International Journal of Tryptophan Research



ORIGINAL RESEARCH

# Rapid Isocratic Liquid Chromatographic Separation and Quantification of Tryptophan and Six kynurenine Metabolites in Biological Samples with Ultraviolet and Fluorimetric Detection

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**Abstract:** A simple, rapid isocratic liquid chromatographic procedure with ultraviolet and fluorimetric detection is described for the separation and quantification of *L*-tryptophan (Trp) and six of its kynurenine metabolites (kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic, kynurenic, xanthurenic and anthranilic acids). Using the Perkin Elmer LC 200 system, a reverse phase Synergi 4 µ fusion-RP80 A column (250 × 4.6 mm) (Phenomenex), and a mobile phase of 10 mM sodium dihydrogen phosphate: methanol (73:27, by vol) at pH 2.8 and a flow rate of 1.0–1.2 ml/min at 37 °C, a run took ~13 min. The run took <7 min at 40 °C and a 1.4 ml/min flow rate. Limits of detection of all 7 analytes were 5–72 nM and their recoveries from human plasma and rat serum and liver varied between 62% and 111%. This simple method is suitable for high throughput work and can be further developed to include quinolinic acid and other Trp metabolites.

**Keywords:** anthranilic acid, high-performance liquid chromatography, 3-hydroxyanthranilic acid, 3-hydroxykynurenine, kynurenic acid, kynurenine, quinolinic acid, tryptophan, xanthurenic acid

*International Journal of Tryptophan Research* 2010:3 175–186

doi: 10.4137/IJTR.S6225

This article is available from http://www.la-press.com.

## Introduction

The amino acid *L*-tryptophan (Trp) is of particular scientific and medical interest, because it is not only essential for protein synthesis and hence growth, but also the precursor of a large number of biologically active metabolites, including the cerebral indolylamine serotonin, (5-hydroxytryptamine), the pineal hormone melatonin and the many other metabolites of the hepatic kynurenine pathway. These include the important redox cofactors $NAD^+(P^+)H$, the B-vitamin-like substance nicotinic acid, the convulsant kynurenine, the *N*-methyl-*D*-aspartate (NMDA) receptor antagonist kynurenic acid and agonist quinolinic acid and the malaria mosquito gametocyte-activating agent xanthurenic acid. Analysis of Trp and its metabolites is therefore an important laboratory aid to research across a wide range of scientific disciplines and medical specialties. Many procedures exist for separation and quantification of groups of the above and other metabolites and derivatives, using gas chromatography-mass spectrometry (GC-MS) or high-performance liquid chromatography (HPLC) with various detection systems (for review and references, see Molnar-Perl[1]). The HPLC technique has received greater popularity in analysis of Trp and its metabolites, largely because of availability of sensitive detection systems and the native fluorescence of some of these metabolites which minimised the requirement for the derivatisation needed in GC analysis.

The presence of a relatively large number of kynurenine metabolites in mammalian biological samples presents a challenge to analysts when developing a rapid procedure suitable for analysis of large numbers of samples. For example, current HPLC methods can determine between 1 and 4 Trp metabolites in serum or urine in 15–70 min[2–7] and, although faster methods have been developed for analysis of up to 6–9 kynurenine metabolites, a run still takes 17–40 min and most require additional pre-column sample deproteinisation and clean-up, and the use of gradient elution.[8–11] More recently, HPLC-MS has been used for more rapid separation of kynurenines in two studies. In one,[12] only Trp, kynurenine and 3-hydroxykynurenine were separated within 8 min using on-line solid-phase extraction, whereas in the other,[13] Trp, 6 kynurenines, B-vitamin markers and others (in total 16 analytes) were separated within 5 min. However, the use of MS was essential for proper identification of this large number of closely eluted peaks. In another earlier, but simple, isocratic HPLC procedure[14] designed for measuring only Trp using UV detection, the authors reported also very close retention times for 8 kynurenine metabolites, 4 indole metabolites and 4 other compounds. In this earlier study,[14] Trp was sufficiently separable from the nearest metabolites to enable the authors to quantify it. In the present work, we have developed this procedure[14] further for successful separation and quantification of Trp and at least 6 of its kynurenine metabolites within 7 min and suggest that further developmental work may widen this simple procedure to include other Trp metabolites.

## Materials and Methods

### Chemicals and other materials

Tryptophan(Trp),kynurenine(K),3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), kynurenic acid (KA), xanthurenic acid (XA), quinolinic acid (QA), indol-3-ylacetic acid (IAA), indol-3-yllactic acid (ILA) and indol-3-ylpyruvic acid (IPA) were purchased from The Sigma-Aldrich Co. Ltd. (Fancy Road, Poole, Dorset BH12 4QH, UK) and were stored as directed by the manufacturer. Water and methanol (HPLC grade) were purchased from either V W R International (Hunter Boulevard, Magna Park, Leicestershire LE17 4XN, UK) or Fisher Scientific UK (Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK). Acids and alkalis were purchased from VWR International, were of the purest commercially available grades and were made up in HPLC-grade water. Filtration, Eppendorf and other tubes were purchased from Fisher or other standard suppliers.

### Standard and other solutions

Individual solutions or mixtures containing Trp and its various metabolites (1 µg/ml each) were prepared in HPLC-grade water without or with the use of a small volume (1 ml) of M-NaOH (in the cases of 3-HAA, KA, QA and XA). AA was first dissolved in 1 ml of pure ethanol before dilution with water. All individual analytes and their mixtures were initially prepared as neutral solutions, but, as perchloric acid extracts of biological samples were to be used throughout,

$HClO_4$ was subsequently added to these aqueous standard solutions at a similar final concentration (6%, w/v) as that in the biological sample extracts. One ml portions were stored at –24 °C and used as calibrants within 2 months. Dilutions of the above standard mixture were made for limits of detection experiments. The mobile phase was initially that described by Gjerde et al,[14] namely methanol: 10 mM sodium dihydrogen phosphate (30:70, by vol), but this ratio was subsequently altered to improve analyte separation. The 10 mM sodium phosphate buffer was prepared in HPLC-grade water and filtered through a cintered-glass funnel before mixing with methanol. The free buffer was stored at 4 °C for up to 1 week, whereas the buffer-methanol mobile phase was kept at room temperature in its reservoir and used within 2 weeks.

