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# Determination of tryptophan and ten of its metabolites in a single analysis by high-performance liquid chromatography with multiple detection

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ABSTRACT

Tryptophan is an essential amino acid whose metabolism involves several pathways. Defects, either inherited or functional, in its transport mechanism or catabolism are related to a large variety of clinical abnormalities. This paper presents a relatively simple and rapid method to determine quantitatively tryptophan and several of its metabolites in biological fluids using reversed-phase high-performance liquid chromatography and multiple detection.

## INTRODUCTION

Tryptophan (Trp) is an essential amino acid whose metabolism involves several different pathways. Two are of fundamental importance in man:

(1) Trp is hydroxylated by a tetrahydrobiopterin-dependent enzyme leading to the production of the neurotransmitter serotonin and of 5-hydroxy-indoleacetic acid.

(2) The kynurenine pathway leads to nicotinic acid and to the synthesis of $NAD^+$ [1]; this main breakdown requires the activity of the pyridoxal-requiring enzyme kynureninase [2].

Moreover, gut bacterial activity causes decomposition of unabsorbed Trp, producing indole compounds [2]. The determination of Trp and its metabolities is of interest in the study of all clinical conditions related to either a block or an enhancement of any of these pathways. This can be due either to a defect in enzyme activities (*i.e.* defects in tetrahydrobiopterin biosynthesis, kynureninase deficiency, vitamin $B_6$ deficiency) or to a transport defect (Hartnup disease). The assay of 5-hydroxy-Trp and of the catecholamines is well established and it is not the object of this work [3]. We report a simple chromatographic method for the determination of Trp and ten of its closely related metabolites (four indole derivatives, three kynurenine-type compounds and xanthurenic, 3-hydroxy-anthranilic and

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kynurenic acids) using reversed-phase high-performance liquid chromatography (HPLC) and combined UV–fluorimetric detection.

## EXPERIMENTAL

### *Materials*

Trp and other standards were obtained from Sigma (St. Louis, MO, USA); potassium phosphate, sulphosalicylic acid (analytical reagent grade), water and solvents (HPLC grade) were obtained from Merck (Darmstadt, Germany), as was the chromatographic column.

### *Sample preparation*

Some compounds are easily degraded by increasing temperature, by light or by low pH [4]; therefore, the standard solutions and biological samples were promptly treated and then assayed, or stored under conditions of light exclusion at −20°C until analysis, which was then performed as soon as possible. Proteins must be immediately removed from plasma and other protein-containing fluids by precipitation with an equal volume of 5% sulphosalicylic acid in water. This method allows the measurement of total Trp. When measurement of free Trp is needed, other methods such as ultracentrifugation or ultrafiltration can be used for deproteinization. As a rule, protein removal is not necessary for urine, but the absence of proteins should be checked. Antioxidants such as sodium metabisulphite or ascorbic acid can be added. Plasma and urine are collected in the basal condition (fasting) and/or after a provocative test (*i.e.* oral Trp load) [2].

### *HPLC equipment*

Analyses were carried out using a Hewlett-Packard (HP) 1090-L liquid chromatograph, equipped with an HP1040-M diode-array detector connected on line with an HP1046-A spectrofluorimetric detector. Signals were acquired with a Pascal workstation (Series 9000, software revision 5.21) which allows, together with the management of analytical conditions, a real-time view of UV absorbance (spectra and traces) and fluorescence emission, data storage and reduction. The injector was a manual Rheodyne valve with a 50-$\mu$l loop. The stationary phase was LiCrospher 100 RP-18 (5 $\mu$m particle size) in a LiChroCART 124-4 cartridge 12 cm long × 4.6 mm I.D.

