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Quantitation of tryptophan, kynurenine and kynurenic acid in human plasma by capillary liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

Concentrations of tryptophan and its metabolites in plasma are of great interest in determining proper diagnosis and medication of several neurological diseases like, for example, Alzheimer's disease. A method of standard addition was developed to determine total level of tryptophan and two of its metabolites, kynurenine and kynurenic acid, in human plasma by capillary liquid chromatography–electrospray ionization tandem mass spectrometry. Plasma samples were simply deproteinized by addition of diluted perchloric acid. Samples were then mixed with trichloroacetic acid and injected onto a capillary column. Analytes were separated by a fast gradient elution of the injected samples. Detection was performed by sheathless electrospray tandem mass spectrometry in the multiple reaction monitoring mode. Linear calibration curves were obtained for spiked plasma sample with up to 100% of the expected analytes concentrations. The determined concentrations were well within ranges previously reported (i.e., 6 n*M*–95 μ*M*) and limit of detections were around 3 n*M* for each analyte.
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Keywords: Tryptophan; Kynurenine; Kynurenic acid

1. Introduction

L-Tryptophan is one of 10 essential amino acids and plays an important role in protein synthesis and as precursor of many biologically active substances such as serotonin (5-HT). Tryptophan is predominately metabolized in the kynurenine pathway (Fig. 1) by mammalian peripheral tissues [1]. Degradation of tryptophan by a cytokine induced indoleamine-2,3-dioxygenase to formyl kynurenine seems to enhance when the cellular immune system is activated [2–4]. Thus monitoring of this enzyme activity is becoming increasingly important. The kynurenine pathway metabolites also seems to be involved in the pathogenesis of several neurologic disorders such as Alzheimer's disease [5], Parkinson's disease [6,7], schizophrenia [8] and epilepsy [9].

Several liquid chromatography (LC) methods for detection and quantitation of tryptophan and its metabolites in various biological samples have been described. These methods have employed electrochemical [10], ultraviolet [11], fluorometric [12] and mass spectrometric [13] detection.

LC coupled with electrospray ionization (ESI) and

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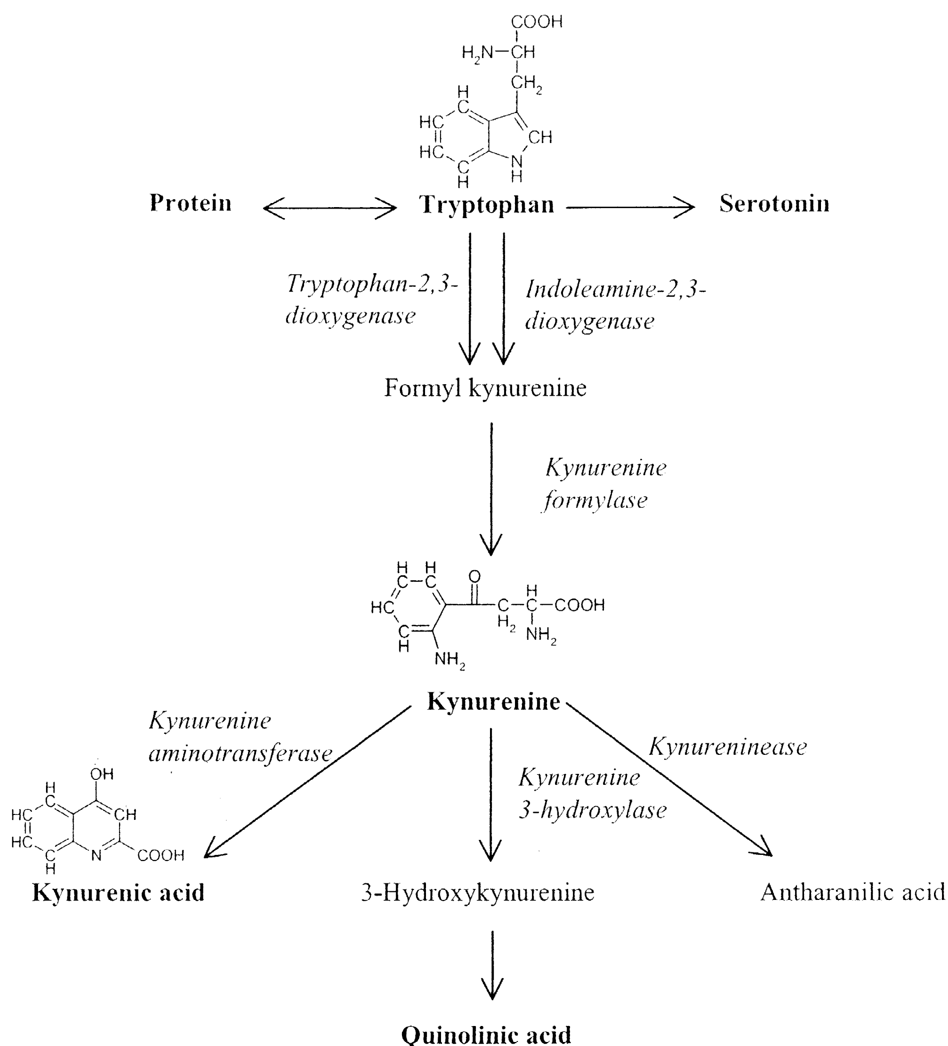

