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Short Communication

Simultaneous measurement of serotonin and 5-hydroxyindoleacetic acid in rat brain using a liquid chromatographic method with electrochemical detection

M. DE VRIES and J. ODINK*

Department of Clinical Biochemistry, TNO-CIVO Toxicology and Nutrition Institute, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

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ABSTRACT

A simple and sensitive method for the simultaneous measurement of 5-hydroxytryptamine and its main metabolite 5-hydroxyindoleacetic acid in rat brain is described. Brain tissue samples were only deproteinated and, without further extraction, were injected directly onto a high-performance liquid chromatography column and detected electrochemically. The detection limit for 5-hydroxytryptamine and 5-hydroxyindoleacetic acid was 16 and 8 g per injection, respectively. Within 8 min, the total separation of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid is achieved. With this method over 100 analyses can be performed in a single working day. Brain samples from young and old, male and female Brown Norway rats were analysed for indoles by this method. The ratio 5-hydroxytryptamine/5-hydroxyindoleacetic acid, an index for serotonin turnover, showed significant differences between age groups and genders in the cortex, and a significant difference between genders in the hypothalamus.

INTRODUCTION

The role of 5-hydroxytryptamine (serotonin, 5-HT) in the brain is complex. 5-HT is involved in many neurological processes and has been used as a parameter in many studies. There appears to be a fundamental relationship between aging and changes in neurotransmitter metabolism in the brain. The concentration of brain catecholamines and indoles has been found to be age-related in rats [1–3], mice [4] and monkeys [5], as is the release of 5-HT from cortical slices [6]. For a further study of the effects of age on 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the brain we have developed a sensitive, simple and rapid method for determining 5-HT and 5-HIAA in small rat brain samples.

Until now various techniques have been employed to determine 5-HT and 5-HIAA in brain homogenates. Fluorescence spectrometry [7–9] and high-per-

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formance liquid chromatography (HPLC) with electrochemical detection (ED) [10–12] provide high sensitivity. These methods, however, either require sample purification by means of ion-exchange extraction or solvent extraction procedures and the use of a long HPLC column resulting in long retention times, or are complicated.

Here we describe a method for the determination of 5-HT and 5-HIAA using HPLC–ED which is very simple and easy to perform and which has a high sensitivity. To restrict analysis time we minimized prepurification steps (only deproteinization) and used a short HPLC reversed-phase column (40 mm).

EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC system comprised a Spectra-Physics SP8810 isocratic pump (Eindhoven, The Netherlands), a Perkins-Elmer ISS 100 autoinjector (Gouda, The Netherlands) and a Bioanalytical Systems amperometric detector (West Lafayette, IN, U.S.A.), consisting of a Model LC-4B control unit equipped with a Model RE-1B Ag/AgCl reference electrode, a Model TG-2M 0.05 mm gasket, a Model TL-5A MF-1000 dual glassy carbon working electrode and a stainless-steel auxiliary electrode.

Stainless-steel Hyperchrome HPLC columns (40 mm × 4.6 mm I.D.) (Salm & Kipp, Breukelen, The Netherlands) were packed with ODS-Hypersil 3 μm (Shandon, Zeist, The Netherlands) at 63 MPa by the balanced-density slurry technique, on a column-packing installation designed at the TNO-CIVO Toxicology and Nutrition Institute, using a Haskel pump Type DSTV-150 (Amman Technik, Stuttgart, Germany). The slurry and packing solvents were 2-propanol and methanol, respectively (Merck, Darmstadt, Germany).

The mobile phase consisted of 75 m*M* potassium dihydrogenphosphate, 5 m*M* ethylenediaminetetraacetic acid (EDTA) and 0.01 m*M* octanesulphonic acid (OSA) as ion-pair reagent, adjusted to pH 4.1, and degassed for 15 min prior to use. The flow-rate was 1.5 ml/min. The column was continuously perfused with the mobile phase at minimum flow-rate when not used for determination.

ED was at a potential difference of +500 mV relative to the Ag/AgCl reference electrode. The full-scale detector sensitivity was 2 nA. The identification and quantification of samples was achieved by comparison with a standard solution containing 30 n*M* 5-HT and 30 n*M* 5-HIAA, and by the measurement of elution time and peak height.

Chemicals and reagents

All reference compounds and clorgyline were obtained from Sigma (St. Louis, MO, U.S.A.). A stock standard solution of each compound was prepared at 10 m*M*. Before use the stock standard was further diluted to 30 n*M*. Methanol, EDTA and potassium dihydrogenphosphate were obtained from Merck. OSA was obtained from Janssen Chemie (Beerse, Belgium).