### *Analytical conditions*

The mobile phase was made up of two eluents: A, phosphate buffer (0.02 *M* $KH_2PO_4$–$H_3PO_4$) pH 5.4; B, acetonitrile–methanol–water (50:10:40, v/v/v). The flow-rate was 1 ml/min. The gradient was as follows: isocratic elution with eluent A for 6 min, then a ramp to 25% eluent B until 20 min and to 75% B until 35 min. The column was cleaned with eluent B for 5 min, then conditioned with 20 ml of eluent A. UV absorption was monitored at the wavelengths 254, 280 and 365 nm, band width 16 nm, using

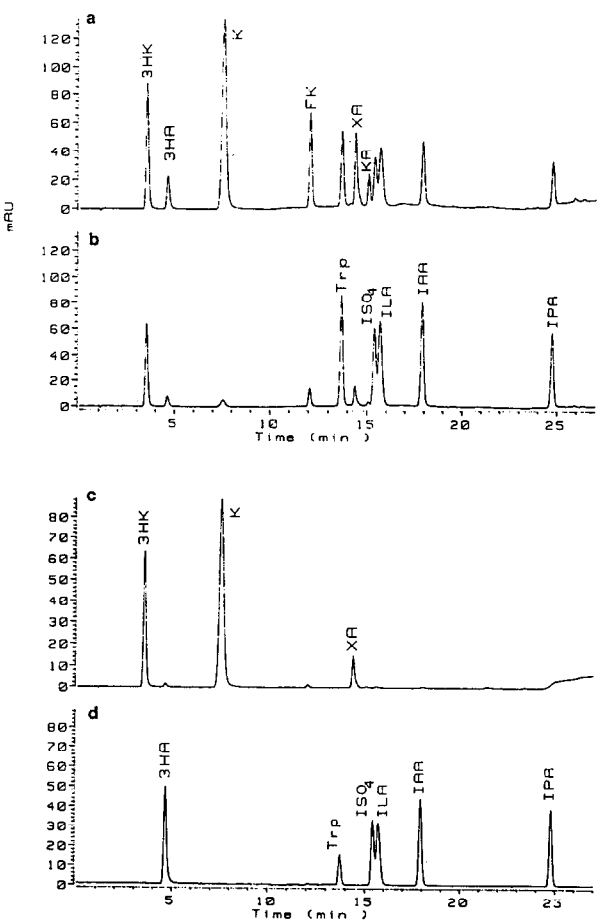


Fig. 1. The four chromatograms [UV at (a) 254, (b) 280, (c) 365 nm and (d) fluorescence] obtained from a standard mixture of the eleven compounds (concentration: 0.5 m*M*).

550 nm, band width 80 nm, as the reference wavelength; additionally UV spectra from 190 to 450 nm were stored. The fluorescence excitation was set to 340 nm and the emission to 440 nm; after 13.5 min, excitation and emission were changed to 280 and 340 nm, respectively.

## RESULTS

The results of the analytical run were obtained as four chromatograms: UV absorbance at 254, 280 and 365 nm and fluorescence emission.

Eleven compounds, of relevant biochemical and clinical significance, were well separated and identified (Fig. 1, Table I).

Quantitation of each compound was possible using peak areas, and calibration curves were obtained from standard solutions at concentrations of 10, 50, 100 and 500 μmol/l. The choice of the chromatogram used for each peak for quantitative measurements depended on considerations of sensitivity and peak resolution (see Discussion).

The responses were linear in the measured range; as an example Table II reports data on linearity for Trp. The sensitivity of the method is enough to determine usual levels in plasma for the majority of the compounds. The lower response limit is about 2.5 μmol/l for Trp as well as for the other metabolites.

## DISCUSSION

Methanol is generally used with different buffers as the organic modifier in reversed-phase liquid chromatography of Trp metabolites [4–6]. In our experience this method is not reliable (even using different gradient profiles) for a full separation of all compounds. Trp/XA and $ISO_4$/KA/ILA are poorly separated. While the use of both fluorescence and UV at 365 nm can resolve the problem well for Trp and XA (the first is fluorescent and does not absorb at 365 mm: see Table I), in the last triplet this method is not reliable because the contribution of the signal at 365 nm in the KA peak is very low and $ISO_4$ and ILA (both fluorescent) absorb considerably also at 254 and 280 nm. The introduction of acetonitrile into the organic eluent can overcome this disadvantage, yielding an acceptable resolution of all the peaks.

