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Note

Simple high-performance liquid chromatographic method with electrochemical detection for the determination of indoleamines in tissue and plasma

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The serotoninergic transmitter system plays an important role in the control and regulation of many brain functions. The aim of the present study was the development of a simple and fast analytical method for the simultaneous determination of 5-hydroxytryptophan (5-HTP), 5-hydroxy-3-indoleacetic acid (5-HIAA) and 5-hydroxytryptamine (5-HT) in tissue samples. Recent studies also show alterations of the plasma serotonin level in acute migraine headache [1,2] and in melancholic (DSM III) patients [3]. This is the reason to devise a methodology that allows the determination of very low concentrations of 5-HT in human plasma. This paper describes a simple, fast and sensitive method developed for this purpose.

EXPERIMENTAL

Reagents

Chemicals were obtained from the following sources: 5-HT, 5-HIAA, 5-HTP, norepinephrine, dopamine, 4-hydroxy-3-methoxyphenylethylene glycol and 3,4-dihydroxyphenylacetic acid from Sigma (St. Louis, MO, U.S.A.), EDTA, sodium metabisulphite and methanol (HPLC grade) from Merck (Darmstadt, F.R.G.), ammonium hydroxide from Farmitalia Carlo Erba (Milan, Italy) and acetic acid from Scharlau (Barcelona, Spain). Water was purified using a Milli Q system.

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Apparatus

A Model 420 solvent-delivery pump from Kontron (Zürich, Switzerland) was used in conjunction with a TL-5A glassy carbon electrode and LC-4B controller, both from Bioanalytical Systems (West Lafayette, IN, U.S.A.). A six-port rotary valve (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl sample loop was used for sample injection. Chromatographic separations were performed using a 15 cm $\times$ 0.4 mm I.D., 5-μm Spherisorb ODS 1 column (Tracer Analitica, Barcelona, Spain).

Chromatography

The mobile phase was a mixture of 0.3 *M* acetic acid, 0.08 *M* ammonium hydroxide, 0.1 m*M* EDTA and 15% methanol (v/v); the apparatus pH was adjusted to 4.5. The mobile phase was filtered through a 0.45-μm filter (GVWP, Millipore) and degassed under vacuum with magnetic agitation. All separations were performed isocratically at a flow-rate of 0.9 ml/min at room temperature.

Standards and calculations

Stock solutions of the external standards were prepared in 0.4 *M* PCA (perchloric acid) and 0.4 m*M* sodium metabisulphite at a concentration of 100 μg/ml, and stored at 4°C. Working solutions (10 ng/ml) were prepared daily by a 1:10 000 dilution of stock solutions in the mobile phase. In order to test the linearity of the detector signal, a series of six standards was diluted from the stock solution containing 9.3–250 pg per injected volume. The tissue and plasma levels of the substances were calculated by comparing the heights of the peaks in the sample with those in the standard solution of known content. The recovery obtained for 5-HT in plasma samples ranged from 98 to 102%.

Sample preparation

Plasma samples. Human volunteer blood was taken by anterocubital vein puncture using a cannula. The first 0.5 ml was discarded, and blood was collected in a plastic tube containing 200 μl of 10% EDTA. The sample used to obtain platelet-free plasma (PFP) was centrifuged (11 000 *g*, 5 min), and a 1-ml aliquot of the supernatant was deproteinized with 60% PCA and centrifuged (6000 *g*, 5 min). An aliquot was taken from the supernatant and diluted 1:10 with the mobile phase. Platelet-rich plasma (PRP) was centrifuged (90 *g*, 10 min) and subsequently processed in the same way as the PFP samples.

Tissue samples. Male Wistar rats (300 $\pm$ 30 g) were sacrified by decapitation, and the brains were rapidly removed. Hypothalamic regions, hippocampus and pineal gland were dissected and homogenized in 0.75–1.00 ml of mobile phase. Brain samples were centrifuged (2500 *g*, 5 min). Tissue and plasma samples were filtered through a 0.22-μm filter (HAFT 01300, Millipore), and subsequently 20 μl were injected.