## Preparation of plasma, serum and liver extracts

To a 0.5 ml portion of human plasma or rat serum, 0.4 ml of HPLC-grade water and 0.1 ml of 60% (w/v) $HClO_4$ were added in ice-cooled tubes. The contents of the tubes were vortexed for 5–10 s and allowed to stand for 5 min before centrifugation at 14,000 g for 10 min at 4 °C, using a Hermle refrigerated high-speed centrifuge (Hermle labortechnik GmbH, Siemensstrasse 25, 78564 Wehingen, Germany). The supernatant was decanted and adjusted to 1 ml with 6% (w/v) $HClO_4$ to correct for minor volume changes due to plasma protein precipitation. A 0.5 ml portion was immediately filtered through a Vectaspin filtration tube by centrifugation at 14,000 g for 10 min at 4 °C and the filtrate was either transferred to an HPLC injection vial for immediate use or stored (along with the unfiltered portion) at –24 °C for subsequent analysis. For rat liver extracts, to a 1 g piece of previously frozen liver, 1 ml of ice-cold HPLC water and 2 ml of ice-cold 12% (w/v) $HClO_4$ were added and the mixture was homogenised for ~1 min using an Ultra-Turrax homogeniser. The homogenates were allowed to stand in ice-cold tubes for 10 min before centrifugation at 6,000 g for 10 min at 4 °C. The supernatants were decanted and adjusted to 4 ml with 6% (w/v) $HClO_4$ to correct for small volume decreases due to tissue precipitation. A 0.5 ml portion of each homogenate was filtered and used or stored as described above for plasma and serum extracts.

## Recovery of Trp and kynurenine metabolites from human plasma and rat liver and serum

A concentrated mixture of Trp and its 6 kynurenine metabolites was prepared in 6% (w/v) $HClO_4$ and small volumes were added to different human plasmas, rat sera and rat livers (n = 4 each) shortly before preparing extracts in 6% (w/v) $HClO_4$, as described above, such that final concentrations of Trp and its 6 kynurenine metabolites were 2, 1 and 0.5 µg/ml. Untreated $HClO_4$ extracts of these biological samples acted as controls. Standards containing these different concentrations were run as calibrants. All standards and extracts were chromatographed under the standard conditions described below.

## Stability of kynurenines after frozen storage of perchloric acid tissue extracts

Stability was tested using perchloric acid extracts of normal human plasma samples prepared as described above. Freshly prepared extracts were analysed on the same day under the standard conditions described below. Portions of these extracts were then frozen at –24 °C for periods of 3 days and 1–4 weeks, after which they were reanalysed. Plasmas were isolated from blood samples taken before and hourly for up to 7 h during the acute Trp loading test in an earlier study.[15] As the concentrations of Trp and its kynurenine metabolites varied widely during this test, we were able to assess stability at different metabolite concentrations. For each of the above storage time points, plasma extracts from two different subjects were used. Stability was expressed as a percentage of the mean values observed in freshly analysed extracts.

## Limits of detection and linearity

A solution containing 2 µg/ml each of Trp and its 6 kynurenine metabolites was prepared in 6% (w/v) $HClO_4$, from which serial dilutions were made down to 0.001 µg/ml. The original and its various dilutions were chromatographed under the standard conditions described below. Linearity was plotted and limit of detection (LOD) noted using both UV and, where appropriate, fluorescence detection. LOD using UV was performed at 220 nm, whereas that for fluorescing metabolites was first examined

at the common 254/404 nm (excitation/emission) wavelength combination, but was followed by using the specific wavelength maxima of each compound. For all other experiments, 220 nm (UV) and the 254/404 nm combination (fluorescence) were adopted. The signal to noise (S/N) ratio in the LOD and all other experiments was 5:1 or higher (see below).

### Between-day variations

A mixture of 7 components (1 μg/ml each) was chromatographed under the standard conditions described below on 16 different days over a 4-week period to determine between-day variations. Within-day variations were not examined, as running the standard mixture more than once daily was very infrequent. When this occurred, variations between duplicate standards were negligible (~<1%).

### Equipment and data handling

A Perkin-Elmer LC 200 quaternary pump with a column oven, a degasser, a liquid autosampler and a UV/VIS and a fluorescence (LS 30) detector in series were used. The system was run isocratically using a Synergi 4 μ reverse-phase Fusion-RP80A column (250 × 4.6 mm) with guard column (Phenomenex). Operation of the system, data processing and handling were all performed by the associated Total Chrome software. A standard mixture containing 1 μg/ml of each component was used as calibrant at the start of each run. UV detection was performed at 220 nm throughout, as lower levels of K and its metabolites could not be detected at higher wavelengths. Fluorimetric detection was performed using the Perkin-Elmer LS 30 spectrophotofluorimeter and a suitable flow cell. The LS 30 has facilities for altering the excitation/emission wavelengths through the timed events function, thus enabling multiple wavelength detection to be operated during a run and this was performed in LOD experiments for detection of Trp and its 3 fluorescing metabolite (3-HAA, KA and AA) at their specific excitation/emission maxima (see the Results section). The total Chrome software used was a versatile and powerful system that was capable of detecting and quantifying very tiny peaks as will be seen from the results in Table 5 and Figure 4. The default setting for the signal/noise (S/N) ratio of this software was 5:1. When a peak was not quantified by the software because of the absence of a baseline, a click on the S/N ratio button prompted the software to suggest a new ratio (usually 5 or higher) to enable area measurement. A further facility of the software is manual integration, which, though not as accurate as the automatic software equivalent, can on occasion elicit a very tiny peak if the baseline is drawn accurately at the relevant retention time. A S/N ratio of 5 was used in almost all experiments, though a higher setting (50:1 or 100:1) was occasionally applied in a few fluorimetric detections. Although the fluorescence of KA is known[16] to be enhanced by addition of $Zn^{2+}$, this cation was not added to the mobile phase to avoid formation of its insoluble phosphate salt.