Sample preparation

Young (6 months) and old (39 months) male and female Brown Norway rats were obtained from IVEG-TNO (Rijswijk, The Netherlands). They were housed in groups of five in our animal facility and given access to food and water *ad libitum*. Rats were kept on a 12–12 h light–dark cycle. Lights were on from 07:00.

Rats were killed by decapitation. Their brains were rapidly removed and dissected on an ice-cooled glass plate. Hypothalamic and cortical halves and one hippocampus were isolated from the right hemisphere. The isolated tissues were rapidly frozen on liquid nitrogen and stored at −80°C until further use.

Tissues were homogenized in ten volumes of 80% (v/v) methanol, 0.1 m*M* EDTA and 5 μM clorgyline using a PTFE glass homogenizer. Homogenates were centrifuged at 14 000 *g* for 5 min. Prior to injection, 10 μl of the clear supernatants were diluted four-fold in the mobile phase solution. Aliquots of 25 μl were directly injected into the HPLC system.

The pellets were resuspended in the original volume and the tissue protein content was measured according to the method of Bradford [13] using the commercially available Bio-Rad Protein kit.

Data analysis

The within-assay and between-assay standard deviations ($S.D._w$ and $S.D._b$) were calculated with analysis of variance (ANOVA) from the data obtained for a pooled homogenated hypothalamic tissue sample, analysed in duplicate on five days over a period over two weeks. The $S.D._0$, *i.e.* the S.D. of a single analysis, was calculated as follows: $(S.D._0)^2 = (S.D._w)^2 + (S.D._b)^2$.

The recovery of 5-HT and 5-HIAA by this method was calculated from the data for a spiked pooled sample, analysed in quadruplicate on five days.

Differences between young and old Brown Norway rats were investigated using regression analysis with age, gender and their interaction as factors.

RESULTS AND DISCUSSION

Quality of the method

A typical chromatogram of a standard solution of 0.75 pmol 5-HT and 5-HIAA is shown in Fig. 1A. All components under study were well resolved. The retention times for 5-HT and 5-HIAA were 5.5 and 8.2 min, respectively. The identity of the peaks was further confirmed by the evidence that the addition of small amounts of 5-HT and 5-HIAA to a pooled sample increased their sizes but not their shapes. In Fig. 1B a typical chromatogram of a pooled homogenated hypothalamus with and without a small amount of standard is shown. The two additional peaks in the chromatograms are dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC). These compounds were too close to the elution front to quantitate them reliably in the tissue homogenates.

The pH was the most critical factor in the composition of the mobile phase. A

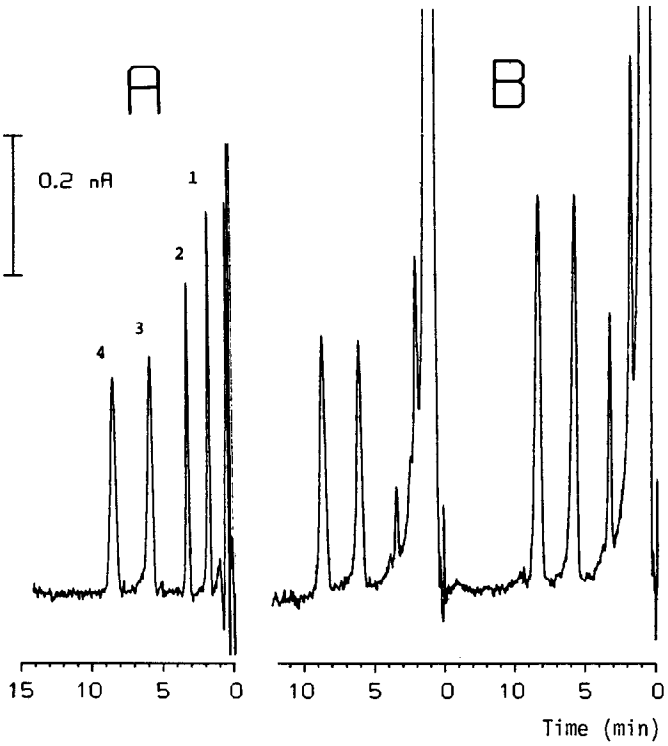

Fig. 1. (A) Chromatogram of a standard solution of 0.75 pmol of dopamine (1), DOPAC (2), 5-HT (3) and 5-HIAA (4). The applied potential was 500 mV relative to an Ag/AgCl reference electrode. The flow-rate was 1.5 ml/min. The detector was set on a gain of 2 nA/V. (B) Chromatograms of a pooled hypothalamic tissue samples and the same sample with an addition of 0.42 pmol of standard. The samples were diluted four-fold in mobile phase solution.

small change in pH value (> 0.10) caused the 5-HT and DOPAC peaks to overlap. At pH 4.1 the peaks of all the constituents were completely separated.

