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DETERMINATION OF 5-HYDROXYTRYPTOPHAN, SEROTONIN AND 5-HYDROXYINDOLEACETIC ACID IN RAT AND HUMAN BRAIN AND BIOLOGICAL FLUIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid and sensitive method for the concurrent determination of 5-hydroxytryptophan, serotonin and 5-hydroxyindole acetic acid by reversed-phase high-performance liquid chromatography with electrochemical detection has been developed. The separation of the indolic compounds was achieved using a phosphate–citric acid eluent containing 5% methanol. Detection limits in the low picogram range were found. The method has been applied to the determination of the indolic compounds in rat and human brain tissues, as well as in human plasma and cerebrospinal fluid. Tissue and plasma preparation required only deproteinization before chromatography, while cerebrospinal fluid was directly applied to the column.

INTRODUCTION

Owing to the role played by serotonin (5-HT)-containing neurons in a variety of physiological functions (*e.g.*, sleep regulation and sexual behaviour) and pathological states (*e.g.*, depression), a large number of methods for the determination of serotonin, its amino acid precursors and metabolites in biological samples have been developed over the past two decades. Fluorometric[1,2], gas chromatographic–mass spectrometric (GC–MS)[3,4], radioenzymatic[5,6] and radioimmunological[7] methods have been successfully employed to measure indolic compounds in brain and biological fluids. However, these techniques either lack adequate sensitivity (*e.g.*, fluorometry) or are expensive, time consuming and require complicated extraction and derivatization procedures (*e.g.*, GC–MS). Moreover, they do not allow the simultaneous detection and quantitation of indolic compounds.

Recently, high-performance liquid chromatography (HPLC) with fluorescence

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or electrochemical detection has been successfully applied to the determination of 5-HT and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) in biological samples (see refs. 8–10). However, most of these methods required ion-exchange sample prepurification prior to the HPLC analysis and did not allow the simultaneous determination of serotonin and its amino acid precursors and metabolites.

We have developed a method for the concurrent measurement of 5-HT, 5-hydroxytryptophan (5-HTP) and 5-HIAA using reversed-phase HPLC with electrochemical detection which is rapid, sensitive, easy to perform and which does not require prepurification of the indolic compounds. This method has been applied to the determination of indolic compounds in rat and human brain areas as well as in human cerebrospinal fluid (CSF) and plasma.

MATERIALS AND METHODS

Reagents and solvents

The standards 5-HT creatinine sulphate, 5-HIAA, 5-HTP, homovanillic acid (HVA), 3,4-dihydroxyphenylethylene glycol (DOPEG), dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylalanine (DOPA), 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG), norepinephrine hydrochloride (NE), epinephrine (E) and dopamine (DA) were purchased from Sigma (St. Louis, MO, U.S.A.) and the aromatic L-amino acid decarboxylase inhibitor, *m*-hydroxybenzylhydrazine (NSD 1015), from Aldrich (Beerse, Belgium). Standard solutions (100 μg/ml) were prepared in distilled water and stored at 4°C (stock solutions). They were diluted to appropriate concentrations in the eluent immediately before use. NSD 1015 was dissolved in a few drops of 1 *M* hydrochloric acid and made up to the desired volume with water.

Water with resistivity greater than 10 MΩ/cm was obtained by double distillation. Methanol, spectroscopic grade, was from Rhône-Poulenc (France). All other chemicals were reagent grade from E. Merck (Darmstadt, G.F.R.). The mobile phase consisted of a 0.1 *M* K_2HPO_4–citric acid buffer (1:0.60, v/v; pH 4.7) containing 5% methanol and the disodium salt of ethylenediaminetetraacetic acid (EDTA) (0.1 m*M*). The solvent mixture was filtered under vacuum through a 0.22-μm Millipore GS type filter before use. The temperature of the solvent mixture was maintained at 30°C by use of a constant-temperature water-bath.