### Standard and other conditions

After confirming the previous observation[14] of the poor separation of kynurenine metabolites when the methanol concentration in the mobile phase was 30%, various attempts were made to improve the separation, first by changing the methanol concentration, then the pH, the flow rate and finally the column oven temperature. Some of these attempts will be described in the Results section below, but, for Trp and 6 of its kynurenine metabolites, we adopted the following standard conditions: a mobile phase of 27% methanol: 73% 10 mM sodium dihydrogen phosphate (final pH 2.8) was used at a flow rate of 1.4 ml/min at 40 °C with UV (220 nm) and fluorescence detection (254/404 nm) in series. All standards and biological sample extracts were in 6% (w/v) $HClO_4$ and 10 μL samples were auto-injected at 4 °C. Retention times of analytes in mixture were determined from those of individual components and, additionally for QA and 3-HK, by spiking biological sample extracts with a range of concentrations of each substance.

## Results

### Separation of Trp and its metabolites

#### Initial attempts and changing the methanol content of the mobile phase

Initially, we hoped to separate most of the components tested in the original procedure[14] and so used Trp, 7 kynurenines (QA, 3-HK, K, 3-HAA, XA, KA and AA) and 3 indole metabolites of the tryptamine and transamination pathways, namely IAA, ILA and IPA. We first tested the mobile phase in its original composition,[14] namely 30/70 methanol/phosphate buffer at a 1 ml/min flow rate, but at 37 °C, rather

than room temperature. Some of these metabolites co-eluted and one or two were not eluted within 20 min. Based on the principle of solvent strength selectivity, the decreased retention of co-eluting substances observed here is due to the mobile phase being less polar (ie, having a higher methanol content than would be desirable). Consequently, it was immediately clear that the methanol content should be decreased. A range of methanol concentrations was tested between 15% and 40% (at 1 ml/min and 37 °C). The best separation was achieved with a 27% methanol concentration, which was adopted in most subsequent studies. Various attempts to improve the separations were made by changing the pH, temperature and flow rate. At a 0.9 ml/min flow rate at 37 °C and using neutralised solutions of analytes for injection, we found that pH 5.6 achieved the best separation of 10 of the above 11 components, within 16 min, with QA escaping in the solvent front. IPA detection was, however, variable, almost certainly due to its keto-enol tautomerisation. As our primary interest was concerned with analysis of kynurenine metabolites in biological samples, all subsequent attempts were made using Trp and the above 7 kynurenines.

### Separation of Trp and kynurenines

This was attempted by changing the pH, column oven temperature and flow rate. At pH 2.8, a flow rate of 1.0–1.2 ml/min and at 37 °C, 6 kynurenines plus Trp were separated within ~13 min. Figure 1 shows a typical such separation with both UV (220 nm) and fluorimetric (254/404 nm) detection. As the UV detector was used first, a difference in retention time of Trp and its 3 fluorescing metabolites 3-HAA, KA and AA of ~ 0.2 min can be seen by comparing Figure 1(a) with (b).

Increasing the temperature to 40 °C and the flow rate to 1.4 ml/min resulted in a faster separation, within 7 min without altering the pattern of elution, as shown in Figure 2. QA was not detectable, having presumably been eluted in the solvent front. These latter (standard) conditions were used for all subsequent experiments for method validation (see also the Materials and Methods section).

### Separation of quinolinic acid

This was achieved by lowering the pH to 2.0, the flow rate to 1.15 ml/min, the methanol content of the mobile phase to 25% (v/v), and the column oven temperature to 37 °C. Under these conditions, QA appeared immediately before 3-HK and the pattern of elution of kynurenines was altered, as shown in Figure 3. QA separation was, however variable and further improvements will be necessary to achieve a more reproducible elution of this metabolite.

## Linearity and limits of detection

Limits of detection (LOD) are shown in Table 1. The UV LOD of the 7 compounds 3-HK, K, Trp, 3-HAA, XA, KA and AA varied between 22 and 72 nM. Using fluorimetric detection at 254/404 nm for the fluorescing compounds Trp, 3-HAA, KA and AA gave LOD not different from those using UV. However, when LOD of these 4 compounds were determined at their respective optimum excitation and emission wavelengths, values of 5, 32, 26 and 7 nM respectively were obtained, thus demonstrating the 2–10-fold superiority of fluorimetric detection. These levels of LOD correspond to concentrations of the 7 compounds of between 1 and 10 ng/ml. Linearity was observed for all concentration ranges between those at the LOD and the highest concentrations tested (1 or 2 μg/ml).

## Between-day variations in analyte determination

Analysis of Trp and its 6 kynurenine metabolites was performed in a freshly prepared standard mixture containing 1 μg/ml of each component and repeatedly for 16 days over a 4-week period on frozen portions of this mixture. The data in Table 2 give the coefficients of variation (CV), as a %, between days for 16 determinations and show the CV's to vary between 1.9% and 7.7%.

## Recovery from human plasma and rat liver and serum

As shown in Table 3, recoveries of Trp and its 6 kynurenine metabolites from human plasma and rat serum and liver varied between 62% and 111%. As there were only modest differences in recoveries of metabolites when added at 2, 1 or 0.5 μg/ml, compared with differences in recoveries between the quadruplicate human or rat samples, the recovery values for all three analyte concentrations were averaged for each of the four individual biological