Indoles have a relatively low redox potential plateau value when compared to catecholamines and other brain constituents. To minimize interference with these constituents we used a potential of $+500$ mV relative to the Ag/AgCl reference electrode. At this potential all compounds of interest were detected with sufficient sensitivity.

The detector response of diluted standards was found to be linear over the range 1.25–40 pmol. To measure in this range, the homogenates were diluted four-fold in mobile phase solution, and an aliquot of 25 μl was injected into the HPLC system. Under these conditions a good separation from the elution front was achieved. The absolute detection limit was 16 and 8 pg per injection for 5-HT and 5-HIAA, respectively (signal-to-noise ratio $= 3$). The recovery calculated for a spiked pooled tissue sample was 98 and 101% for 5-HT and 5-HIAA, respectively.

TABLE I

WITHIN-ASSAY, BETWEEN-ASSAY AND OVERALL STANDARD DEVIATIONS OF THE METHOD CALCULATED FOR A POOLED TISSUE SAMPLE

Compound	Concentration (nmol/l)				C.V.[a] (%)
	Mean	$S.D._0$	$S.D._b$	$S.D._w$	
5-HT	36.2	1.6	0.0	1.6	4.4
5-HIAA	50.4	2.1	1.9	0.9	4.2

[a] Coefficient of variation of a single assay.

The within-assay, between-assay and overall standard deviations calculated for a pooled sample are summarized in Table I. For 5-HT the $(S.D._b)^2$ was calculated to be negative as a consequence of the relatively high $S.D._w$. This is perhaps due to the limited number of analyses we performed with the pooled sample. The overall results, however, showed a good precision of the method.

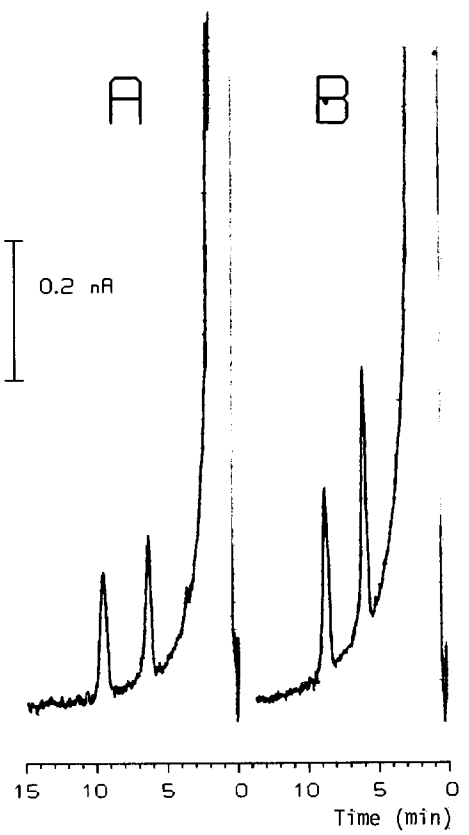

Fig. 2. Chromatograms of cortex (A) and hippocampus (B). Samples were diluted four-fold diluted in mobile phase solution. A 25-μl sample was injected into the HPLC column.

Brain tissue preparation

The tissue samples were deproteinized in 80% (v/v) methanol containing clorgyline and EDTA. Clorgyline, a monoamine oxidase inhibitor, was added to the homogenizing solvent to prevent degradation of 5-HT in the tissue sample during purification. We chose methanol instead of the commonly used perchloric acid because our findings indicate that the indoles are more stable in methanol (no data shown). Over a period of five months no degradation of the indoles in the pooled sample could be detected using methanol as a homogenizing solvent.

Under the conditions described in this paper, no further purification was necessary to obtain a complete separation of the indoles. Over 100 analyses can be done in one working day.

