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Note

Rapid analysis of tryptophan metabolites using reversed-phase high-performance liquid chromatography with fluorometric detection

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The importance of tryptophan and its metabolites is a subject of increasing interest. The role of tryptophan as a precursor of serotonin and its implications in mental disturbances [1, 2], as well as in the mode of action of anti-rheumatic non-steroidal, anti-inflammatory drugs has been discussed in the literature [3, 4].

Tryptophan is metabolized via two major pathways: the kynurenine pathway and the serotonin pathway. An abnormal metabolism of tryptophan has been observed in patients suffering from a wide variety of diseases; altered levels of its metabolites have been found in patients with cancer of the breast [5, 6], bladder [7] and in Hodgkin's disease [8]. Patients afflicted by these diseases have higher than normal amounts of several metabolites along the kynurenine pathway while sub-normal levels of tryptophan metabolites have been detected in serum from uremic patients [9].

The tremendous physiological importance of tryptophan and its metabolites has stimulated the development of many analytical methods [10—13], most of which are either laborious or of inadequate sensitivity. In addition, the close structural and chemical similarity of these compounds has often resulted in poor separations.

Recent developments in microparticulate, chemically-bonded nonpolar phases have made possible rapid and reproducible high-performance liquid chromatographic (HPLC) analyses. Because of the complexity of the retention mechanism, it is possible to separate simultaneously compounds of a wide polarity range. The determination of biologically-important compounds at levels at which they occur naturally places great demands on HPLC detection systems. Fluorometric measurements offer great advantages over other commonly used detection systems in terms of sensitivity and selectivity. In addition, since few naturally-occurring compounds possess native fluorescence, interferences are not encountered as often as with the less selective detection systems.

Reported in this paper is the use of a reversed-phase partition mode of HPLC, coupled with a fluorometric detection system in the analysis of tryptophan and its metabolites.

EXPERIMENTAL

A Model 6000 A solvent delivery system, Model 660 solvent programmer and Model U6K universal injector, all from Waters Assoc. (Milford, Mass., U.S.A.), were used in all determinations. An FS 970 fluorescence monitor and an SF 770 Spectroflow monitor (Kratos Inc., Schoeffel Instrument Division, Westwood, N.J., U.S.A.) were connected in series and used for monitoring column effluents. The fluorescence monitor was equipped with an SFA 339 wavelength drive and an MM 700 memory module which were used for obtaining the excitation spectra.

A prepacked, stainless-steel column, μBondapak C_{18} (10 μm), was purchased from Waters Assoc.

Reagents

All reagents used were of the highest purity (A.C.S. certified grade). Reference compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.), and methanol, distilled in glass, from Burdic & Jackson (Muskegon, Mich., U.S.A.).

Chromatographic conditions

The low concentration eluent was a 0.02 *M* KH_2PO_4, pH 3.7, and the high concentration eluent was a mixture of anhydrous methanol and water (6:4, v/v). Eluents were filtered through Millipore membrane filters, Type HA, pore size 0.45 μm (Millipore, Bedford, Mass., U.S.A.) and degassed before use. A 35-min linear gradient from 0 to 100% of the high concentration eluent was used. The flow-rate was 1.5 ml/min, and the temperature was ambient in all cases.

Preparation of the serum samples

Freshly drawn human blood was collected in a tube without anticoagulant. The blood was allowed to clot spontaneously for 10—15 min at room temperature. The serum sample was deproteinated by the addition of 1 ml of cold, 6% (w/w) trichloroacetic acid (TCA) to each ml of serum. Samples were vortexed at moderate speed for 1 min and centrifuged at 630 *g* for 5 min. Excess acid was neutralized with solid tris(hydroxymethyl)aminomethane.

Identification of peaks in serum sample

Initial identification of chromatographic peaks in the serum sample was based on retention times and co-chromatography with the reference compounds. Further proof of the peak identity was obtained by comparing the stopped-flow excitation spectra of the reference compounds and the peaks under study [14].

RESULTS AND DISCUSSION

The reversed-phase HPLC separation of the synthetic mixture of tryptophan metabolites, detected by measuring their native fluorescence and UV absorbance is shown in Fig. 1. In spite of the fact that the fluorescence signals were highly attenuated, the enhancement in sensitivity compared to the UV absorption is obvious. Only kynurenine does not fluoresce naturally under the conditions used, and UV monitoring is mandatory for its detection.