Chromatography

Chromatography was performed using a Micromeritics (Model 750) pump, an automatic sample injection system WISP (Model 710 B, Waters Assoc.) and a stainless-steel column (15 cm × 4.6 mm I.D.) packed with C_{18} reversed-phase, Spherisorb ODS (5 μm). The flow-rate was usually 1 ml/min and the column was operated at 30°C. Electrochemical detection was accomplished using a Model LC-4 amperometric detector from Bio-analytical Systems (West Lafayette, IN, U.S.A.) and a carbon paste (silicone oil/graphite) electrode maintained at +0.6 V *versus* a silver–silver chloride reference electrode. The signal was recorded and the peak areas determined with a SP 4100 computing integrator from Spectra-Physics (Santa Clara, CA, U.S.A.). Quantitations were made automatically by comparison of the peak areas of the samples with those given by known concentrations of standards.

Sample preparation

Rat brain samples. Male Sprague Dawley rats (COBS CD strain; Charles River, France) weighing 140–160 g were used. They were maintained on a 12-h light/dark cycle with free access to food and water.

Animals were sacrificed by decapitation and the brain was immediately removed. Brain regions were dissected out in the cold, immediately frozen on solid carbon dioxide and kept at $-80°C$ until analysis. For 5-HT and 5-HIAA measurements, tissues were homogenized in 0.1 *M* perchloric acid (1:10 w/v); after centrifugation, 100 μl of the supernatant were applied to the HPLC column. For 5-HTP measurements, rats received NSD 1015 (100 mg/kg i.p.) and were sacrificed 30 min thereafter. The tissues were homogenized in ten volumes of 0.1 *M* perchloric acid and after centrifugation (28,000 *g* for 10 min) the supernatant was directly applied to the HPLC column. In some cases, catechol compounds were removed as follows: the supernatant was adjusted to pH 8.4 with 2.8 *M* Tris (pH 9.6) and 50 mg of acid-washed alumina were added. The vials were shaken for 10 min, centrifuged and 150 μl of the supernatants were then transferred into minivials of the automatic injector for 5-HTP determination.

Human brain samples. Post-mortem human brain samples were collected from subjects with no history of psychiatric or neurological disorders, then frozen and dissected as described by Gaspar *et al.*[11]. Tissues were homogenized in ten volumes of 0.1 *M* perchloric acid and centrifuged; 100 μl of the supernatant were used for chromatographic analysis.

Plasma samples. Plasma samples (500 μl) were deproteinized by adding 10 μl of 70% perchloric acid. After centrifugation, 50 μl of the supernatant were removed and diluted twelve-fold in the running buffer before chromatographic analysis (injection volume 50 μl).

CSF samples. Human CSF was obtained via lumbar puncture from schizophrenic patients and immediately stored at $-80°C$ in polypropylene tubes. Chromatographic analysis was performed by direct injection of 100 μl CSF.

Recoveries

Recovery of 5-HT and 5-HIAA was determined by adding a known amount of the indolic compounds to duplicate samples of homogenates. Recovery of 5-HTP was estimated by adding 1 nCi (brain tissue) or 5 nCi (plasma) of [U-^{3}H]-L-5-HTP (specific activity 12.1 Ci/mmole, New England Nuclear) to the samples prior to deproteinization. Radioactivity was measured by liquid scintillation. The 5-HTP recoveries (mean $\pm$ S.E.M.) were 85 $\pm$ 1 and 78 $\pm$ 2% for brain tissue and plasma samples, respectively.

RESULTS AND DISCUSSION

Chromatographic conditions

The optimal separation of 5-HT, 5-HTP and 5-HIAA was achieved using a K_2HPO_4–citric acid (0.1 *M*, pH 4.7) buffer containing 5% methanol and 0.1 m*M* EDTA.