Fig. 1. Abbreviated pathway of tryptophan metabolism showing pathways to protein, serotonin and kynurenine.

tandem mass spectrometry (MS–MS) is a specific and sensitive method for detection of many endogenous compounds in biological matrices [14]. However, polar compounds like tryptophan and its metabolites generally have lower ESI responses than less polar compounds probably due to their lower surface activity during the electrospray droplet formation [15]. Another aspect is that the biological matrixes can dramatically effect ionization and severely limit detection and thus challenge the quantitation. The matrix effect is again more emphasized for polar compounds [16]. In addition lower retention times of polar compound in reversed-phase liquid chromatography causes more interference from the injection bulk, e.g., salts [17].

An ideal calibration standard should be prepared in a blank matrix, however when analyzing endogenous substances in plasma it is not always possible to prepare a "true" blank [18]. The method of standard addition can thus be used to provide a good calibration plot for quantitative analyses.

In this paper, we present a method of standard addition for the analysis of tryptophan and kynurenine and kynurenic acid by capillary LC–ESI-MS–MS. As far as we know this is the first method published using LC in combination with electrospray

tandem mass spectrometry for simultaneous quantitation of tryptophan and its metabolites in human plasma. The selectivity enabled by tandem mass spectrometry in comparison with previously referred detection methods was evaluated.

2. Experimental

2.1. Materials

L-Tryptophan was purchased from Fluka (Buchs, Switzerland). Kynurenine, kynurenic acid and trichloroacetic acid (100%, w/v, solution) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, formic acid, perchloric acid, diiodomethane and trichloroethylene were purchased from Merck (Darmstadt, Germany). All used chemicals are of analytical grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). Human plasma was purchased from the blood bank at the University Hospital (Uppsala, Sweden). All fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Preparation of capillary column

The column, 150×0.5 mm, was slurry packed in the laboratory with 3 μm C_{18} particles (ODS AQ, YMC Europe, Scermbeck/Weselerwald, Germany) in fused-silica. A 5% (w/w) slurry of the packing material in diiodomethane–trichloroethylene (38:62, v/v) was homogenized in an ultrasonic bath for about 1 min. A packing reservoir connected to an empty capillary was filled with the slurry. At the other end the capillary was secured with two layers of frits, a piece of glass fiber paper (Whatman GF/A W & R, Balston, UK) and a metal screen filter with 2 μm porosity (VICI, Schenkon, Switzerland) in a union. The packing procedure began with delivering 0.1 ml/min acetonitrile by an LC pump (PU-980, Jasco, Tokyo, Japan) into the reservoir until the pressure reached 250 bar, which was maintained on the system for 1 h using constant pressure mode followed by a slow pressure release through the column.

2.3. Standard and sample preparation

Micromolar stock solutions of tryptophan, kynurenine and kynurenic acid were prepared in water and stored at −80 °C. Working multiple standards solutions were prepared in water by using freshly thawed stock solutions.

Frozen plasma was thawed at room temperature. The plasma sample (500 μl) was spiked with the working standards solution and water to have desired concentrations of analytes and a total volume of 550 μl. The spiked plasma sample was deproteinized by adding 1/10 (v/v) of 2.4 *M* perchloric acid. The acidified plasma was vortexed at high speed for 1 min and centrifuged for 5 min at 3500 *g* and 5 °C. The supernatant (350 μl) was transferred to another capped tube and vortex-mixed with 70 μl of 6% trichloroacetic acid (TCA) to obtain a total concentration of 1% (w/v) TCA. The tube was vortexed and centrifuged for 5 min at 23 500 *g* and 5 °C. The sample was directly injected onto the separation column. The sample was kept in darkness at 5 °C for later injections.

