Plasma tryptophan, kynurenine and 3-hydroxykynurenine measurement using automated on-line solid-phase extraction HPLC–tandem mass spectrometry


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Abstract

Tryptophan metabolism plays a key role in several (patho) physiological conditions. In order to study the clinical importance of tryptophan and its predominant metabolites (kynurenines), it is important to be able to measure large series of samples with high accuracy and reliability. We aimed to develop a high-throughput on-line solid-phase extraction-liquid chromatographic–tandem mass spectrometric (XLC–MS/MS) method that enables the measurement of tryptophan and its metabolites kynurenine and 3-hydroxykynurenine in plasma. Fifty microliters plasma equivalent was pre-purified by automated on-line solid-phase extraction, using strong cation exchange (PRS, propylsulphonic) cartridges. Chromatographic separation of the analytes and deuterated analogues occurred by C18 reversed phase chromatography. Mass spectrometric detection was performed in the multiple reaction-monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionization. Total run-time including sample clean-up was 8 min. Intra- and inter-assay analytical variations were less than 9%. Linearity in the 0.11–1200 (tryptophan) and 0.050 and 0.023–45 μmol/L (kynurenine and 3-hydroxykynurenine, respectively) calibration range was excellent (R > 0.99). Detection limits were 30 nmol/L for tryptophan, 1 nmol/L for kynurenine and 5 nmol/L for 3-hydroxykynurenine. Reference intervals for 120 healthy adults were 45.5–83.1 μmol/L (tryptophan), 1.14–3.02 μmol/L (kynurenine), <0.13 μmol/L (3-hydroxykynurenine) and 19.0–49.8 for tryptophan-to-kynurenine ratio. Blood sampling for tryptophan and tryptophan-to-kynurenine ratio should be performed before breakfast, due to biological variation during the day. This study describes how plasma tryptophan, kynurenine and 3-hydroxykynurenine can be measured accurately and precisely by automated high-throughput XLC–MS/MS.

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1. Introduction

The essential amino acid L-tryptophan is in the body for the greater part used for protein synthesis. Tryptophan metabolism highly regulates levels of important biogenic amines such as serotonin, kynurenes, melatonin and trace amines. Tryptophan is predominantly metabolized towards neuroactive kynurenines following the kynurenine pathway. Alternative tryptophan pathways are the conversion to serotonin and melatonin, or to tryptamine and kynuramines [1,2]. A simplified scheme of the tryptophan metabolism is shown in Fig. 1.

The first metabolizing step in the kynurenine pathway is the oxidative opening of the indole ring. This reaction is catalyzed by two haem-dependent enzymes namely tryptophan 2,3-dioxygenase (TDO; EC 1.13.11.11) and indoleamine-2,3-dioxygenase (IDO; EC 1.13.11.17). TDO is almost entirely localized in the liver, while IDO is found in both the periphery (macrophages) and the central nervous system (astrocytes, infiltrating macrophages, microglia and dendritic cells). The kynurenine-to-tryptoehan ratio reflects the IDO activity [3] and is therefore often used in research to monitor the tryptophan metabolism. The expression of IDO is induced during inflammation and infectious diseases by proinflammatory stimuli and T-helper-cell (Th1-cell) cytokines such as interferon-γ. Furthermore IDO appears to play a role in immunoregulation and communication between the immune and nervous systems [1,2,4–6] and is suggested to mediate the tumor immune escape [7]. Another kynurenine pathway metabolite, 3-hydroxykynurenine,
causes neuronal damage and cell death mediated by free radicals in a neuronal hybrid cell line and the rat striatum [2,8]. In addition, it activates Th1-cells in immune activation.

Tryptophan metabolism plays a key role in several (patho)physiological processes, such as the endocrine manifestations of neuroendocrine tumors, i.e., carcinoid tumors with excessive serotonin production [9], mood disorders such as depression with decreased serotonin production [10], inflammation, immune-activation, transplantation [1,2,4–6] and pregnancy [10]. The metabolic fate of tryptophan (Fig. 1) is dependent on factors such as tryptophan availability and enzyme activities, and determines the synthesis of tryptophan-derived indoles, i.e., serotonin and melatonin. In order to study the (patho)physiological mechanisms of this metabolism it is important that tryptophan and its major degradative metabolite kynurenine can be easily quantified in small sample volumes with high accuracy and reliability.