The separation of indolic compounds on the C_{18} reversed-phase column depended on the pH, the ionic strength and the amount of methanol in the mobile phase. As expected from the pK_a of 5-HIAA (4.7), the retention time of the acid

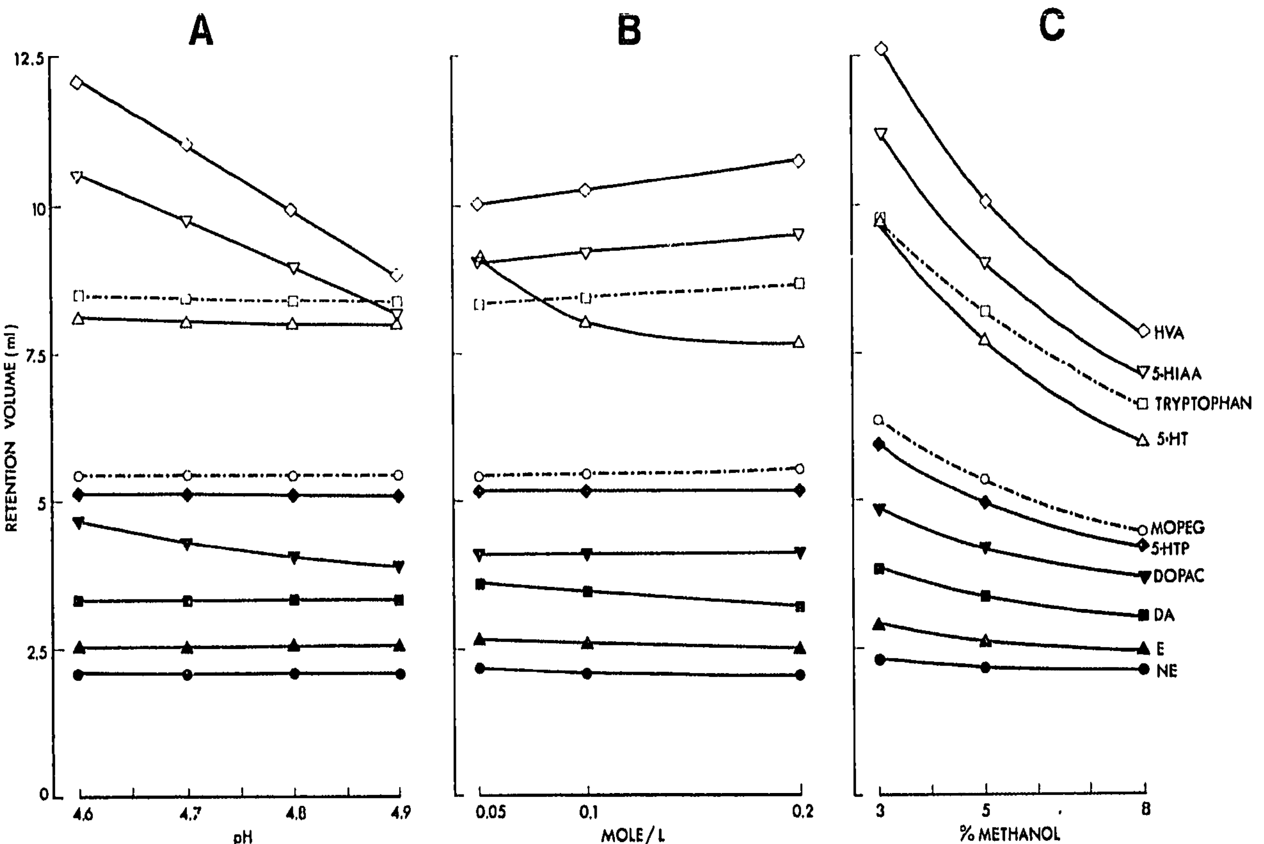

Fig. 1. Relationship between retention time and pH (A), ionic strength (B) and methanol concentration (C). Stationary phase: C_{18} Spherisorb ODS (5 μm); Flow-rate 1 ml/min; temperature 30°C. Electrode potential: +0.6 V (straight line); +0.88 V (dotted line). Mobile phase: K_2HPO_4–citrate containing methanol and 0.1 m*M* EDTA; ionic strength 0.1 *M*, 5% methanol (A); pH 4.7, 5% methanol (B); ionic strength 0.1 *M*, pH 4.7 (C).