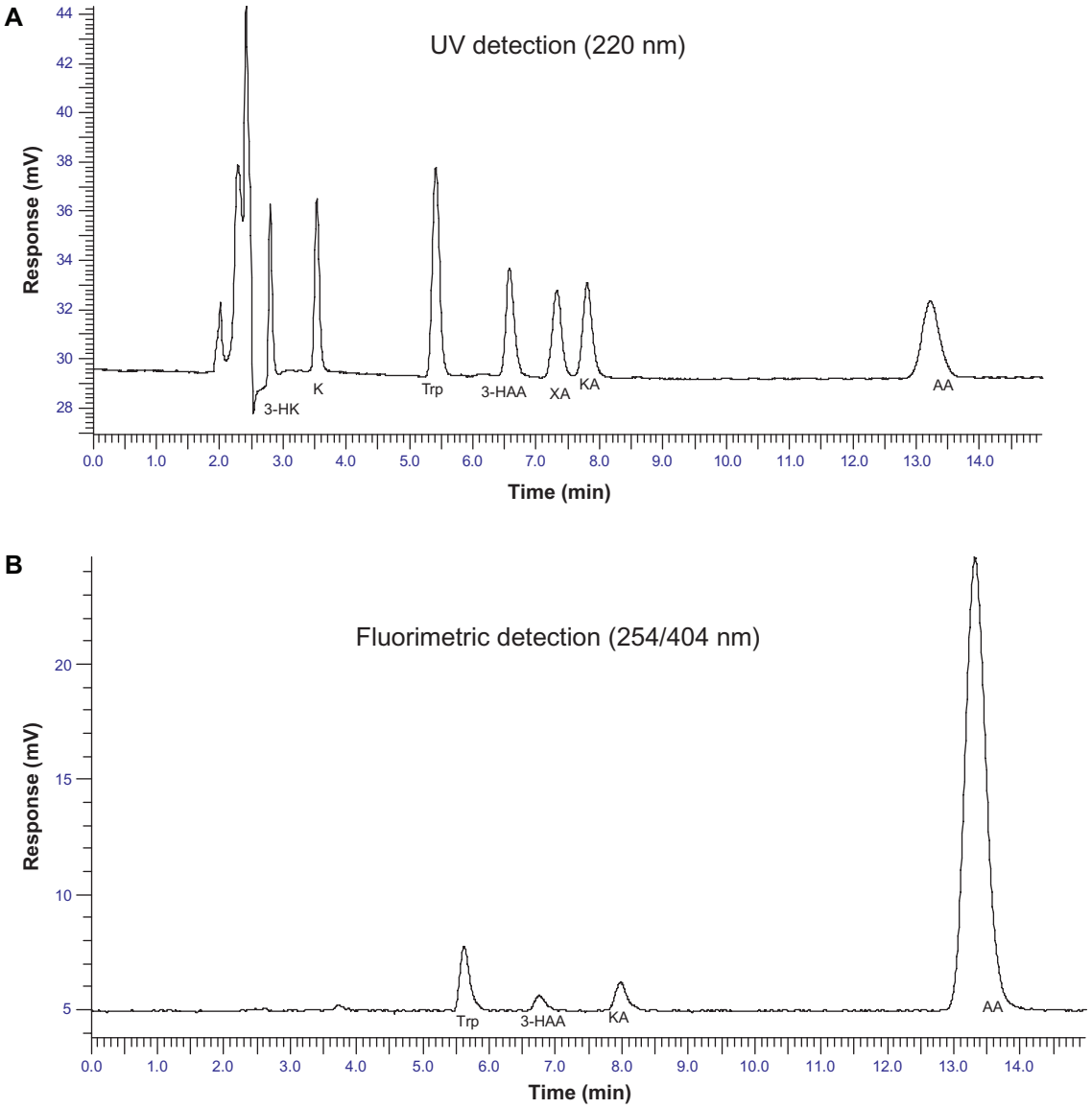


**Figure 1.** HPLC chromatograms of tryptophan and six kynurenines with ultraviolet (UV) and fluorimetric detection.
**Notes:** A mixture containing 1 μg/ml each of tryptophan (Trp) and 6 kynurenines [kynurenine (K), 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), kynurenic acid (KA), xanthurenic acid (XA) and anthranilic acid (AA)] was chromatographed isocratically using a mobile phase of methanol: 10 mM sodium dihydrogen phosphate (27:73 by vol) at a final pH of 2.8, a flow rate of 1.15/min, and a column oven temperature of 37 °C. Other details are as described in the Materials and Methods section. Peaks were UV detected at 220 nm (**a**) or fluorimetrically at an excitation/emission wavelength combination of 254/404 nm (b). Note that UV preceded fluorimetric detection, hence the ~0.2 min lag in peak retention time in (**b**) for fluorescing compounds.

samples and presented in Table 2 as the means for each parameter. As shown, recoveries of Trp and its 6 kynurenine metabolites from human plasma, rat serum and rat liver varied between 76%–95%, 62%–103% and 75%–111% respectively.

## Stability of perchloric acid extracts of human plasma samples upon frozen storage

Stability was examined in 6% (w/v) $HClO_4$ extracts of human plasma samples stored at –24 °C for up to 4 weeks. As the results in Table 4 show, stability of Trp and 5 of its kynurenine metabolites was maintained for up to 4 weeks of frozen storage. Only 3-HAA showed a small loss of 7%–8% after 3–4 weeks. Stability was not influenced by the presence of larger amounts of Trp or its metabolites in samples derived from subjects receiving acute Trp loads.

## Normal values in human plasma

The results in Table 5 show the normal fasting values for Trp and 6 kynurenine metabolites