Several methods for detection and quantification of tryptophan and its metabolites have been described using high performance liquid chromatography (HPLC) with different detectors [3,11–16], including mass spectrometry (MS) [5,17,18]. Most of the published HPLC methods are time consuming and require large sample volumes, because separate methods are required to measure all components. HPLC coupled with electrospray ionization and tandem MS (MS/MS) is a specific and sensitive method for the detection of many endogenous compounds in biological matrices [19]. Capillary LC–MS/MS has been described for multicomponent analyses [5,18]. However, potentially considerable improvements can be made in sample throughput and accuracy by reducing the total analysis time by automation of the extraction process and coupling it directly to the chromatographic system. This process is termed on-line solid-phase extraction-liquid chromatography (XLC).

This study describes a high-throughput, sensitive, specific, and automated XLC–MS/MS method that enables simultaneous extraction, concentration, separation, and mass selective detection of tryptophan and its metabolites kynurenine and 3-hydroxykynurenine in plasma. The tryptophan-to-kynurenine ratio in plasma has been used as a diagnostic tool in several (patho)physiological conditions, i.e., leukemia [20], irritable bowel syndrome [21] and prostate cancer [22], without taking its biological variation into account. Since tryptophan plasma levels follow a circadian rhythm [23], this study also includes the measurement of the biological intra- and inter-day variation of tryptophan, kynurenine and their ratio.

Fig. 1. Schematic view of tryptophan metabolism. Abbreviations: IDO: indoleamine-2,3-dioxygenase; TDO: tryptophan-2,3-dioxygenase; IFN-γ: interferon-gamma; MAO: monoamine oxidase; KAT: kynurenine amino-transferase; QPRT: quinolinic acid phosphoribosyltransferase; NAD+: nicotinamide adenine dinucleotide.
2. Experimental

2.1. Reagents

HPLC-grade acetonitrile and methanol were obtained from Rathburn Chemicals Ltd.; ammonium formate 99.995% from Sigma–Aldrich Ltd.; formic acid 98–100% ultrapure from BDH Laboratory Supplies; isopropanol, ammonium acetate, hydrochloric acid and glacial acetic acid from Merck KGaA; and ammoniumhydroxide from ICN Biomedicals BV. Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure.

1-tryptophan, p-l-kynurenine and p,l-3-hydroxykynurenine were purchased from Sigma–Aldrich Ltd. The deuterated internal standard 1-tryptophan-2′,4′,5′,6′,7′-d5 was from C/D/N Isotopes and l-kynurenine-3′,4′,5′,6′-d4 was purchased from Buchem BV. 3-hydroxykynurenine-d2 was homemade [24].

2.2. Stock solutions and samples

We prepared stock solutions in 0.08 mol/L acetic acid. Stock solutions were serially diluted and used to form calibrators and low, medium, and high quality-control samples in pooled plasma via enrichment. Blank plasma was obtained by dialysis, based on a previously described method [25] of pooled plasma from healthy volunteers.

Plasma samples were obtained by venipuncture in 10 mL vacutainer tubes (Becton Dickinson) containing K2EDTA solution as anticoagulant. After centrifugation, plasma was transferred to glass tubes, and samples were stored at −20 °C until analysis.

Before analysis, we mixed aliquots of plasma samples (250 μL) with 50 μL internal standard working solution (300 μmol/L in diluted acetic acid for tryptophan and 5 μmol/L for kynurenine and 3-hydroxykynurenine) and diluted them with 200 μL water. We placed sample vials in the autosampler, and 50 μL of each sample (equivalent to 25 μL of plasma) was injected. Required sample volume for automatic injection can be scaled down to 50 μL by using μL injection mode.

2.3. Instrumentation

We used a Spark Holland Symbiosis® on-line SPE system for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (2 high-pressure dispensers), and an HPLC pump, as explained previously [26,27]. The ACE module contains 2 connectable 6-way valves and a SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and clean-up. The integrated HPLC pump was a binary high-pressure gradient pump.

We used Isolute® PRS (propylsulphonic acid based strong cation exchange) 10 mm × 1 mm SPE cartridges (Argonaut) for sample extraction and performed HPLC by use of an Atlantis dC18 column (particle size 3 μm, 2.1 mm internal diameter by 100 mm; Waters). Column temperature was controlled at 25 °C with a Mistral Column Oven (Spark Holland). Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters). All aspects of system operation and data acquisition were controlled using Masslynx v4.1 software with automated data processing using the Quanlynx Application Manager (Waters).

