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Short Communication

Improved method for the determination of 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine in tissue using high-performance liquid chromatography with electrochemical detection

Lena Nyström*, Gabriella Salén and Thomas Hedner

Department of Pharmacology, University of Gothenburg, Box 330 31, S-400 33 Gothenburg (Sweden)

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ABSTRACT

A liquid chromatographic method with electrochemical detection is described for the determination of the 5-hydroxytryptamine (5-HT) neurotoxins 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT) in rat brain tissue. This method has also been used for the determination of 5-hydroxyindoleacetic acid, homovanillic acid and 5-HT in other tissue samples. The method is based on extraction of the indoles from brain samples with perchloric acid followed by reversed-phase liquid chromatography with electrochemical detection. The detection limit is 1 ng per 100 mg of tissue. This paper describes a quick and reliable method of assaying the 5-HT neurotoxins 5,6-DHT and 5,7-DHT in brain tissue, which is improved compared to currently available assays.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) neurotoxins have been used extensively in neuropharmacology in order to study the structure and function of the serotonin system in the brain [1–3]. 5,6-Dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT) are the most well known currently available serotonergic neurotoxins [4–6]. These agents, which have only very limited effects on catecholamine neurons, presumably act via the formation of highly reactive and cytotoxic oxygen radicals such as the superoxide radical, the hydroxy radical and singlet oxygen [1]. Although both 5-HT neurotoxins are highly selective for 5-HT neurons, 5,7-DHT appears to be a better tool than 5,6-DHT because it has a higher neurotoxic potency and more limited non-specific cytotoxic effects on non-monoamine neurons [1].

As some 5-HT neurotoxins may occur endogenously in the brain in amounts smaller than those administered [7], there is a need for a selective and sensitive method for the assay of, *e.g.*, 5,6-DHT and 5,7-DHT. High-performance liquid chromatographic (HPLC) methods for the determination of biogenic amines have been described in many reports [7,8]. If minute amounts of 5,6-DHT and 5,7-DHT are to be quantified in nervous tissue, a good separation of the neurotoxins from other 5-HT-like structures must be ensured.

In this work we describe a new, selective method for the determination of 5,6-DHT and 5,7-DHT with a shorter retention time, a better separation and a better sensitivity than those of earlier published methods. This method includes the

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use of an octadecylsilane-bonded HPLC column, sodium octylsulphate as ion-pairing agent and isocratic elution with acetate buffer.

EXPERIMENTAL

Chemicals

The following reference substances were used: 5,6-dihydroxytryptamine–creatinine sulphate (5,6-DHT), 5,7-dihydroxytryptamine–creatinine sulphate (5,7-DHT), 5-hydroxyindole-3-acetic acid (5-HIAA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), 5-hydroxytryptamine–creatinine sulphate (5-HT) and 3-hydroxy-4-methoxybenzoic acid (IVA, the internal standard), all from Sigma (St. Louis, MO, USA).

Preparation of the mobile phase included the use of deionized water (Millipore, Bedford, MA, USA), sodium acetate (Merck, Darmstadt, Germany), methanol p.a. (May&Baker, Dagenham, UK), *n*-octyl sodium sulphate (OSS) (Merck), acetic acid p.a. (May&Baker) and methylenediaminetetraacetic acid disodium salt p.a. (Na_2EDTA) (Merck). For the preparation of samples and standard solutions Na_2EDTA, sodium metabisulphite p.a. ($Na_2S_2O_5$) (Merck), 70% perchloric acid (PCA) p.a. (Merck) and hydrochloric acid p.a. (J. T. Baker, Phillipsburg, NJ, USA) were used.

Spherisorb ODS 2, 5 μm particle size (Phase Separations, Queensferry, UK), was used as column packing material.

Chromatography

The chromatographic system consisted of a Milton Roy (Riviera Beach, FL, USA) mini pump, equipped with a pulse dampener, an injection valve (Model 7125, Rheodyne, Berkeley, CA, USA) and a 100-μl loop, a precolumn (Upchurch Scientific, Oak Harbour, WA, USA) and a stainless-steel column (250 mm × 4.6 mm I.D.) packed with ODS 2, 5 μm particle size (Phase Separations). A Model LC-4A electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA), a TL-5A glassy carbon working electrode (Bioanalytical Systems) and an RE-1 Ag/AgCl reference electrode (Bioanalytical Systems) was used. The potential of the detector was set at +0.80 V and a sensitivity of 5–20 nA per 10 mV output was used. An SP 4290 integrator (Spectra-Physics, San José, CA, USA) was used to monitor the signals at a chart speed of 0.25 cm/min. The mobile phase contained 0.055 *M* sodium acetate, 8–8.5% methanol, 0.129 m*M* OSS, 4% acetic acid and 0.403 m*M* Na_2EDTA, and the pH was adjusted to 3.80. The buffer was then vacuum-filtered and degassed through a Type HA filter with 0.45-μm pores (Millipore). The mobile phase was pumped through the column at 1.0–1.25 ml/min at ambient temperature (21–22°C) and a pressure of 140 bar.