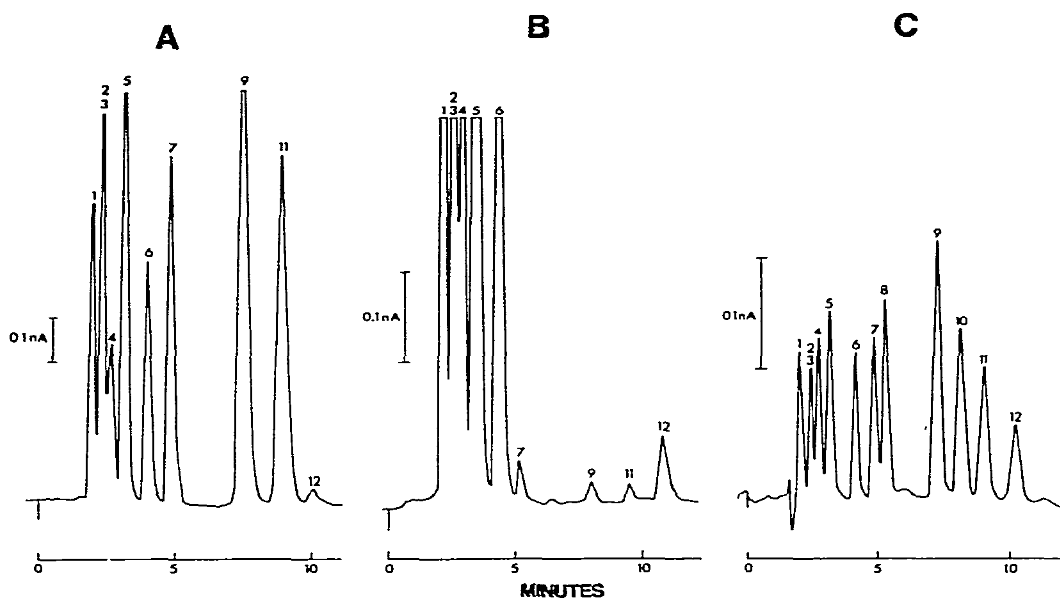

Fig. 2. Chromatogram of a standard mixture of indolic compounds and catechol derivatives. Peaks: 1 = NE; 2 = E; 3 = DOPA; 4 = DOPEG; 5 = DA; 6 = DOPAC; 7 = 5-HTP; 8 = MOPEG; 9 = 5-HT; 10 = tryptophan; 11 = 5-HIAA; 12 = HVA. Chromatographic conditions; column C_{18} Spherisorb ODS (5 μm); flow-rate 1 ml/min; temperature 30°C. A and B, Eluent 0.1 *M* K_2HPO_4–citrate, pH 4.73, containing 5% methanol and 0.1 m*M* EDTA; electrode potential +0.6 V. C, eluent 0.2 *M* K_2HPO_4–citrate, pH 4.73, containing 5% methanol and 0.1 m*M* EDTA; electrode potential +0.88 V. Traces A and C correspond to 20 ng of compound 10 and 1 ng of the other compounds. B corresponds to 2.5 ng of compounds 1, 2, 3, 4, 5, 6, 8 and 12, 50 pg of 7, 15 pg of 9 and 25 pg of 11.

metabolite on the column increased with decreasing pH (Fig. 1A). In contrast, the retention times of 5-HT and 5-HTP were independent of the pH of the eluent in the range 4.6–5, probably due to the fact that in this pH range the amino groups are completely protonated. An increase in the ionic strength of the running buffer decreased the retention time of 5-HT but left unaffected those of 5-HTP and 5-HIAA (Fig. 1B). As indoles are relatively non-polar compounds, the addition of methanol to the buffer was necessary to reduce elution times. In our system, 5% methanol was found to be sufficient to achieve adequate resolution of the indolic compounds without unduly prolonging the retention of HVA, the last compound to be eluted (Fig. 1C). Addition of a small amount of EDTA, which strongly complexes metal ions, improved the baseline stability.

Fig. 2A shows that, under the conditions outlined above, the three indolic compounds (1 ng of each) could be resolved within less than 10 min. At a flow-rate of 1 ml/min the retention times for 5-HTP, 5-HT and 5-HIAA were 4.8, 7.6 and 8.9 min, respectively. A constant column temperature (30°C) was found to be necessary in order to obtain constant retention times. The detector response was linear over the range 0.01–50 ng for each of the indoles. The detection limits (signal-to-noise ratio of 2) were found to be 15, 10 and 20 pg for 5-HTP, 5-HT and 5-HIAA, respectively (see Fig. 2B), which are comparable to or even better than those obtained with existing GC–MS[3,4], HPLC with fluorescence[12–14] or electrochemical[10,15–19] detection or

radioenzymatic techniques[5,6]. No variation of the detector response was found over (at least) 24 h, making unnecessary the use of internal standards.