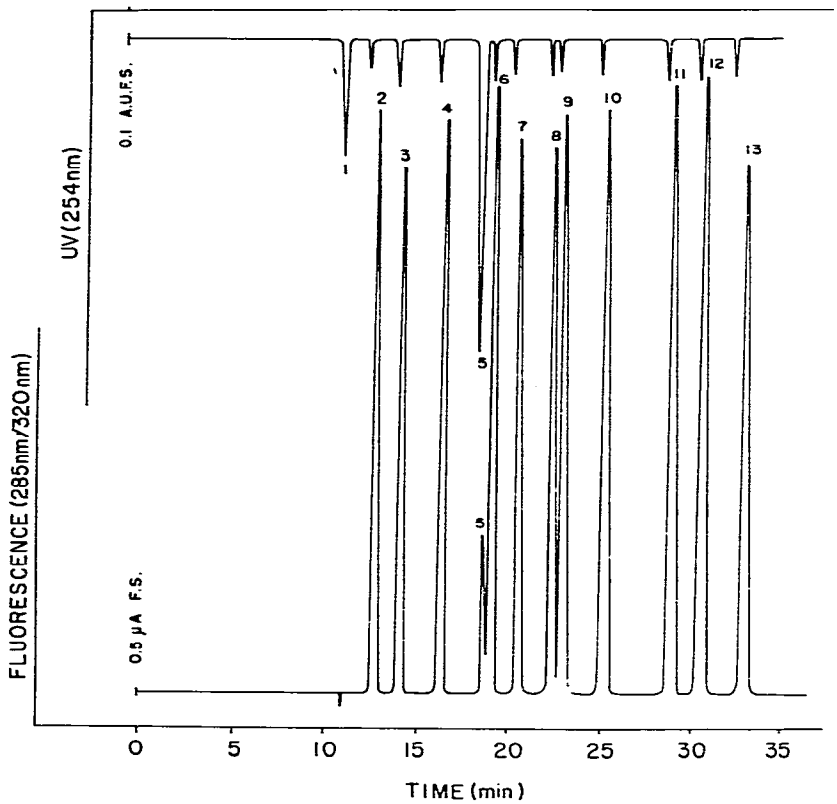

Fig. 1. Separation of tryptophan metabolites detected by a native fluorescence with an excitation wavelength of 285 nm and the emission cut-off filter of 320 nm. Column: μBondapak C_{18} (Waters Assoc.). Mobile phase: low concentration eluent, 0.02 *M* KH_2PO_4, pH 3.7; high concentration eluent, amhydrous methanol—water, 6:4 (v/v). Gradient: linear from 0 to 100% of the high concentration eluent in 35 min. Flow-rate, 1.2 ml/min at ambient temperature. Detection: fluorescence, 285 nm excitation, 320 nm cut-off filter; UV, 254 nm. Peaks: 1 = kynurenine (1.37 nm); 2 = 5-hydroxytryptophan (1.11 nm); 3 = serotonin (0.026 nm); 4 = tryptophan (1.73 nm); 5 = kynurenic acid (1.37 nm); 6 = tryptamine (0.995 nm); 7 = 5-hydroxyindole-3-acetic acid (2.766 nm), 8 = anthranilic acid (6.33 nm), 9 = indoleacetamide (1.62 nm), 10 = indole-3-lactic acid (1.36 nm), 11 = indole-3-acetic acid (1.74 nm), 12 = indole (2.10 nm) and 13 = indole-3-propionic acid (1.15 nm).

