a mean of the two methods was taken. Standard addition studies were performed by adding approximately 1—10 times normal levels of the compounds. The compounds were recovered linearly in yields of 60—92%, depending upon the particular compound, the brain area, and the w/v ratio used for homogenation. The recoveries for whole brain were: 5-HT 82%, TRP 71%, 5-HIAA 72%, and HVA 91%.

Most previous HPLC methods for determining catechols and/or indoles in brain require an extractive and/or chromatographic clean-up before analysis. With our method, the determinations are made easily and sensitively after simple homogenization. Special care must be taken to establish the identity of peaks observed when an unpurified homogenate is directly injected. The peaks observed here were identified on the basis of their relative fluorometric and amperometric response, as well as by chromatographing brain samples in solvent systems differing in pH (3.5, 4.5, and 5.5), and in percent and type of organic modifier (0—15% methanol and acetonitrile). A recent report [5] of the HPLC—fluorometric determination of several catechols and indoles in an unpurified brain homogenate contains several apparently misidentified peaks. Specifically, the peaks assigned to norepinephrine, dopamine, tyramine, 5-hydroxytryptophan, and indolelactic acid all failed to behave similarly to standards in our tests [25]. The peaks observed for tyramine, norepinephrine and dopamine were also several orders of magnitude too large. The observation of large levels of tyramine (>1000 ng/g brain) was of special interest as tyramine is generally considered to be a trace amine with brain concentrations of ca. 1 ng/g [26]. When the brain homogenate was chromatographed using a solvent system similar to that reported, a fluorometric peak did co-chromatograph with the tyramine standard; however, the peak was not electro-active, in contrast to the tyramine standard which was easily detected amperometrically. This reconfirms tyramine's status as a trace amine in rat brain and illustrates the power of the combined fluorometric/amperometric detection system.

Determinations of several indoles in rat pineal are shown in Figs. 3 and 4. In Fig. 3, TRP, 5-HT, 5-HIAA, and a peak tentatively identified as 5-hydroxytryptophol (5-HTOL), were determined by injecting 20 μl of a centrifuged 200-μl 0.1 *M* $HClO_4$ pineal homogenate. In Fig. 4, the important pineal hormone, melatonin, was determined by injecting 100 μl of the same homogenate. A solvent system with a higher percentage of methanol was used to elute this relatively lipophilic compound. Thus, in two runs on the chromatograph, five indoles can be measured in a single pineal. These compounds include melatonin, a hormone with a well established anti-gonadotrophic effect, and 5-HTOL, a compound about which very little is known, but which may have hormonal actions similar to melatonin [27]. The pineal shown was obtained from a young (ca. 30 g) Sprague-Dawley rat sacrificed at 12:00 noon when melatonin is at its lowest level [28, 29]. The compounds determined in pineal have all been recovered in >90% yield from spiked samples.

In conclusion, we have found that the combination of fluorometric and

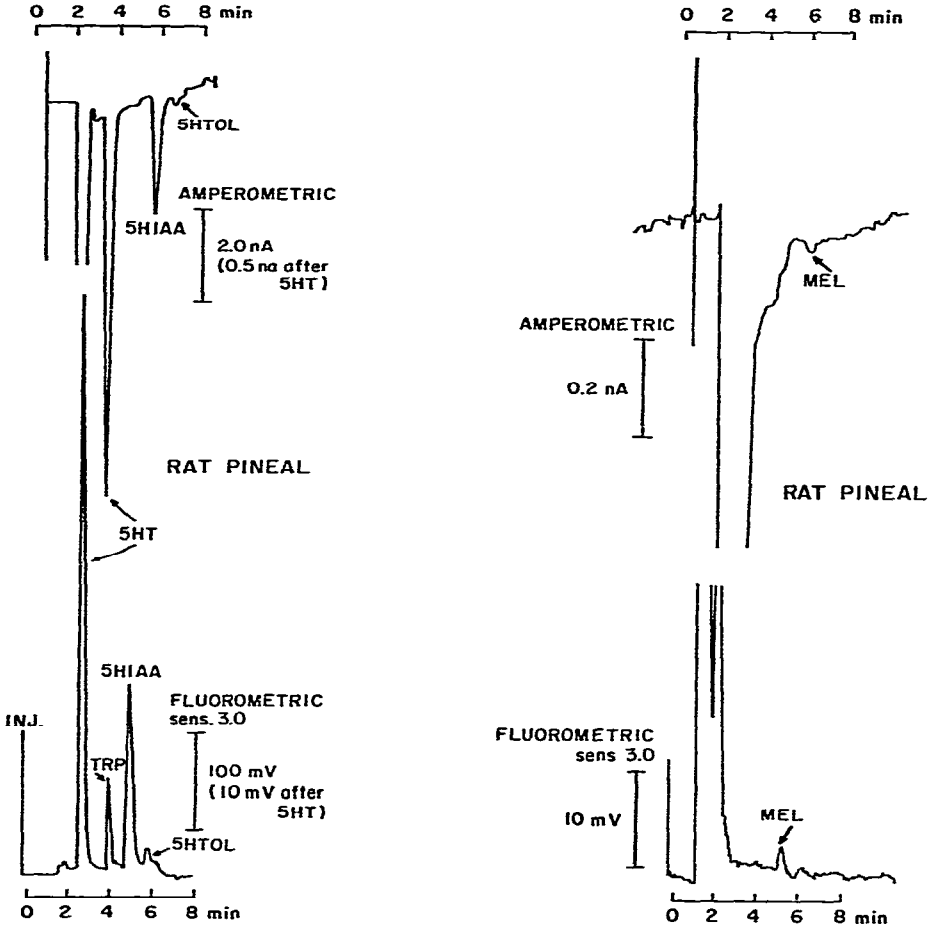

Fig. 3. Determination of 5-HT (100 ng/pineal), TRP (3.1 ng/pineal), 5-HIAA (8.6 ng/pineal), and 5-HTOL (0.74 ng/pineal) in a single rat pineal. Injection volume 20 μl of a 200-μl homogenate. Chromatographic condition 2 (see Table I).

Fig. 4. Determination of melatonin (MEL) in a single rat pineal (MEL, 0.2 ng/pineal). Injection 100 μl of a 200-μl homogenate from a rat pineal obtained at the time of the lowest diurnal MEL level.

amperometric detectors with HPLC has allowed determination of indoles and catechols in rat brain and pineal to be made easily and with a great deal of specificity and sensitivity. The dual detection system affords an extra measure of certainty concerning the identity of the peaks observed. For routine use, we have found fluorometric detection to be practically problem-free, as opposed to amperometric detection, where an occasional problem due to bubble formation, electrode passivation, and non-specific instrumental noise is to be expected.

ACKNOWLEDGEMENTS

This research was supported by MHCRC grant MH 30929, NICHD grant HD-03008, the Solomon R. and Rebecca D. Baker Foundation, and the Gateposts Foundation.

NOTE ADDED IN PROOF

A recent method [30] uses combined fluorometric and amperometric detection to determine several of the compounds in brain extracts.

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