RESULTS AND DISCUSSION

Chromatographic conditions

Alterations in the nature and relative concentrations of the mobile phase components allowed the selectivity to be optimized and the retention times to be minimized. The effect of the mobile phase composition on the capacity factors of the dopaminergic and noradrenergic systems was studied to prevent the coelution of compounds that could interfere with the serotoninergic compounds under study. Thus 5-HTP, 5-HT and 5-HIAA were well resolved with a mobile phase of pH 4.5 containing 15% methanol (v/v) in less than 7 min. The detector was set to +0.5 or +0.45 V to analyse tissue and plasma samples, respectively. In plasma samples, we were interested only in the serotonin determination. This compound was totally oxidized at the selected detection potential.

Sensitivity and within-assay reproductibility

The peaks were first identified by comparison of their retention times with those of the standards under different chromatographic conditions. The limit of detection of 5-HT calculated at a signal-to-noise ratio of 2:1 was 6 pg. The within-assay precision was determined by processing and assaying ten 20-μl aliquots of a sample obtained by homogenizing a single rat hypothalamus. The estimated coefficients of variation ranged from 2.8% (5-HTP) to 3.3% (5-HIAA).

Quantitative analysis

Fig. 1 shows chromatograms of a standard solution and of small regions of rat brain. Under our experimental conditions, 5-HTP was not detected in rat hippocampus samples; however, it was detected in the brain hypothalamic regions. Fig. 2 show chromatograms of serotonin determination in PRP and PFP samples. The values obtained for PRP (774 ± 19 ng/ml, $n=30$) are in agreement with those of other authors [2,4–7]. The values found for PFP (1.87 ± 0.66 ng/ml, $n=8$) are lower than those previously reported [7,8]. However, they are in agreement with the more recent publications [4,9]. Artigas et al. [4] suggested that the higher levels reported earlier were caused by artefacts. Nevertheless, like the procedure without prior extraction into an organic phase developed by Anderson et al. [10], our technique offers an advantage with respect to others [4,10,11] in that sample extraction is not necessary.

In summary, the present method permits a simultaneous determination of 5-HT and its deaminated metabolite (5-HIAA), as well as 5-HTP (the immediate precursor of serotonin), from samples of small regions of the brain without prior purification. Moreover, the determination of 5-HT in PRP and PFP human samples is possible under the same conditions.