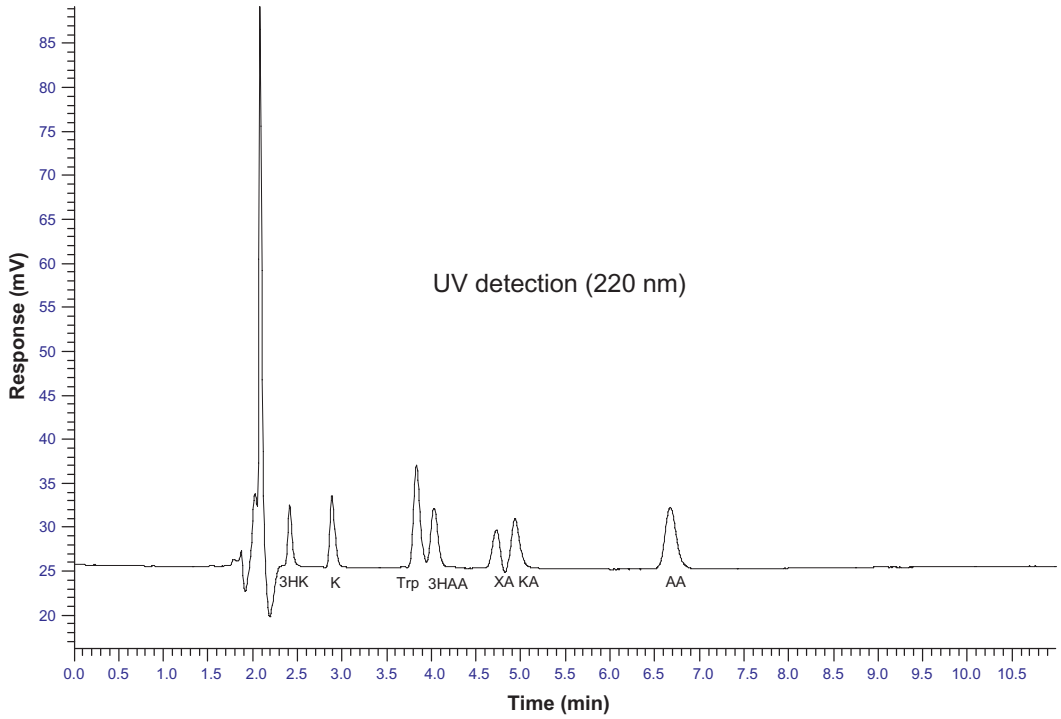


**Figure 2.** HPLC chromatogram of tryptophan and six kynurenines under the standard conditions.
**Notes:** A mixture containing 1 μg/ml each of tryptophan and 6 kynurenines was chromatographed under the standard conditions in the Materials and Methods section, namely a mobile phase of methanol: 10 mM sodium dihydrogen phosphate (27:73 by vol) at a final pH of 2.8, a flow rate of 1.4 ml/min, and a column oven temperature of 40 °C. Other details are as described in the Materials and Methods section. Peaks were detected at 220 nm (UV detector). For abbreviations, see Figure 1.

for 114 healthy US subjects of diverse ethnicity corrected for full recovery. These data will be discussed below.

The chromatograms in Figure 4 depict the profile of a fasting plasma sample from a healthy US subject. Trp and all 6 kynurenines were quantified using UV and fluorimetric detection. Concentrations (in μM) of 3-HK, K, Trp, 3-HAA, XA, KA and AA were 1.75, 3.28, 47, 1.44, 0.75, 0.0117 and 0.0027 respectively. The ability of the Total Chrome software to detect and

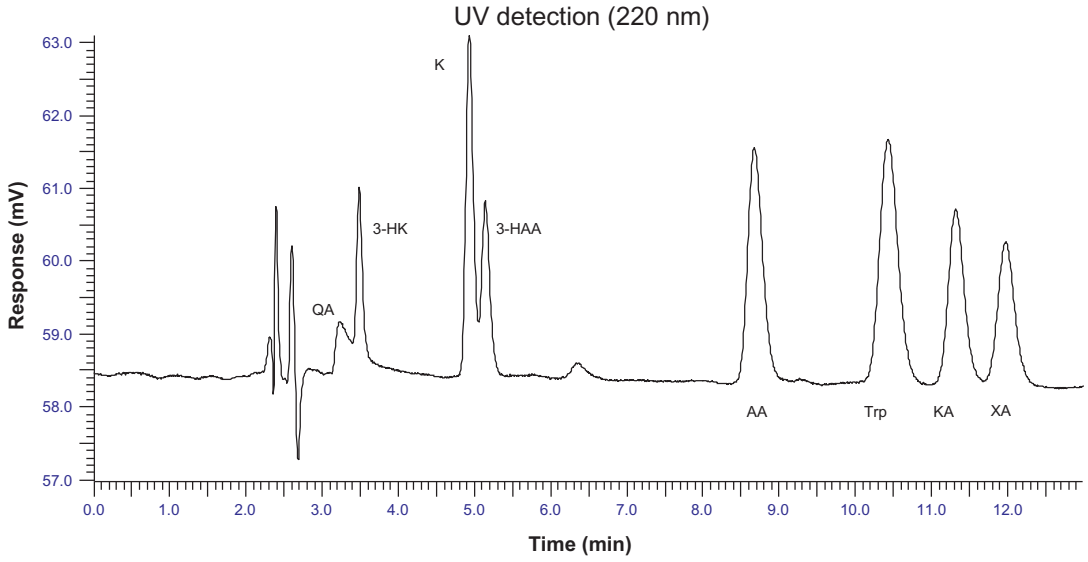


**Figure 3.** HPLC chromatogram of tryptophan and 7 kynurenines, including quinolinic acid.
**Notes:** Details and abbreviations are as in Figure 1, with the following exceptions: 1) quinolinic acid (QA) was present in the mixture (also at 1 μg/ml); 2) the methanol content of the mobile phase was 25% (v/v); 3) the final pH was 2.0; 4) the flow rate was 1.15 ml/min; 5) the column oven temperature was 37 °C.

**Table 1.** Limits of detection (LOD) of tryptophan and six kynurenines.

| Analyte | UV-LOD (nM) | FL-LOD (nM) |
|---|---|---|
| 3-HK | 22 | – |
| K | 24 | – |
| Trp | 24 | 5 |
| 3-HAA | 65 | 32 |
| XA | 49 | – |
| KA | 53 | 26 |
| AA | 72 | 7 |

**Notes:** Limits of detection (LOD) were determined for 3-hydroxykynurenine (3-HK), kynurenine (K), tryptophan (Trp), 3-hydroxyanthranilic acid (3-HAA), xanthurenic acid (XA), kynurenic acid (KA) and anthranilic acid (AA) using both UV and fluorescence (FL) detection. UV detection was performed at 220 nm, whereas fluorescence (FL) detection was at the excitation/emission wavelength optima of 285/390 nm (Trp), 340/420 nm (3-HAA), 344/390 nm (KA), 313/420 nm (AA). The standard conditions used and other details are given in the Materials and Methods section.

quantify tiny UV peaks is clearly seen in Figure 4a. With fluorimetric detection at 254/404 (Fig. 4b), only AA among fluorescing compounds could not be quantified at the above sub-optimal wavelength combination.