2.3.1. On-line SPE

We performed on-line SPE following a similar method as described before [26,27]. The Symbiosis system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted. The analytes were eluted directly on the analytical column. After conditioning, the sample was passed on to the extraction cartridge using 0.01 mol/L HCl as the loading solvent, and wash solvents were applied. Elution was performed by 300 μL 50 mM ammoniumformate pH 3, delivered by one of the high-pressure dispensers. The eluate is directly mixed with the chromatographic mobile phase and transported to the analytical column for chromatographic separation. Simultaneously, the cartridge was flushed and prepared for reuse. Processing of subsequent plasma samples was carried out in parallel.

2.3.2. Liquid chromatography

The binary gradient system consisted of 0.2% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Gradient elution was performed according to the elution program as shown in Table 1. Gradients applied were linear; flow rate was 0.30 mL/min. During SPE elution with a flow rate of 0.25 mL/min, the chromatographic flow rate was decreased to 0.05 mL/min to maintain a similar total flow rate of 0.30 mL/min. Column temperature was kept at 25 °C.

2.3.3. Mass spectrometry

In positive ionization mode, tryptophan, kynurenine and 3-hydroxykynurenine (and their respective deuterated internal standards) were protonated to produce ions at the form [M + H]+: tryptophan: m/z 205, tryptophan-d5: m/z 210; kynurenine: m/z 209, kynurenine-d4: 213, 3-hydroxykynurenine: m/z 225 and 3-hydroxykynurenine-d2: 227. Upon collision-induced dissociation (CID), these precursor ions produced characteristic product ions of m/z 188, m/z 192, m/z 94, m/z 98, m/z 110 and m/z 111, respectively. We developed a multiple reaction monitoring (MRM) method using a dwell time of 10 ms and an interchannel delay of 5 ms. Recently, the use of additional MRM transitions for absolute confirmation of the presence of a compound in an analytical method has been proposed (e.g., EU directive 2002/657/EC). For this reason, we used mass transitions m/z 205 → 146 and 205 → 118 (tryptophan), 209 → 192 and 209 → 146 (kynurenine), and 225 → 208 and 225 → 162 (3-hydroxykynurenine) as qualifiers.

2.4. Quality control and method validation

2.4.1. Selectivity

We verified the identities of sample tryptophan, kynurenine, and 3-hydroxykynurenine peaks by analysis of the compound specific mass spectra after addition of calibrator (standard addition).

2.4.2. Detection limits

For plasma, we determined detection limits [limit of detection (LOD)] and quantification limits [limit of quantification (LOQ)] by injecting serially diluted samples containing tryptophan, kynurenine and 3-hydroxykynurenine. LOD was defined as the injected

<table>
<thead>
<tr>
<th>Time (mm:ss)</th>
<th>Flow (mL/min)</th>
<th>Solvent A%</th>
<th>Solvent B %</th>
</tr>
</thead>
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<td>0</td>
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<td>0</td>
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<tr>
<td>08:00</td>
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<td>0</td>
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</table>

Solvent A: 0.2% formic acid; Solvent B: acetonitrile.
amount that produced a signal-to-noise ratio of 3 and LLOQ as the injected amount that produced a signal-to-noise ratio of 10. We estimated the percentage of carryover between sequential analyses performed on new SPE cartridges by alternating injections of blanks and plasma samples with high concentrations of the compounds.

### 2.4.3. Linearity and imprecision

We plotted the ratios of analyte peak area to internal standard peak area against metanephrine at the following 8 concentrations: 4.85, 9.70, 19.39, 38.78, 77.57, 290.88 and 1163.54 μmol/L for tryptophan, 0.19, 0.38, 0.76, 1.52, 3.04, 11.41 and 45.65 μmol/L for kynurenine, and 0.18, 0.35, 0.71, 1.41, 2.83, 10.60 and 42.39 μmol/L for 3-hydroxykynurenine. On 20 different days, we prepared and measured fresh calibration lines. The lines were calculated by use of QuanLynx software and least-squares linear regression. We applied the Clinical and Laboratory Standards Institute (CLSI) EP-6P protocol [28] to test the linearity of the method. The dilutional linearity of the assay was performed in duplicate by serial dilution of enriched plasma samples with water.