As a result of this procedure, each compound is well separated and can be measured in at least one of the four chromatograms. The choice of

TABLE I

RETENTION TIMES AND RELATIVE ABSORBANCES AT DIFFERENT WAVELENGTHS OF THE ELEVEN METABOLITES

The underlined signals are used for quantitation. For some compounds more than one signal can be used (see text).

| Compound | | $t_R$ (min) | Fluorescence ($\lambda_{ex.}/\lambda_{em.}$) | UV signals[a] (nm) | | |
|---|---|---|---|---|---|---|
| | | | | 254 | 280 | 365 nm |
| 3-Hydroxykynurenine | 3HK | 3.9 | None | 41.2 | 27.2 | 31.6 |
| 3-Hydroxyanthranilic acid | 3HA | 8.09 | 340/440 | 69.1 | 23.4 | 7.4 |
| Kynurenine | K | 8.63 | None | 58.0 | 2.8 | 39.2 |
| Formyl-kynurenine | FK | 12.99 | 340/440 | 79.6 | 18.5 | 1.9 |
| Tryptophan | Trp | 14.6 | 280/340 | 34.4 | 65.6 | None |
| Xanthurenic acid | XA | 14.7 | None | 57.7 | 21.7 | 20.6 |
| Kynurenic acid | KA | 15.45 | None | 84.0 | 13.0 | 3.0 |
| Indoxyl-sulphate | $ISO_4$ | 16.47 | 280/340 | 34.7 | 65.3 | None |
| Indol-lactic acid | ILA | 17.13 | 280/340 | 32.6 | 67.4 | None |
| Indol-acetic acid | IAA | 22.35 | 280/340 | 32.1 | 67.9 | None |
| Indol-propionic acid | IPA | 28.6 | 280/340 | 31.5 | 68.5 | None |

[a] The numbers indicate the relative mAU for the three wavelengths (percentage of the sum of the three absolute absorbances).

TABLE II

DATA ON RESPONSE LINEARITY AND MINIMUM DETECTION LEVEL FOR Trp USING UV (280 nm) AND FLUORESCENCE (FL) EMISSION (EXCITATION 280 nm, EMISSION 340 nm) SIGNALS

The equation of the calibration curve is $y = a + bx + cx^2$. $r^2$ = linear regression coefficient.

| $x$ | | $y$ response (area) | |
|---|---|---|---|
| | | FL (280/340 nm) | UV (280 nm) |
| 10 | μmol/l | 30.4 | 194.8 |
| 50 | μmol/l | 173.3 | 970.1 |
| 100 | μmol/l | 367.8 | 2192.1 |
| 500 | μmol/l | 1835.2 | 10938.0 |
| A | | −4.52 | −30.9 |
| B | | 3.68 | 21.7 |
| C | | $5.69e^{-6}$ | $5.61e^{-3}$ |
| $r^2$ | | 0.99996 | 0.99988 |
| Minimum detectable (μmol/l) | | Below 5 | Below 1 |

the signal used for quantitation is based on either specificity or sensitivity considerations. The fluorescence trace with excitation at 280 nm is particularly useful for the identification of Trp and indole derivatives.

The UV spectra acquired during the run are a relevant additional help in peak identification. However, special care must be taken comparing the spectra from biological samples with those from authentic compounds, since the elution is in gradient mode and the UV spectra are critically affected by the actual percentage of methanol and acetonitrile in the mobile phase: even the slightest alteration in the retention time can lead to a substantial modification of the UV spectra.

In conclusion, the present method is reliable for the simultaneous determination of several biochemically linked compounds, using a relatively simple and rapid procedure.

Its clinical applications are considerable because of the wide clinical relevance of Trp and Trp-related compounds. Their involvement in several clinical conditions and pathologies, such as Hartnup disease, $B_6$ deficiency, dermatological changes, cancers and mental disturbances, is well known [2,4].

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