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Note

Determination of urinary serotonin using liquid chromatography with electrochemical detection

J. JOUVE

Laboratoire de Biochimie Médicale, CHU Bretonneau, 37044 Tours Cédex (France)

J. MARTINEAU

Laboratoire de Neurophysiologie et de Thérapeutique en Pédopsychiatrie, CHU Bretonneau, 37044 Tours Cédex (France)

N. MARIOTTE

Laboratoire de Biochimie Médicale, CHU Bretonneau, 37044 Tours Cédex (France)

C. BARTHELEMY

Laboratoire de Neurophysiologie et de Thérapeutique en Pédopsychiatrie, CHU Bretonneau, 37044 Tours Cédex (France)

J.P. MUH*

Laboratoire de Biochimie Médicale, CHU Bretonneau, 37044 Tours Cedex (France)

and

G. LELORD

Laboratoire de Neurophysiologie et de Thérapeutique en Pédopsychiatrie, CHU Bretonneau, 37044 Tours Cedex (France)

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High-performance liquid chromatography coupled with electrochemical detection (HPLC—ED) is a technique of increasing importance in neurochemical research. Serotonin (5-HT)-containing neurons play an important role in a variety of physiological functions (sleep regulation and sexual behaviour) and pathological states (psychiatric disorders, depression, mental retardation, infantile autism).

Various analytical methods have been employed to measure 5-HT in biological tissues and fluids, including thin-layer chromatography [1],

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fluorimetry [2, 3], ultraviolet spectrophotometry [4], gas chromatography—mass spectrometry (GC—MS) [5, 6], radioenzymatic assay [7, 8] and radioimmunoassay [9]. These methods either lack selectivity and/or sensitivity or they are too expensive or time-consuming for routine analyses.

Recently, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [10, 11], fluorimetric detection [12] or combined fluorimetric—electrochemical detection [13, 14] has been applied to the analysis of indoles. However, these methods require extensive sample pretreatment, analysis times are rather long, and the compounds of interest are not completely separated from each other or from interfering compounds.

We have developed a method for the measurement of urinary 5-HT, using reversed-phase HPLC—ED. This method is rapid, sensitive, easy to perform and requires only rapid prepurification of indolic compounds. The small gravity-fed liquid—solid extraction columns used for the clean-up step decrease the overall time required for the assay and afford improved selectivity.

EXPERIMENTAL

Apparatus

The analyses were performed using a Hewlett-Packard 1080 A chromatograph (Hewlett-Packard, King of Prussia, PA, U.S.A.). The electrochemical detector (Tacussel Systems) consisted of a Faraday cage enclosing a Tacussel DELC cell with a glassy carbon working electrode, a platinum auxiliary electrode, an Ag/AgCl reference electrode and a polarographic analyser Tacussel PRG-DEL (Tacussel Lyon, Villeurbanne, France). The analytical column was an Ultrasphere ODS (5 μm particle size; 250 mm × 4.6 mm I.D.) from Beckman (Berkeley, CA, U.S.A.), protected by a guard column of μBondapak C_{18}—Corasil (30 × 3.9 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.). Peak areas were measured using a Hewlett-Packard 79850 A LC terminal (Hewlett-Packard).

Reagents and chemicals

The standard 5-hydroxytryptamine creatinine sulphate and internal standard 5-hydroxymethyltryptamine oxalate (5-HMT) were purchased from Aldrich (Beerse, Belgium). Stock solutions of reference compounds and the internal standard (100 μg/ml) were prepared in hydrochloric acid (150 mmol/l) and stored in amber bottles at 4° C. All concentrations are reported as the free base. All other chemicals were of analytical-reagent grade and were obtained from Merck (Darmstadt, F.R.G.). Methanol for HPLC was obtained from Carlo Erba (Milan, Italy). The cation-exchange resin was Amberlite CG-50 (H^+), 200—400 mesh, from Fluka (Buchs, Switzerland). The resin Amberlite CG-50 was first washed repeatedly with glass-distilled water, then regenerated by washing successively with 4 *M* hydrochloric acid, 2 *M* sodium hydroxide, 4 *M* hydrochloric acid and water, between each solvent. Finally, it was equilibrated with 0.1 *M* hydrochloric acid. The resin was poured into a polypropylene column (4 cm × 0.8 cm I.D.) with an integral reservoir (10 ml) (Bio-Rad Labs., Richmond, CA, U.S.A.).