We determined intra- and inter-assay variation by the use of 3 pooled samples with tryptophan, kynurenine and 3-hydroxykynurenine in low, medium, and high concentrations and obtained intra-assay imprecision from 20 replicates measured in a single series and inter-assay imprecision from 20 different assays over a 3-week period.

### 2.4.4. Recovery

We estimated mean relative recoveries by the addition of tryptophan, kynurenine and 3-hydroxykynurenine to plasma in low, medium, and high concentrations and measured recoveries in 8 replicates of these samples by using 2 cartridges placed in series. Furthermore XLC versus LC recovery was measured using aqueous standards.

### 2.4.5. Stability

Samples with low, medium, and high concentrations of added tryptophan, kynurenine and 3-hydroxykynurenine were measured in triplicate after different storage conditions. The 1st set was assayed immediately and served as reference point; other sets were stored at 10 °C (autosampler temperature), 4 °C and room temperature for 16, 24, 48 and 72 h and 7 days. The remaining samples were frozen at −20 °C, and stability was investigated after 1–3 freeze–thaw cycles.

### 2.4.6. Biological variation, reference values, and patient samples

We determined biological inter-day variation by analyzing plasma obtained from 16 healthy individuals (12 men, 6 women, age range 20–56 years, median age 35 years), on 5 consecutive days (at 09:00 h). We determined biological intra-day variation at 5 times during 1 day (08:30 (before breakfast), 10:30, 12:30, 14:30, and 16:30 h) using plasma from 26 healthy individuals (13 men, 13 women, age range 21–59 years, median age 38 years) following a serotonin-low standardized diet. Calculations of the mean plasma concentrations and their 95% confidence intervals (non-parametric) were performed according to standard procedures. The plasma sample collected before breakfast (08:30 h) served as baseline concentration. Paired Student’s t-tests were used to determine the significance of longitudinal changes in absolute analyte concentrations, with respect to the baseline concentration as well as the preceding concentration. One-sided t-tests were considered significant at P<0.05. Statistics were calculated using SPSS version 14.

Tryptophan, kynurenine, 3-hydroxykynurenine and tryptophan-to-kynurenine ratio reference intervals were based on the analysis of 120 plasma samples derived from healthy individuals (36 men, 84 women, age range 38–83 years, median age 55), during the PREVEND study [29,30]. These studies were approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

### 3. Results

#### 3.1. Quality control and method validation

##### 3.1.1. Chromatography and selectivity

Total sample analysis time, including extraction, was 8 min. Complete chromatographic separation can be achieved by reversed phase chromatography. Deuterated internal standards can be used, since the mass spectrometer monitors precursor as well as product ions with high analytical specificity. Fig. 2 shows mass chromatograms of a plasma sample derived from a healthy subject as total ion current (A) of all MRM transitions used and as the separate MRM transitions for 3-hydroxykynurenine (B), kynurenine (C), tryptophan (D) and their respective internal standards obtained by XLC–MS/MS in MRM. We confirmed the identities of the compounds by standard addition of the plasma sample, resulting in the total ion current chromatogram in Fig. 2E and the specific mass spectra in Fig. 2F–H.

##### 3.1.2. Detection limits

LLOD was 30 nmol/L for tryptophan, 1 nmol/L for kynurenine, and 5 nmol/L for 3-hydroxykynurenine. Respective quantification limits (at a signal-to-noise ratio of 10) were 110, 50, and 23 nmol/L, with CVs of 13.4, 18.8, and 19.7%, respectively.

Cartridges could be reused up to 15 times, with carryover <0.1% observed between sequential analyses performed on reused SPE cartridges, by applying additional washing steps in the method.

##### 3.1.3. Linearity and imprecision

Plasma calibration curves and control samples were run with every batch of patient samples. Linearity was excellent over the respective calibration ranges, with corresponding correlation coefficients (R²) consistently >0.99 for all 3 compounds. Plasma calibration curves were reproducible between days, with R² > 0.99. Mean analytical intra- and inter-assay repeatability and reproducibility for enriched pooled plasma in low, medium, and high concentrations are shown in Table 2. Intra-assay CV (n=20) was 1.7–3.6% (tryptophan), 3.8–7.2% (kynurenine), and 4.3–8.8% (3-hydroxykynurenine). Inter-assay CV (n=20) was 1.7–7.0% (tryptophan), 2.9–5.6% (kynurenine), and 5.3–8.2% (3-hydroxykynurenine). Plasma samples with high tryptophan, kynurenine and/or 3-hydroxykynurenine concentrations that exceed the calibration range could be diluted up to 100 times.