## Discussion

Analysis of tryptophan and its kynurenine metabolites presents a challenge to researchers across many scientific disciplines and medical specialties. Most current analytical procedures involve relatively long runs of between 15 and 70 min for Trp and a small number of metabolites (up to 4),[2–7] or 17–40 min for a larger number (6–9).[8–11] In more recent studies using LC-MS, faster separations were achieved. Thus, using solid-phase extraction, Trp and 2 kynurenines were separated in 8 min,[12] whereas in another study,[13] Trp, 6 kynurenines and 9 other compounds were separated in 5 min. In this latter study, MS was essential to identify and quantify 16 very closely eluted substances. Thus, no method exists for simple and rapid separation of Trp and its kynurenine metabolites in a large number of samples without the need for a relatively expensive MS or complicated procedure. The method reported in the present study fulfils these requirements, as Trp and 6 of its kynurenine metabolites have been successfully separated and quantified within 7 min using a simple isocratic procedure (Fig. 2). Our procedure is based on an earlier method[14] developed for quantification of Trp, in which 10 Trp metabolites were too closely eluted to achieve their satisfactory separation from one another. By lowering the methanol content of the mobile phase from the 30% (v/v) level in the original method[14] to 27%, successful separation was achieved in the present study for Trp and 6 kynurenines (K, 3-HK, 3-HAA, KA, XA and AA). The run time was as short as $<7$ min at a column oven temperature of 40 °C, a flow rate of 1.4 ml/min and a final pH of the mobile phase of 2.8. As the UV chromatogram in Figure 1 (a) shows, all 7 components were resolved, including KA and XA, the resolution of which is not always possible isocratically.[17,18]

Decreasing the pH to 2.0, the methanol content to 25% (v/v), the flow rate to 1.15 ml/min and the column oven temperature to 37 °C resulted in detection of quinolinic acid (Fig. 3), but a longer run ($>12$ min). However, QA detection was variable in subsequent experiments. At higher pH, we were able initially to separate Trp, 6 kynurenines, excluding QA, and

**Table 2.** Between-day coefficients of variation for tryptophan and kynurenine metabolites.

| Analyte | CV (%) |
|---|---|
| 3-HK | 7.7 |
| K | 4.3 |
| Trp | 4.1 |
| 3-HAA | 4.4 |
| KA | 2.2 |
| XA | 3.5 |
| AA | 1.9 |

**Notes:** CV = SD/mean. Sixteen portions of a frozen (–24 °C) standard mixture containing 1 μg/ml of each of the above 7 analytes were analysed daily over a 4-week period under the standard conditions in the Materials and Methods section. Abbreviations are as in Table 1.

**Table 3.** Recovery of tryptophan and kynurenine metabolites from biological material.

| Metabolite | Human plasma | Rat serum | Rat liver |
|---|---|---|---|
| 3-HK | 83.5 ± 9.5 | 88.2 ± 2.7 | 95.4 ± 10.8 |
| K | 76.8 ± 5.9 | 87.4 ± 4.0 | 91.6 ± 2.3 |
| Trp | 95.0 ± 3.6 | 99.0 ± 7.1 | 96.8 ± 5.93 |
| HAA | 91.5 ± 5.7 | 62.3 ± 2.6 | 74.9 ± 2.1 |
| XA | 76.5 ± 9.7 | 103.3 ± 8.8 | 111.1 ± 7.6 |
| KA | 82.8 ± 3.9 | 88.7 ± 6.6 | 105.7 ± 7.3 |
| AA | 80.4 ± 7.7 | 99.9 ± 11.9 | 88.2 ± 4.2 |

**Notes:** Values are means ± SEM for each group of 4 samples, each of which represents the average of 3 different concentrations of metabolites (2, 1 and 0.5 μg/ml). Analyses were performed under the standard conditions in the Materials and Methods section. For abbreviations, see Table 1.

**Table 4.** Effects of frozen storage of perchloric acid extracts of human plasma on stability of kynurenine metabolites.

| | Percentage of values in fresh extracts | | | | |
|---|---|---|---|---|---|
| **Duration …. analyte** | **3 days** | **1 week** | **2 weeks** | **3 weeks** | **4 weeks** |
| 3-HK | 100.9 ± 1.2 | 100.6 ± 2.3 | 99.0 ± 8.8 | 100.1 ± 1.9 | 99.3 ± 1.9 |
| K | 102.6 ± 3.8 | 95.0 ± 5.6* | 103.6 ± 3.4 | 105.7 ± 2.2 | 95.7 ± 5.2 |
| Trp | 99.4 ± 0.8 | 101.0 ± 0.7 | 104.1 ± 0.7 | 100.3 ± 0.4 | 99.4 ± 4.33 |
| 3-HAA | 90.0 ± 13.0* | 99.6 ± 0.6 | 101.1 ± 3.8 | 92.6 ± 8.5* | 92.0 ± 9.6* |
| KA | 102.2 ± 3.8 | 98.5 ± 8.4* | 101.5 ± 4.1 | 106.5 ± 2.3 | 103.4 ± 2.5 |
| XA | 101.7 ±1.5 | 94.2 ± 7.2* | 98.5 ± 6.3 | 104.4 ± 15.2* | 106.4 ± 27.8* |
| AA | ND | 95.7 ± 8.6* | 103.8 ± 4.9* | 104.5 ± 4.8 | 106.0 ± 6.2 |

**Notes:** Plasmas from blood samples taken hourly over a 7 h period from 10 normal subjects undergoing the acute tryptophan loading test in a previous study[15] were used. Perchloric acid extracts of plasma were prepared as described in the Materials and Methods section and were analysed immediately. Portions of these extracts were reanalysed following storage at –24 °C for the various durations listed above. All analyses were performed under the standard conditions in the Materials and Methods section. Tests for each of the above durations were conducted on samples from 2 different subjects. Values are means ± SEM for each determination of 12 samples, except those marked with an*, which were means of 6–10 samples, due to loss of sample or undetectable levels in the fresh extracts. ND denotes not determined. For other abbreviations, see Table 1.