Sample preparation

Rat brain tissue was quickly removed, placed on an ice-chilled Petri dish (± 0°C) for dissection and stored in liquid nitrogen until analysed. After careful thawing in a refrigerator at 4°C, the brain tissue was homogenized with a Sonifer Type B-30 sonic disruptor (Branson Sonic Power, Danbury, CT, USA) in ice-chilled 0.1 *M* PCA containing 5.37 m*M* Na_2EDTA and 2.63 m*M* $Na_2S_2O_5$ at a ratio of 1:4 [brain weight (g)/extraction volume (ml)]. After centrifugation (10 000 *g*, 10 min, 4°C), 20 ng of internal standard (IVA) were added to 200 μl of the supernatant and 100 μl were injected into the loop.

Standard preparation

A stock solution of 500 μg/ml 5,6-DHT–creatinine sulphate in 0.1 *M* PCA containing 5.37 m*M* Na_2EDTA and 2.63 m*M* $Na_2S_2O_5$ was used. The 1 μg/ml internal standard (IVA) solution was obtained by diluting a stock solution of 500 μg/ml with 0.01 *M* hydrochloric acid.

RESULTS AND DISCUSSION

Extraction

Liquid extraction of the brain samples using PCA results in a complete recovery of indoleamines and catecholamines in the filtered supernatant. Both 5,6-DHT and 5,7-DHT molecules are highly reactive when added to biological tissues. In order to avoid spontaneous oxidation after tissue sampling, the brain tissue parts were immediately transferred to and stored in liquid nitrogen. In the prepared standard solutions, using $Na_2S_2O_5$ as an antioxidant in order to im-

prove stability, both 5,6-DHT and 5,7-DHT remained stable during the day. Without $Na_2S_2O_5$ there was a decrease in detector response of approximately 10% over 2 h (data not shown).

Separation and quantification

As shown in Figs. 1–4 the separation of 5,6-DHT from amines and indoles was complete. The retention time (t_R) for 5,6-DHT was approximately 9 min, and the compound was well separated from 5-HIAA (13.8 min), 5-HT (16.7 min) and HVA (20.0 min). The internal standard, IVA, appeared at 24 min. The retention times are considerably shorter than those reported in earlier published methods [9–11]. In addition, the indoles were well separated when using the present method (Figs. 1–4).

As shown in Fig. 5, it is also possible to assay 5,7-DHT with this technique. The t_R for 5,7-DHT was approximately 10.72 min, and, like 5,6-DHT, it was well separated from 5-HIAA, 5-HT

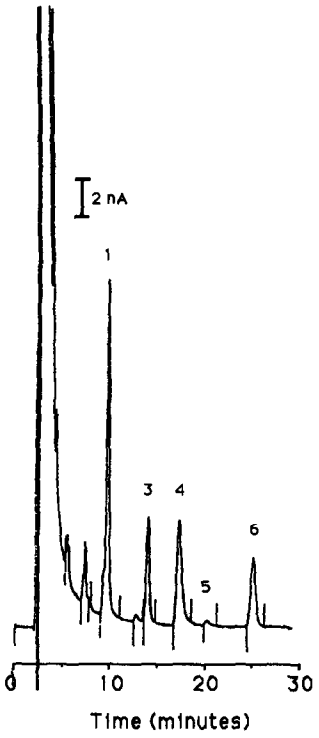

Fig. 2. Chromatogram of a rat brain stem treated with 5,6-DHT i.c.v. For peak identification, see Fig. 1.

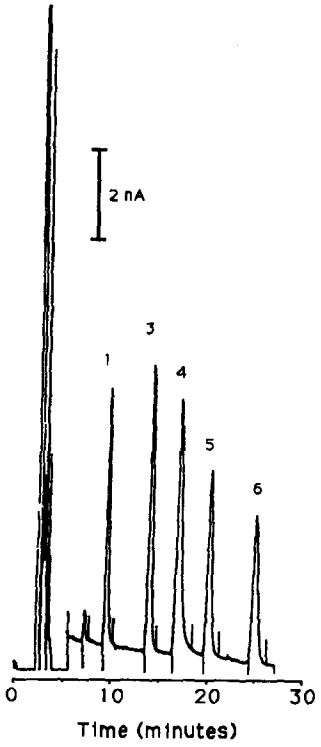

Fig. 1. Chromatogram of standard amines and indoles and the internal standard (IVA). Peaks: 1 = 5,6-DHT (t_R = 9.44 min); 3 = 5-HIAA (t_R = 13.81 min); 4 = 5-HT (t_R = 16.74 min); 5 = HVA (t_R = 20.01 min); 6 = IVA (t_R = 24.77 min).