##### 3.1.4. Recovery

Recoveries ranged from 3.3 to 4.1% (tryptophan), 37.8 to 48.7% (kynurenine), and 30.9 to 47.6% (3-hydroxykynurenine).

##### 3.1.5. Stability

Tryptophan and kynurenine were stable in plasma stored up to 7 days at 10, 4 °C or room temperature. 3-Hydroxykynurenine is stable in plasma up to 3 days at the same conditions. No changes in measured concentrations were observed in plasma that had been subjected to 1, 2, or 3 freeze–thaw cycles. Stability data (n=3) are not shown.

##### 3.1.6. Biological variation, reference values, and patient samples

Biological intra-day CVs (n=26) were 4.4–15.1% (tryptophan), 2.4–17.1% (kynurenine), and 33.3–116.7% (3-hydroxykynurenine), as shown in Table 2. Mean tryptophan, kynurenine, tryptophan-to-kynurenine ratio and 3-hydroxykynurenine concentrations, with
Fig. 2. Total ion current mass chromatograms and mass specific chromatograms obtained in multiple reaction monitoring (MRM) mode by XLC–MS/MS for plasma tryptophan (Trp), kynurenine (Kyn), and 3-hydroxykynurenine (3-HK) and their deuterated internal standards. (A–D) Chromatograms of plasma from a healthy subject. (E–H) Chromatograms of plasma from a healthy subject spiked with calibrators. (A and E) Total ion current mass chromatograms of all MRM transitions used. Retention times are 4.8, 4.5 and 3.0 min for Trp, Kyn and 3-HK, respectively; (B and F) Mass specific chromatograms of 3-HK and its deuterated internal standard 3-HK-d2 (m/z 225 → 110 and 227 → 111, respectively); (C and G) Mass specific chromatograms of Kyn and its deuterated internal standard Kyn-d4 (m/z 209 → 94 and 213 → 98, respectively); (D and H) Mass specific chromatogram of Trp and its deuterated internal standard Trp-d5 (m/z 205 → 188 and 210 → 192, respectively). All signals are normalized to full scale for the highest peak in the window. Retention time is indicated in min. The y-axis shows the peak abundance which is normalized to percentages relative to the highest peak in the chromatogram.

Table 2
Intra- and inter-assay imprecision of the XLC–MS/MS method for plasma tryptophan (TRP), kynurenine (KYN), and 3-hydroxykynurenine (3HK).

<table>
<thead>
<tr>
<th></th>
<th>Mean analytical variation (n=20)</th>
<th>Mean biological variation (n=16)</th>
<th>Mean biological variation (n=26)</th>
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<tr>
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<td>Inter-assay</td>
<td>Intra-day (n=16)</td>
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<tr>
<td></td>
<td>Mean (μmol/L)</td>
<td>SD (μmol/L)</td>
<td>CV (%)</td>
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<tr>
<td>TRP</td>
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<tr>
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<tr>
<td>High</td>
<td>29.63</td>
<td>1.28</td>
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Analytical variation was calculated by measuring each sample 20 times per day (intra-assay) and in 20 different assays (inter-assay). Intra- and inter-day mean biological variation was calculated from results from healthy individuals at 5 times during a day (08:30, 10:30, 12:30, 14:30, and 16:30 h) and on 5 consecutive days at 05:00 h.
concentrations decrease during the day is new and should be considered. As far as we know, the finding that kynurenine concentrations decrease during the day with significant changes \( (P < 0.05) \), because of the tryptophan content of food products. Kynurenine concentrations decrease significantly \( (P < 0.05) \) during the day. Consequently, the tryptophan-to-kynurenine ratio follows the same pattern as the tryptophan concentration during the day with significant changes \( (P < 0.05) \). 3-Hydroxykynurenine concentration shows a not significant decrease in concentration during the day. Biological inter-day CVs \( (n = 16) \) were 2.7–10.6%, 5.1–15.7% and 33.3–169.9%, respectively. Concentrations remained constant during the 5 days (data not shown).