Concentration of 5-HT and 5-HIAA in rat brain regions

The levels of 5-HT and 5-HIAA and the 5-HIAA/5-HT ratio in various brain samples found with our method are summarized in Table II. No significant differ-

TABLE II

CONCENTRATIONS AND TURNOVER OF 5-HT AND 5-HIAA IN DIFFERENT REGIONS OF RAT BRAIN OF YOUNG AND AGED, MALE AND FEMALE BROWN NORWAY RATS

Values are means of five measurements, expressed in μg/g of protein. N.S. = not significant.

Area	Variable	Age (months)	Gender		$S.D._{res}$[a]	*P*		
			Male	Female		Age	Gender	Age · Gender[b]
Hypothalamus	5-HT	6	32.5	32.8	8.38	N.S.	N.S.	N.S.
		39	39.3	28.5				
	5-HIAA	6	13.5	17.6	3.71	N.S.	N.S.	N.S.
		39	15.0	12.3				
	5-HIAA/5-HT	6	0.414	0.534	0.082	N.S.	<0.05	N.S.
		39	0.414	0.394				
Hippocampus	5-HT	6	17.1	19.1	6.94	N.S.	N.S.	N.S.
		39	13.0	11.7				
	5-HIAA	6	14.2	20.9	7.20	N.S.	N.S.	N.S.
		39	10.0	10.0				
	5-HIAA/5-HT	6	0.909	1.126	0.239	N.S.	N.S.	N.S.
		39	0.804	0.863				
Cortex	5-HT	6	10.8	7.4	2.59	N.S.	N.S.	N.S.
		39	10.8	5.9				
	5-HIAA	6	3.7	3.8	1.22	N.S.	N.S.	N.S.
		39	5.3	2.9				
	5-HIAA/5-HT	6	0.355	0.505	0.058	<0.01	<0.01	<0.02
		39	0.491	0.496				

[a] Standard deviation of the residual.

[b] Interaction between age and gender.

TABLE III

COMPARISON OF 5-HT AND 5-HIAA LEVELS IN THE BRAIN STRUCTURES BETWEEN OUR RESULTS AND THE LITERATURE

Results are expressed in μg/g of protein.

Area	Strain	Gender	Age (months)	5-HT	5-HIAA	5-HIAA/ 5-HT	Ref. No.
Hypothalamus	Mice	Female	7	30.7	9.40	0.308	4
			18	27.4	9.40	0.343	
	Brown Norway	Female	6	32.8	17.6	0.534	Our results
			39	28.5	12.3	0.394	
	Zucker	Male	4			0.067	14
Hippocampus	Sprague–Dawley	Male	3	2.25			15
			32	2.12			
	Sprague–Dawley	Male	3			3.08	3
			27			3.46	
	Zucker	Male	4			0.0296	14
	Brown Norway	Male	6	17.1		0.909	Our results
			39	13.0		0.804	
Cortex	Wistar	Female	2	2.7	5.4	1.92	16
	Brown Norway	Female	6	7.4	3.8	0.505	Our results
	Sprague–Dawley	Male	3	2.12			15
			32	2.01			
	Sprague–Dawley	Male	3			2.29	3
			27			2.56	
	Zucker	Male	4			0.191	14
	Brown Norway	Male	6	10.8		0.355	Our results
			39	10.8		0.491	

ences could be detected in the levels of 5-HT and 5-HIAA between age groups and genders in the three brain regions studied. In the female rat, however, the levels of all the indoles in the three brain regions studied appeared to be lower. This is in agreement with results from other workers who have found a significantly lower 5-HIAA concentration in aged mice [4]. In contrast, other authors describe no change or a level which increases with age [2,3].

A marked difference in 5-HT metabolism, as revealed by the 5-HIAA/5-HT ratio, was observed in the hypothalamus and the cortex. A significant effect of age, gender and the interaction between age and gender was found in the cortex, and a significant effect of gender was measured in the hypothalamus. The significant age-dependent 5-HT turnover in the cortex agreed with the findings of other authors [3,4].

To confirm the validity of the method we compared our results with the findings of other authors (Table III). Relatively few studies with Brown Norway rats are described in the literature. No data on the levels of 5-HT and 5-HIAA in the

brain regions of these rats have been described. Studies with other strains show that our results are roughly in the same range.

We conclude that the method presented in this paper is a good alternative to the rather complicated methods used to date for the determination of 5-HT, its main metabolite 5-HIAA and the ratio 5-HIAA/5-HT in regions of rat brains. The method is easy to perform, rapid and sensitive.

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