The chromatographic behaviour of a variety of monoamines and their metabolites which may coexist with the indoles in brain tissue or biological fluids has also been examined (Figs. 1 and 2). A large number of these metabolites could be resolved with the present system (Fig. 2). However, DOPEG, NE, E and DOPA eluted close to the solvent front and could not be reliably assessed. As expected from its oxidation potential (+0.88 V), tryptophan was not detectable at the usual working potential (Fig. 2A). However, it could easily be detected using a higher potential (Fig. 2C), although the detection limits were worse than for the other indolic compounds due to the increase in baseline noise. Also, at this potential, tryptophan was found to slightly interfere with the detection of 5-HT, but this could easily be resolved by increasing the ionic strength of the running buffer to 0.2 M (Figs. 1B and 2C). An increase in the oxidation potential resulted in an increase in the electrochemical response and improved the detection limits for HVA and MOPEG (compare Fig. 2A and C). This was expected as O-methylated catechols are known to be less susceptible to oxidation than free catechols.

Applications

The chromatographic conditions described have been applied to the determination of 5-HT, 5-HTP and 5-HIAA in rat and human brain tissue and human lumbar CSF and plasma with only minimal sample pre-treatment. Representative chromatograms are shown in Fig. 3.

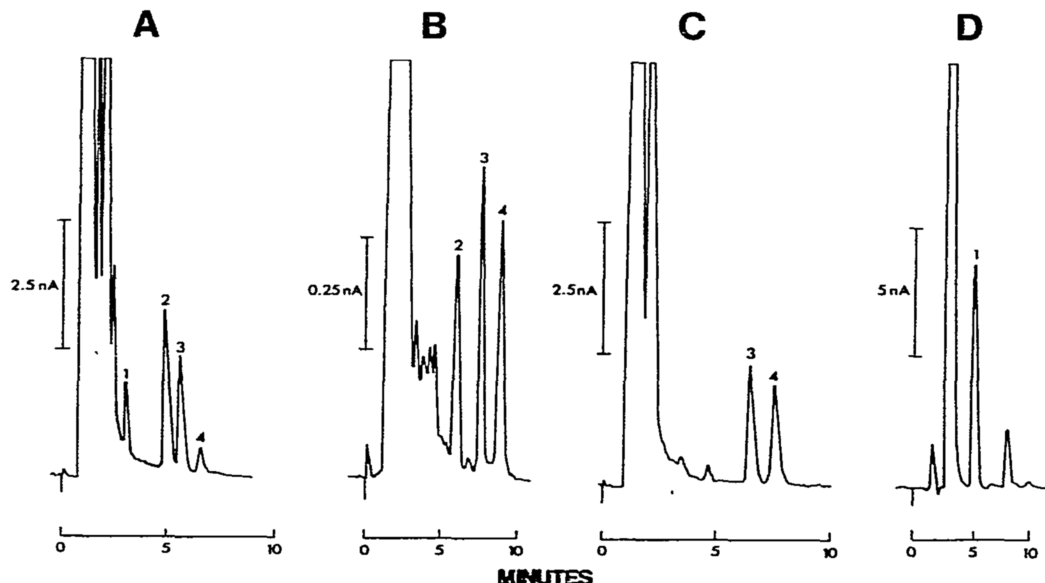

Fig. 3. Chromatograms from rat striatum (after injection of NSD 1015) (A), human hippocampus (B), human CSF (C) and human plasma (D). Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = 5-HIAA; 4 = HVA. Chromatographic conditions: column C_{18} Spherisorb ODS (5 μm); flow-rate 1.5 ml/min (A and C), 1 ml/min (B and D); temperature 30°C. Eluent: 0.1 M K_2HPO_4–citrate, pH 4.7, containing 5% methanol and 0.1 mM EDTA. Electrode potential: +0.6 V.