For 120 reference samples, reference intervals were calculated with EP evaluator in a transformed parametric manner according to CLSI C28-A2 [32]. Reference intervals were 45.5–83.1 \( \mu \)mol/L (tryptophan), 1.14–3.02 \( \mu \)mol/L (kynurenine), and <0.13 \( \mu \)mol/L (3-hydroxykynurenine). Tryptophan-to-kynurenine ratio reference values were 19.0–49.8, calculated in a parametric manner.

4. Discussion

Variation in tryptophan concentration due to inducible IDO/TDO degradation influences the formation of important metabolites such as serotonin. Measurement of tryptophan and its kynurenine metabolites was shown to be relevant in many pathological conditions, i.e., carcinoid tumors with excessive serotonin production [9], mood disorders such as depression with decreased serotonin production [10], inflammation, immune-activation, transplantation [1,2,4–6] and pregnancy [10]. This study shows that tryptophan, kynurenine and 3-hydroxykynurenine can be measured accurately and precisely and with high-throughput using on-line SPE coupled to HPLC with MS/MS detection. In addition, the study shows that for using tryptophan and the tryptophan-to-kynurenine ratio in diagnostics or research, blood sampling has to take place before breakfast, since both markers have significant biological variation during the day. For tryptophan concentrations in plasma this circadian rhythm has been shown before [23], which is probably caused by the tryptophan content of food. As far as we know, the finding that kynurenine concentrations decrease during the day is new and should be subjected to future research on tryptophan, kynurenine and its ratio.

Measurement of plasma tryptophan and kynurenine has been executed earlier, especially with LC. The main problems for this analysis are the amphoteric characteristics of tryptophan and the concentration range difference between tryptophan and kynurenine and especially 3-hydroxykynurenine. Previously described methods, including HPLC with electrochemical, UV and fluorescence detection [3,12,16], have certain drawbacks such as labor intensity and long analysis times due to manual sample preparation. Recently, analysis times have been reduced [11] and more specific and sensitive tandem mass spectrometric detection methods have been developed [5,17,18] with reduced manual sample preparation steps. However, for the simultaneous measurement of tryptophan, kynurenine and 3-hydroxykynurenine no fast and sensitive detection method was available [15]. Since 3-hydroxykynurenine seems a potential interesting marker for the clinic and has the same characteristics as kynurenine, this component was added to our analysis. Main advantages of XLC–MS/MS are ease of handling, portability, and reduction of cost per sample, because of reduced sample preparation time, high-throughput, cheaper cartridges, and reuse of cartridges [26,27]. In addition, automated sample preparation reduces analysis time and analytical variation caused by differences in manual sample pretreatment.

Because tryptophan, kynurenine and 3-hydroxykynurenine contain the same functional charged amino group, a selective SPE process can be achieved using cation exchange. Strong cation exchange media are especially suitable for these weak bases. Isolute PRS (SCX2) cartridges (Argonaut) maintain a permanent negative charge on the sorbent. PRS has very little non-polar character and is the cationic exchange sorbent (propylsulphonic acid) of choice, since the elution solvent is totally aqueous. Analytes are eluted by increasing the ion strength. For complete separation of the three components, reversed phase chromatography is used with care, since the gradient used 100% water phase, which can damage the column. Furthermore, desolvation is more difficult than with an organic solvent. However, for this purpose MS/MS detection provided enough sensitivity. Unique precursor–product ions are used for qualification and quantification. Interference with the same MRM transitions is eliminated chromatographically, whereas additional qualifiers enhance specificity of the detection method.
The XLC–MS/MS method shows excellent analytical performance. Recoveries are consistent, although below 5% for tryptophan, since the method has been optimized for kynurenine and 3-hydroxykynurenine analysis in the same run. However, tryptophan concentrations are much higher than that of kynurenine and 3-hydroxykynurenine, which means the peak abundance is still higher than that of kynurenine and 3-hydroxykynurenine. In addition, LLOQ of tryptophan is less low, although limits of quantification are still better in comparison with non-mass spectrometric methods.

The method allows reproducible quantification of plasma tryptophan, kynurenine and 3-hydroxykynurenine. Analytical variation is <9%, owing to automation of sample preparation. In addition, tophane, kynurenine and 3-hydroxykynurenine. Analytical variation methods.

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References