3 indoles (IAA, ILA and IPA) at pH 5.6 when the 10-component mixture was injected at neutral pH. These preliminary observations strongly suggest that it is possible to develop this method further to include QA (currently best determined by GC-MS[19]), other kynurenine pathway metabolites (eg, picolinic, nicotinic and quinaldic acids) and indole metabolites, although a longer run time may be necessary. Such separation has already been achieved using gradient elution over 18–50 min[9,10] and it therefore remains to be seen if this can be achieved isocratically in a shorter run.

Our rapid isocratic procedure was subjected to a number of validation criteria. Thus, the limits of detection of Trp and 6 of its kynurenine metabolites varied between 1 and 10 ng/ml (5–72 nM) (Table 1).

**Table 5.** Normal fasting human plasma tryptophan and six kynurenine values.

| **Parameter** | **Mean ± SEM (μM)** | **Range (μM)** |
|---|---|---|
| Trp | 63.3 ± 1.9 | 27–127 |
| 3HK | 9.20 ± 0.36 | 1.7–17.13 |
| K | 2.15 ± 0.12 | 0.61–6.96 |
| 3HAA | 0.28 ± 0.03 | 0.001–0.971 |
| XA | 0.16 ± 0.03 | 0.001–0.962 |
| KA | 0.12 ± 0.02 | 0.0003–0.9280 |
| AA | 0.069 ± 0.014 | 0.0005–0.8790 |

**Notes:** Values are means ± SEM with ranges for n = 114. Perchloric acid extracts were analysed under the following conditions: a methanol: sodium dihydrogen phosphate (27:73, by vol) mobile phase at pH 2.8 and a flow rate of 1.2 ml/min at 37 °C. For abbreviations, see Table 1. The data in this Table are based on the results of a previous study.[15]

This was superior to some (500–1000 nM[3] and 450–730 nM[11]), but similar to, or smaller than, other (1–30 nM[12] and 7–400nM[13]) previously reported findings. As can be seen from the ranges in Table 5, our versatile Total Chrome software was capable of detecting much smaller concentrations using its own integration facility. Linearity was observed between concentrations of pure compounds at the above limits of detection and the highest tested (7.3–13.1 μM; 1–2 μg/ml). Higher concentrations were not tested, as this would have affected the peak characteristics. Coefficients of variation between days (Table 2) varied between 1.9%–7.7%, in agreement with values in the literature (3.3%,[7] 0.9%–2.4%,[9] 3.1%,[11] <9%,[12] 4.9%–16.9%[13]). Recoveries from human plasma, rat serum and rat liver were 76.5%–90%, 62.3%–103.3% and 74.9%–111.1% respectively (Table 3). Our human plasma recovery values are in agreement with those previously reported for human plasma (98%–101%,[8] 75%–123%[13]), serum (91%–98%[7]) or urine (64%–100%[11]). Analytes in perchloric acid extracts of human plasma were stable following frozen storage at –24 °C (Table 4) for at least 4 weeks.

The non-fluorescing compounds K, 3-HK and XA were detected and quantified only by UV. Because Trp exists in biological material at relatively high concentrations, it was also detectable by UV, without the need for fluorimetric detection. The latter was used for quantification of 3-HAA, KA and AA if their levels were not sufficiently detectable by UV. Although elution of 3-HAA followed closely that of Trp, our method showed that the relatively small