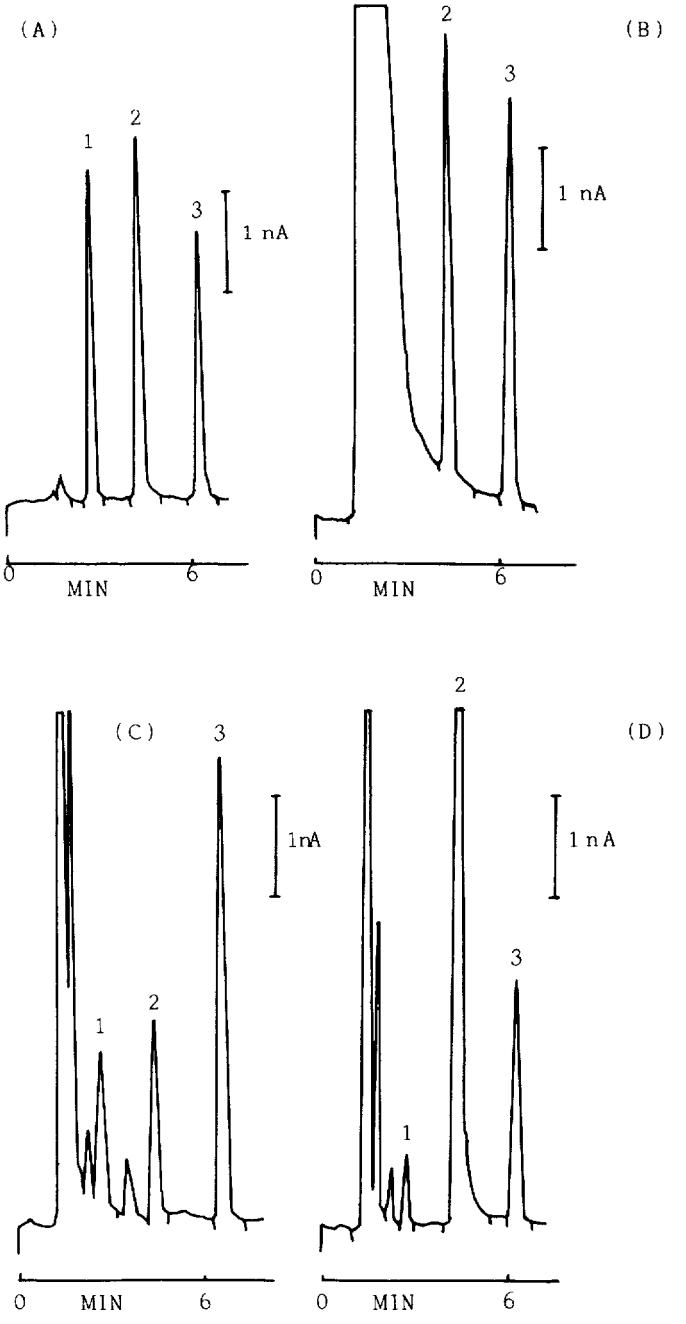

Fig. 1. (A) Chromatogram of a standard solution; 10 ng/ml of each standard dissolved in the mobile phase; (B) chromatogram of hippocampus sample; (C) chromatogram of hypothalamus sample; (D) chromatogram of pineal gland sample. Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = 5-HIAA. Mobile phase, ammonium acetate (pH 4.5) with 15% methanol (v/v); detector potential, 0.5 V vs. Ag/AgCl.

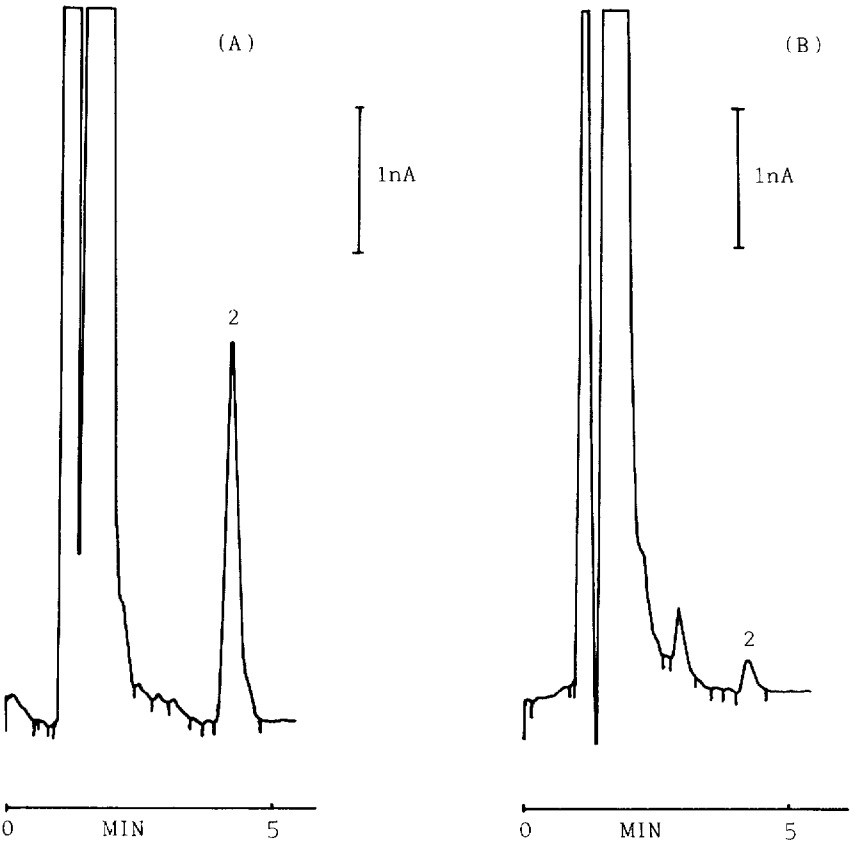

Fig. 2. Chromatograms of (A) human PRP sample and (B) human PFP sample. Detector potential, 0.45 V vs. Ag/AgCl; other conditions as in Fig. 1.

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