Chromatographic conditions

All separations were performed isocratically at room temperature with a flow-rate of 1.0 ml/min. The potential of the working electrode was maintained at + 0.6 V vs. Ag/AgCl. The mobile phase was a modified version of that by Koch and Kissinger [10]. It consisted of methanol—0.5 *M* ammonium acetate pH 5.1, (5:95), prepared in deionized water (Continental Waters Systems, El Paso, TX, U.S.A.). It was deaerated under vacuum at room temperature for a minimum of 1 h before use.

Preparation of ion-exchange columns

Micro isolation columns were prepared from polypropylene columns using

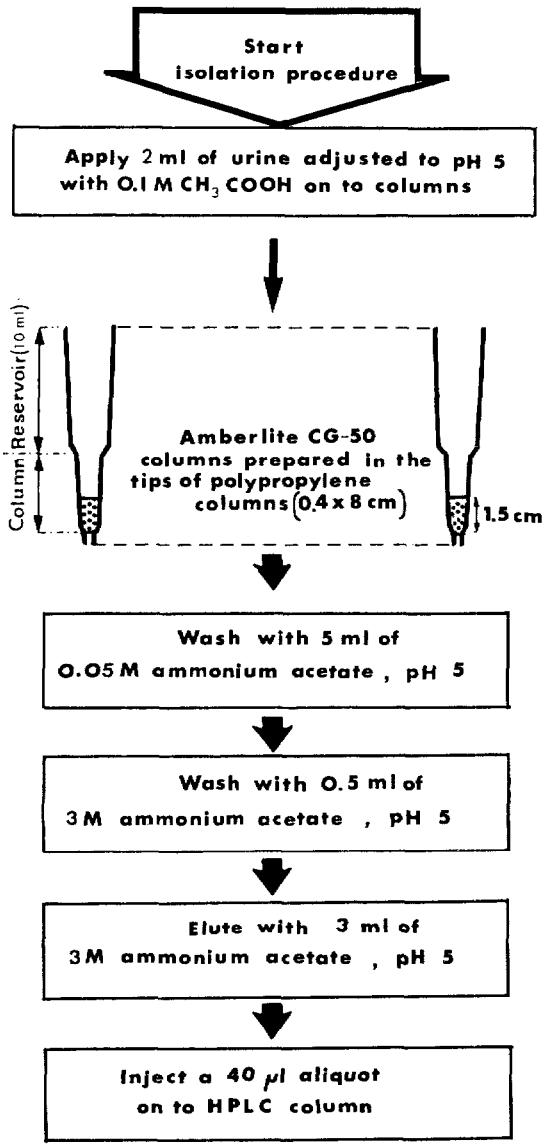

Fig. 1. Flow chart for serotonin isolation.

a slurry of Amberlite CG-50 [15, 16] in the H^+ form, equilibrated in 0.1 *M* hydrochloric acid. The bulbs of the columns were cut off, the tips packed to a height of 1.5 cm and a 4-ml aliquot of 0.2 *M* phosphate buffer (pH 6.5) was passed through each column. The isolation process is depicted in Fig. 1.