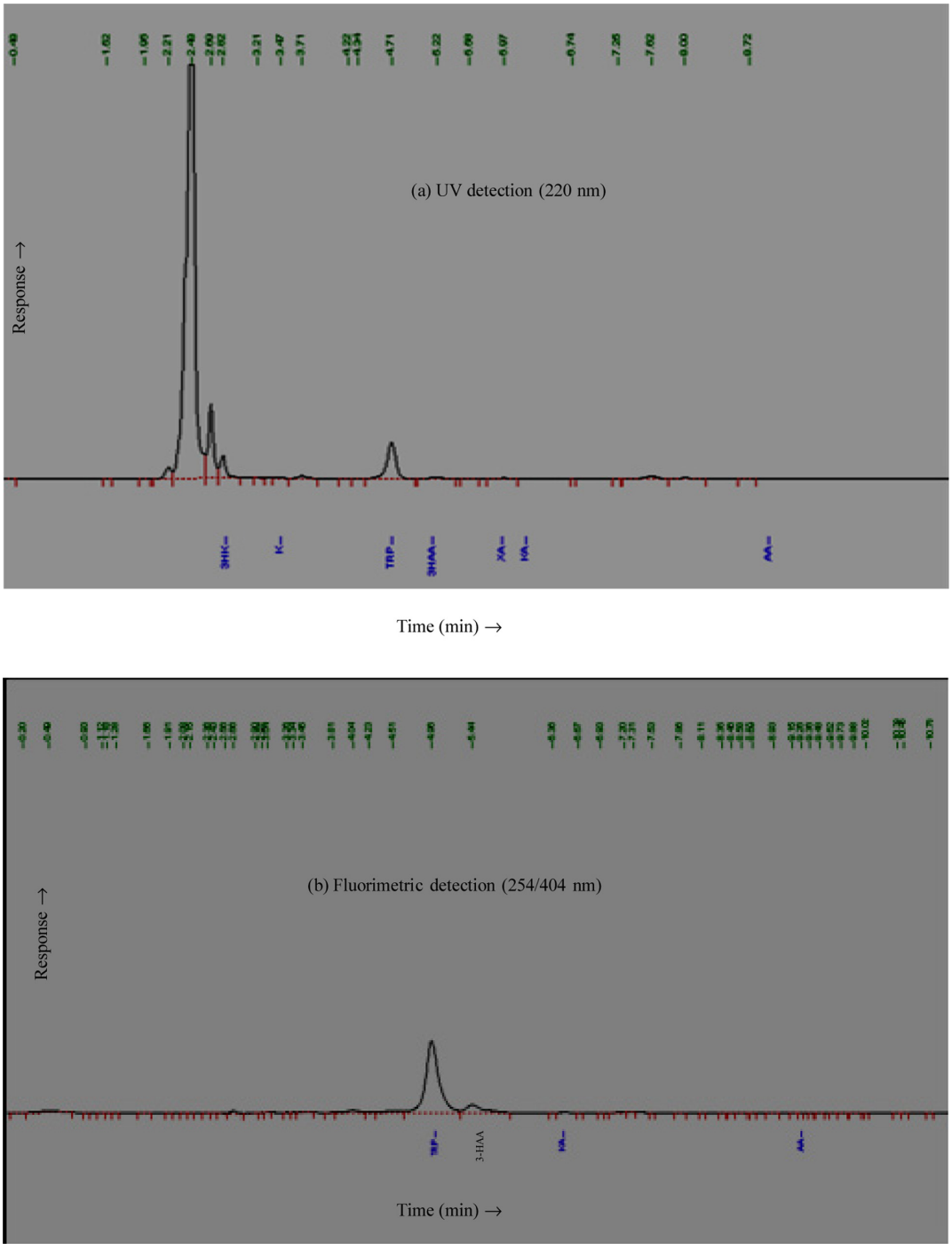


**Figure 4.** Chromatograms of a fasting human plasma sample with UV and fluorimetric detection.
**Notes:** The fasting plasma sample profiles shown are from a previous study,[15] the analyses of which were conducted at an earlier stage of development of this method. Conditions are as in Figure 1, except that the flow rate was 1.2 ml/min. The original graph format drawn by the software is given here to emphasize the small peaks. For abbreviations, see Figure 1. Note that, in this sample, KA was not detectable by UV (**a**), and AA was not detectable fluorimetrically (**b**).

3-HAA, was not covered by the much larger Trp, peak and that both compounds were clearly quantifiable separately (Fig. 4).

The normal values and ranges for Trp and 6 kynurenines in fasting human plasma shown in Table 5 represent an update of the provisional values reported previously[15] at an earlier stage of development of this method. Normal values in rat liver and serum will be reported elsewhere in studies of the effects of kynurenine metabolite administration on alcohol

consumption and metabolism. The mean human plasma values in Table 5 of the present work have been compared with those in the literature, which have been the subject of a recent review.[20] Our Trp and K values agree with previous reports in many publications (for references, see[20]). Our mean values for 3-HAA, XA, KA and AA are somewhat higher than those in several previous reports. However, this may be due to our relatively larger sample size, compared to those in previous studies. Thus, simple observations of data from our 114 subjects showed that as many as 20–36 individual subject values matched previously reported means for these 4 metabolites. Although we do not suggest that previous studies with smaller numbers of subjects have underestimated normal values, investigators may well observe larger mean values for these metabolites in larger study samples. Although, as stated above, our sample was of diverse ethnicity, the few ethnic differences observed do not explain the higher values in some of our sample subjects.

As regards 3-HK, our means are far higher than literature values, which are $<1$ µM.[12,13,21–23] This cannot be satisfactorily explained at present. The most likely explanation is that of another substance(s) co-eluting with 3-HK and the nature of such potential co-eluant clearly requires investigation. Alternatively, it is also possible that plasma [3-HK] is higher than previously reported in the literature. This higher plasma [3-HK] cannot be explained by the method of extraction, because low levels are observed using a similar $HClO_4$-based extraction procedure.[22,23] A puzzling aspect in this regard is that plasma 3-HK values previously reported do not reflect urinary excretion levels. Thus, in three studies reporting 24 h urinary excretion data of kynurenine and its metabolites under basal conditions (ie, before acute Trp loading), the first[24] reported values (in µmol/24 h) of 21.9, 78.2, 59.8, 29.7, 26.4, and 13.4 for K, 3-HK, 3-HAA, XA, KA and AA respectively, or (assuming a 24 h-urine volume of 2 L) 10.95, 39.1, 29.9, 14.85, 13.2 and 6.7 µM respectively. In the second study,[25] values of 11.65, 20.35, 13.25, 9.10 and 4.80 µmol/24 h were reported for K, 3-HK, KA, XA and AA glucuronide respectively, or 5.82, 10.17, 6.62, 4.55 and 2.4 µM respectively. The third study[11] reported values for K, 3-HK, 3-HAA, KA, XA and AA of 5.8, 37.9, 44.4, 34.4, 29.7 and 8.0 µM respectively. From these data, it is clear that, among others, 3-HK and 3-HAA are excreted in urine in greater amounts than K, in contrast to their much lower plasma levels. The ratio of [3-HK]/[K] (µM/µM) in urine in these three studies therefore were 3.57, 1.75 and 6.53 respectively. The corresponding ratio in plasma or serum estimated from studies in which both K and 3-HK data have been reported (see refs[12,13,20,23] and references cited therein) were much lower (varying between 0.043 and 0.19). This represents a huge difference between urinary and plasma ratios varying between at least 9.2-fold and at most 151.9-fold. By contrast, the plasma [3-HK]/[K] ratio in the present work (4.35) resembles more closely the above corresponding ratio in urine. As plasma levels reflect the steady state, and in view of the fact that kynurenine hydroxylase is the main enzyme of kynurenine metabolism and is not rate-limiting,[26] it is difficult to reconcile the low plasma [3-HK] levels previously reported with the much higher urinary values. While we do not question the accuracy of previous estimates and recognise that, in all probability, our values for plasma 3-HK may be unjustifiably high, nevertheless further work is clearly required to resolve this plasma/urine discrepancy. This is the more important given the recent discussion[27] of the importance of accurate analytical methodology in scientific research.

In conclusion, we believe that the present rapid and simple isocratic HPLC procedure for separation and quantification of tryptophan and 6 of its kynurenine metabolites will contribute to the analytical methodology in the tryptophan field. In particular, its speed renders this method suitable for processing large numbers of samples in high throughput work. Our preliminary studies with other Trp metabolites strongly suggest that this method could be developed further to include other kynurenines and indole metabolites and we encourage other investigators to contribute to this development.

## Acknowledgements

This study was supported by a project grant from the Wellcome Trust (069301). The experimental work of this study was initiated at the Cardiff and Vale NHS Trust's Biomedical Research Laboratory, Whitchurch Hospital, Cardiff, Wales, which has since been decommissioned, but this work was continued at the University of Wales Institute Cardiff (UWIC). We thank Cardiff University and its Department of

Psychological Medicine for provision of facilities and Jennifer A Turner and Alex Steptoe for skilful technical assistance.

## Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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