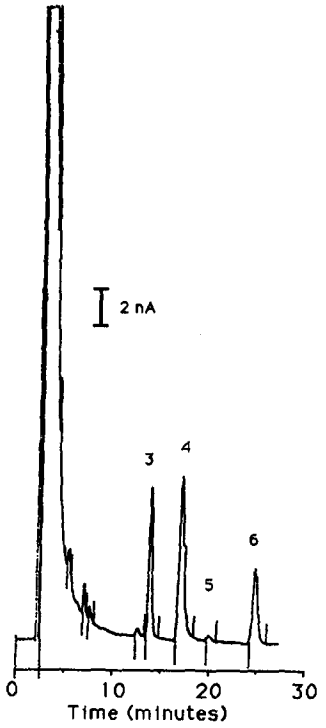

Fig. 3. Chromatogram of a rat brain treated with saline i.c.v. Internal standard (IVA) was added to the sample. For peak identification, see Fig. 1.

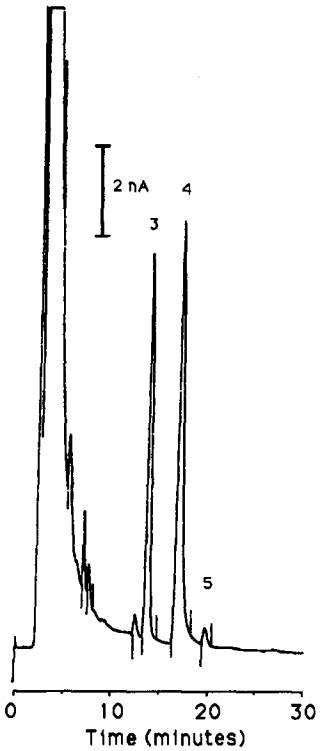

Fig. 4. Chromatogram of a rat brain treated with saline i.c.v., without internal standard. For peak identification, see Fig. 1.

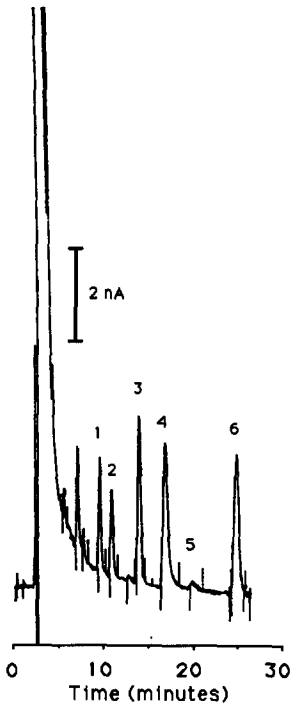

Fig. 5. Chromatogram of a spiked sample, with internal standard (IVA). Peaks 1 = 5,6-DHT (t_R = 9.37 min); 2 = 5,7-DHT (t_R = 10.72 min); 3 = 5-HIAA (t_R = 13.74 min); 4 = 5-HT (t_R = 16.58 min); 5 = HVA (t_R = 19.59 min); 6 = IVA (t_R = 24.56 min).

and HVA. The detection limit was 1 ng per 100 mg of tissue. After repeated injections of four different concentrations ranging from 0.25 to 15 ng 5,6-DHT, an r^2 of 0.997 was found.

Precision and repeatability

One rat was injected intracerebroventricularly (i.c.v.) with 10 μg of 5,6-DHT and decapitated 5 min later. The brain was dissected and assayed for 5,6-DHT. Repeated injections of this sample during the day resulted in a concentration of 80 ng/g and a relative standard deviation (R.S.D.) of 1.41% (n = 5). The concentrations of 5,6-DHT were calculated by comparing the 5,6-DHT/internal standard peak-height ratios of external standards and samples. Under optimal conditions and using commercially pure samples, the sensitivity of this method was 0.25 ng per 100-μl injection at a detector sensitivity of 20 nA per 10 mV output. When tissue samples are analysed, the detection limit has to be related to the sample volume, *i.e.* the extraction volume used. Thus, compared with previously published methods for 5,6-DHT [9,10], the present technique exhibits detection limits in tissue that are approximately 50–100 times better. The improved detection limit using our present method may be explained by the use of antioxidants during sample preparation and the use of a mobile phase different from those normally employed in indole assays [7,8,12,13]. The detection limit of 5,7-DHT determined by HPLC was essentially similar to that of 5,6-DHT (Fig. 5).

CONCLUSIONS

As the 5-HT neurotoxins 5,6-DHT and 5,7-DHT occur in brain tissue endogenously during certain pathological conditions or after intake of some psychotropic drugs, it is essential to have a highly sensitive method for detection and quantification of these compounds. The present analytical method emphasizes the need for a rigid sample handling, including freezing in liquid nitro-

gen, careful thawing and the use of antioxidants to prevent spontaneous degradation of 5,6-DHT and 5,7-DHT in the samples. With the present method, the detection of minute amounts of 5,6-DHT and 5,7-DHT in tissue samples has been considerably improved.

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