Sample preparation, storage and isolation procedure

Night urine specimens (12-h) were preserved with sodium metabisulphite (1 g/l) and stored at −60°C as suggested by Petrucelli et al. [16]. Just before the analysis, the urine samples were brought to room temperature and centrifuged at 1500 *g* (15 min, 4°C). A 2-ml aliquot was adjusted to pH 5 with 0.1 *M* acetic acid, to which 400 ng of 5-hydroxymethyltryptamine (internal standard) were added and the mixture was applied to the Amberlite CG-50 gravity-fed isolation column. The column was washed with 5.0 ml of 0.05 *M* ammonium acetate (pH 5) and 0.5 ml of 3 *M* ammonium acetate (pH 5), of which 40 μl were injected onto the liquid chromatograph for determination of serotonin. A standard (2 ml of 0.1 *M* ammonium acetate, pH 5, containing 400 ng of 5-HT) was extracted along with the urine using the same amount of internal standard.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

Optimal separation of 5-HT was achieved using 0.5 *M* ammonium acetate containing 5% methanol. The separation of 5-HT on the C_{18} reversed-phase column depended on the pH and the amount of methanol in the mobile phase. The mobile phase was delivered at 1.0 ml/min. The electrode potential of +0.6 V vs. Ag/AgCl provided sufficient sensitivity for the determination of picogram quantities of 5-HT with minimal interference from solvent effects and electrical noise.

Fig. 2 shows that, under the conditions outlined above, 5-HT is well resolved from the internal standard and the endogenous urinary compounds.

A constant column temperature (25°C) was found to be necessary to obtain reproducible results.

Quantification of serotonin concentrations

The concentrations of 5-HT in each sample were calculated by determining peak-area ratios relative to 5-HMT and comparing them with those obtained with synthetic standards prepared in 0.1 *M* ammonium acetate (pH 5).

Linear range and limits of detection

The linearity of both the extraction procedure and detector response (determined from peak areas) was verified for 5-HT over the anticipated concentration range of the assay. The former was investigated by assaying pooled urine to which known amounts of serotonin had been added. A linear relationship between 5-HT concentration and peak area was observed over the concentration range studied (0—100 pmol). The equation for the calibration curve was $y = 0.043x - 0.006$ and the coefficient of correlation was $r = 0.99983$. The detection limit (signal-to-noise ratio of 2) was 5.625 pmol/ml, which is well below the concentration levels encountered in human urine specimens.

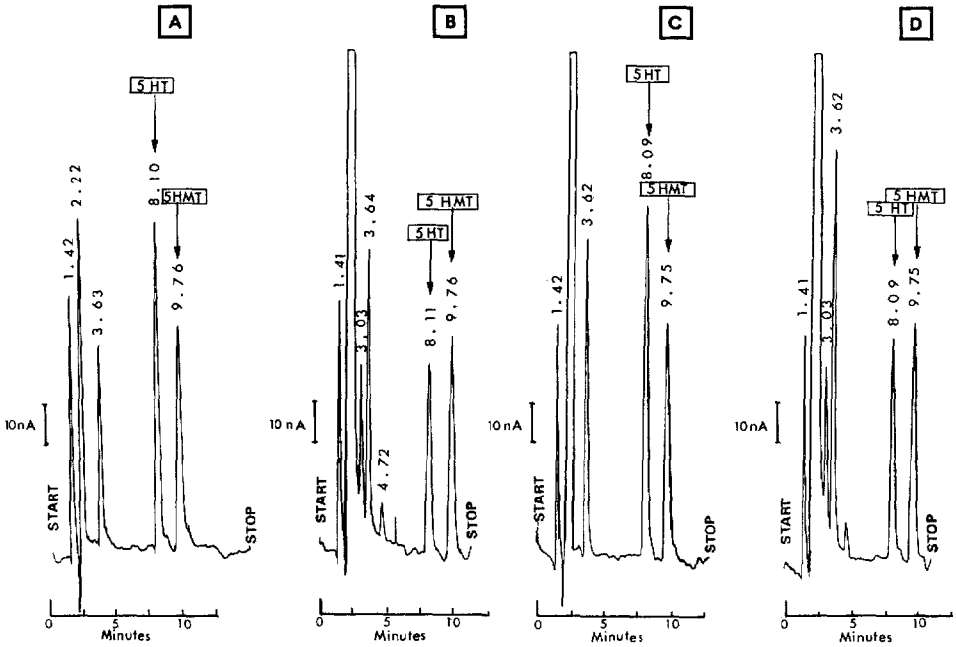

Fig. 2. (A) Chromatogram of a standard mixture of 37.49 pmol of serotonin (5HT) and 37.03 pmol of 5-hydroxymethyltryptamine (5HMT, internal standard). (B) Chromatogram of a urine sample from a seven-year-old normal child. (C) Chromatogram of a urine sample from a seven-year-old mentally retarded child. (D) Chromatogram of a urine sample from a seven-year-old autistic child.