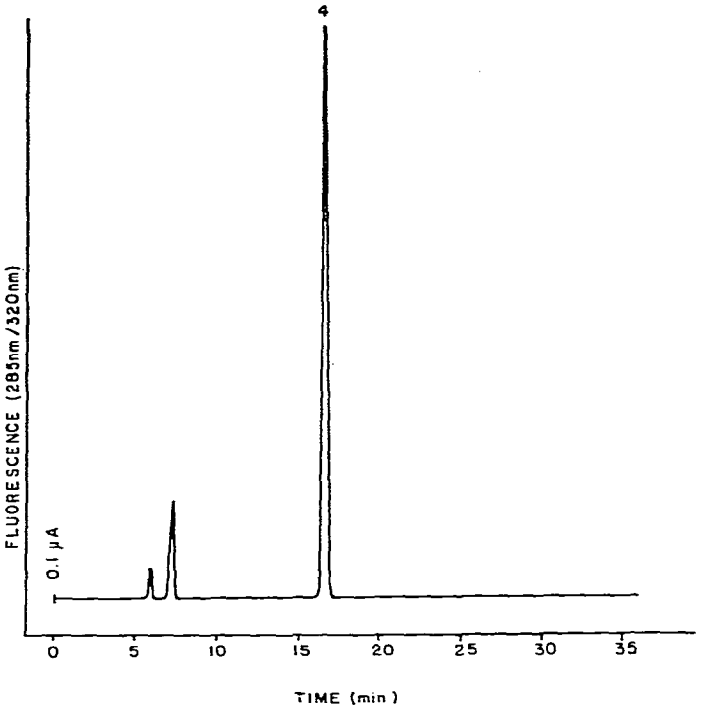

Fig. 2. Chromatogram of a serum sample from a normal subject. Chromatographic conditions and peak identity same as in Fig. 1. Volume of the TCA extract injected: 50 μl (25 μl of serum).

The described separation and detection methods were tested in the analysis of a serum sample from a normal subject (Fig. 2) and a patient with bladder cancer (Fig. 3). It should be noted that because of the use of an acidic protein precipitant (TCA), the total, rather than the free tryptophan was monitored.

This assay was also tested in the analysis of rat brain constituents where no interference with the other naturally-fluorescing compounds were observed.

In conclusion, the described HPLC method is fast, quantitative and the detection is sensitive and selective. The assay is well suited for routine testing of tryptophan metabolites in biological samples, and we believe that it will circumvent many of the problems commonly encountered in current methods of analysis.

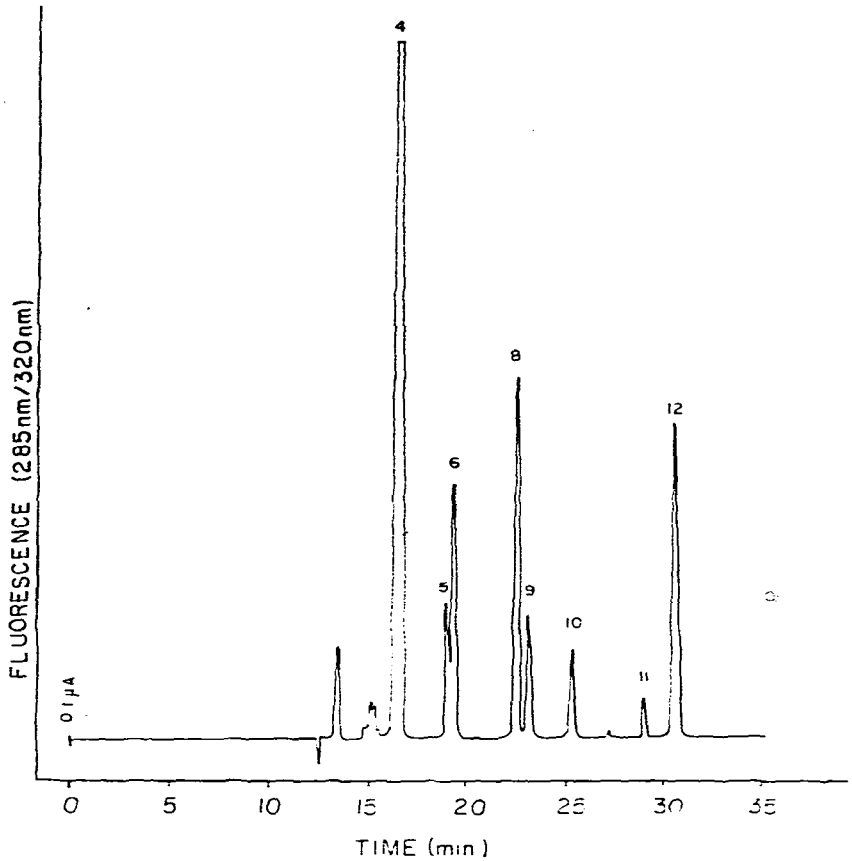

Fig. 3. Chromatogram of a serum sample from a patient with bladder cancer. Chromatographic conditions and peak identity as in Fig. 1. Volume of the TCA extract injected = 50 μl (25 μl of serum).

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