TABLE I

5-HT AND 5-HIAA LEVELS IN HUMAN BRAIN

Values are means ± S.E.M. for 6–12 determinations. N.D. = Not detectable.

Brain area	*5-HT (ng/g)*	*5-HIAA (ng/g)*
Frontal cortex	N.D.	104 ± 16
Cingulate cortex	52 ± 6	275 ± 30
Entorhinal cortex	53 ± 8	171 ± 42
Hippocampus	55 ± 8	197 ± 43
Caudate nucleus	624 ± 62	410 ± 83

5-HT and 5-HIAA in brain tissues and CSF. For these determinations, tissue preparation required only homogenization in 0.1 *M* perchloric acid followed by centrifugation prior to application, while CSF samples were directly applied to the HPLC column.

The concentrations of 5-HT and 5-HIAA found in discrete regions of human brain (Table I) using this method are in agreement with those recently reported by other investigators[16]. 5-HIAA levels measured in the lumbar CSF of schizophrenic patients not treated with neuroleptics (16.7 ± 2.0 ng/ml, n = 6) are also in the range previously reported[20]. Under the same experimental conditions, HVA could also be simultaneously quantitated (21 ± 5 ng/ml, n = 6).

5-HTP in rat brain tissues and human plasma. 5-HT is formed in the brain via hydroxylation of tryptophan into 5-HTP by tryptophan hydroxylase and subsequent decarboxylation of 5-HTP by aromatic L-amino acid decarboxylase. 5-HTP is not present in brain tissue under normal conditions owing to the high activity of the decarboxylase. However, after inhibition of this enzyme by NSD 1015, large amounts of 5-HTP could be detected in rat brain (Fig. 3A). The concentrations of 5-HTP were 187 ± 6,104 ± 8 and 205 ± 13 ng/g in the striatum, cerebral cortex and limbic areas respectively, in good agreement with previously published results obtained by a conventional fluorescence technique[21]. For most of the brain regions studied, sample preparation required only deproteinization by 0.1 *M* perchloric acid followed by centrifugation, but for some dopamine-rich brain areas, such as the striatum, it was preferable to remove catechol compounds by alumina adsorption (see Materials and methods) before chromatographic analysis in order to obtain more accurate determinations.

L-5-HTP levels were also measured in plasma from myoclonic patients upon oral administration of 100 mg carbidopa, an aromatic amino acid decarboxylase inhibitor, followed by intravenous infusion of L-5-HTP (100 mg). The plasma preparation prior to analysis required only deproteinization by perchloric acid. Fig. 3D shows a typical chromatogram. The plasma levels of L-5-HTP in these patients were previously undetectable, but amounted to 1.0 ± 0.2, 2.1 ± 0.2 and 1.3 ± 0.1 μg/ml at 30, 60 and 120 min, respectively, after drug administration (mean ± S.E.M. for data obtained with five patients).

The present method for the separation and quantification of indoles has several advantages over previously published methods:

It requires only minimal sample handling and preparation (direct injection of perchloric acid extracts) as opposed to the more complicated and time-consuming

sample preparations (ion-exchange chromatography or organic extraction) previously described[9,21].

In spite of its simplicity, the detection limits are better than or comparable to those of previous HPLC with fluorescence or electrochemical detection techniques[8-10,22].

The rapidity (less than 10 min for the separation of all compounds), and resolution of the indoles (particularly 5-HTP), are superior to those of previously published methods[8,10].

To our knowledge, no previously reported method allows the simultaneous detection of indoles (including tryptophan and 5-HTP) and the major metabolites of dopamine and noradrenaline without interference.

Finally, it has been found to be practical and reliable in the measurement of indoles, not only in brain tissue but also in a variety of biological fluids.

In conclusion, the present method appears to be rapid, sensitive, reliable and requires minimal sample handling and preparation. By using an automatic injector and a digital peak integrator, 50 samples could be analyzed per day. This method has been successfully applied to the determination of 5-HT, 5-HIAA and 5-HTP in brain tissue and body fluids. The major metabolites of dopamine and norepinephrine can be measured concurrently.

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