Precision and relative recovery

The reproducibility of the method was evaluated from multiple analyses of pooled urine containing 200 ng/ml 5-HT. The within-day and between-day coefficients of variation determined from six measurements were 5.3 and 11.9%, respectively. The recovery was 86 ± 2% (mean ± S.D., n = 5) at the concentrations given above. Using the internal standard, all values approached 95%.

Reference values

Urine specimens (12-h) were obtained from 25 healthy children in age groups of 1—3, 3—5, 5—7, 7—9 and 9—13 years. They were asked to follow dietary conditions based on ingestion of moderate amounts of animal proteins (eggs, meat, fish), white bread and rice, while avoiding vegetables and fruits (bananas, pineapples, grapefruits, walnuts, plums, eggplants, tomatoes, avocados). Medications were suppressed. The restricted diet was followed for two days, and the 12-h urine samples were collected only during the second night of dietary restriction. A typical chromatogram of a urine sample from a normal child is illustrated in Fig. 2. The reference values presented in Table I are given in nmol/mmol of creatinine. Creatinine was assayed by automated colorimetry using a creatinine analyser Model 2 from Beckman. This method makes use of the Jaffé reaction. The mean excretion was found to be decreased between the ages of 1 and 13 years.

TABLE I

URINARY SEROTONIN CONCENTRATIONS IN 25 NORMAL CHILDREN, 25 AUTISTIC CHILDREN AND 19 MENTALLY RETARDED CHILDREN

Age group (years)	Concentration (mean ± S.D.) (nmol/mmol of creatinine)		
	Normal children	Autistic children	Mentally retarded children
1—3	196 ± 73 (n = 7)	155 ± 7 (n = 6)	252 ± 23 (n = 3)
3—5	145 ± 19 (n = 5)	145 ± 14 (n = 5)	157 ± 17 (n = 7)
5—7	92 ± 19 (n = 3)	119 ± 6 (n = 5)	130 ± 14 (n = 7)
7—9	78 ± 19 (n = 3)	101 ± 7 (n = 6)	133 (n = 1)
9—13	75 ± 16 (n = 7)	100 ± 16 (n = 3)	140 (n = 1)

Pathology

Twenty-five urine specimens from autistic children and nineteen urine specimens from mentally retarded children from age groups of 1—3, 3—5, 5—7, 7—9 and 9—13 years, without any other pathology susceptible to modify 5-HT metabolism, and all on a normal diet, were analysed (Table I). The results of the longitidinal study for the age groups between 1 and 13 years demonstrate that the decrease of 5-HT was more pronounced in the controls (2.6 times) than in the autistic or mentally retarded children. For autistic children, 5-HT decreased by a factor of 1.5 between 1 and 13 years; for mentally retarded children, the decrease was 1.8 times. These results confirm that the decrease of 5-HT excretion is related to cerebral maturation and mental development.

CONCLUSION

The described method of analysis for urinary serotonin is simple and rapid. HPLC—ED possesses the advantage of speed of sample processing (individual samples may be processed in less than 10 min). Furthermore, the extraction procedure on Amberlite CG-50 columns can be carried out in a short period of time. The chromatograms revealed practically no extraneous peaks. This method is suitable for psychiatric research applications and routine clinical analysis. Although the use of this technique was illustrated with the determination of urinary 5-HT, it is currently being extended to the quantification of 5-HT in whole blood and brain tissue samples.

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