2.4. Chromatography

An LC system (LC Packings Ultimate capillary HPLC system, Amsterdam, The Netherlands) was used for delivery of the mobile phases A (2.1% formic acid, pH 2), B (2.1% formic acid, 40% acetonitrile) and C (2.1% formic acid, 90% acetonitrile). An electrically actuated six-port switching valve from Valco (Schenkon, Switzerland) with a 7 μl sample loop was used as the injector valve and a second electrically actuated four-port switching valve from Valco was used as the flow direction valve. An active program written in Ultichrom software (Amsterdam, The Netherlands) controlled the LC system and the switching valves. The program sends also a start signal to the mass spectrometer. The LC system delivered 12 μl/min after split of the mobile phases to the capillary column via the injector valve. Immediately after start of the program (at 1 s) the gradient program, the sample injection and collection of data from the mass spectrometer was started. Because of delay time for the gradient to reach the column the analytes were pre-concentrated and desalted on the head of the column. The eluent from

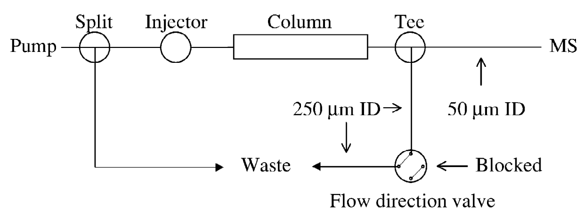

Fig. 2. Experimental set-up of the LC–MS–MS system. Column: 150×0.5 mm packed with 3 μm C_{18} particles (ODS AQ, YMC). Capillary connecting the tee to the electrospray source: 50 μm I.D. Capillary connecting the tee to the waste: 250 μm I.D. Other details as in Section 2.

the column diverted from the mass spectrometer by the flow direction valve for the initial desalting step. The set-up is shown in Fig. 2. The tee between the column and the transfer capillary to the ion source is connected to the flow direction valve. The inner diameter (250 μm) of the tubes connecting the tee to the waste via the flow direction valve was larger than the inner diameter (50 μm) of the fused-silica capillary connecting the tee to the ion source. Because of difference in inner diameter of the tubes the eluent in the initial desalting step diverted from the mass spectrometer and directed into the waste. After desalting for 5.75 min the flow direction valve blocked the flow path to the waste and the eluent forced into the ion source. A linear gradient of 100% A to 100% B under 5 min was applied for rapid separation of the analytes. The program continues with pumping mobile phase B for 3 min and mobile phase C for 5 min to clean the system from more hydrophobic substances present in the plasma and finally pumping A for 10 min to reach equilibrium in the column for the next injection.

Ten minutes after starting the gradient the flow-rate was increased to 15 μl/min for 11 min to reduce cleaning and equilibrium time.

2.5. Mass spectrometry

A PE-Sciex API 365 triple quadrupole mass spectrometer (PE-Sciex, Concord, Canada) equipped with pneumatically assisted electrospray ionization interface (Ionspray) was used for detection of analytes.

Reproducible retention times and well separated peaks by the chromatographic system made it possible to synchronize detection of the ions with their retention times. Detection of one ion gave a better signal-to-noise ratio and decreased detection limit of the analytes. The dwell time was set to 400 ms using multiple reaction monitoring (MRM). The mass spectrometer was operated in the positive ion mode. The first quadruple was set to transmit the protonated molecular ions at m/z 206 for the second most abundant isotope of tryptophan, at m/z 209 for kynurenine and at m/z 190 for kynurenic acid. The second most abundant isotope of tryptophan was selected because of the relatively high concentration of tryptophan (the most abundant isotope would saturate the detector). The protonated molecular ions were fragmented by collision-induced dissociation (CID) with nitrogen as gas.

The most abundant fragment ions, m/z 189 for tryptophan, m/z 192 for kynurenine and m/z 162 for kynurenic acid, were selected for detection. The orifice and ring potentials were optimized to get highest signals for each analyte.

2.6. Quantitation

The plasma sample was spiked with up to 100% of the expected analytes concentrations; tryptophan 62 μ*M*, kynurenine 2.7 μ*M* and kynurenic acid 50 n*M*.

Integration of peaks areas and calculations of the data were performed with MacQuan software (PE-Sciex).

Linear least-squares regression of added volumes of the analytes into the plasma samples were used for the construction of calibration curves.

The correlation coefficients were typically between 0.987 and 0.998.

3. Results and discussion

3.1. Sample

Precipitation of proteins with perchloric acid is a fast and effective sample pretreatment. Precipitation with TCA was also tested however, in comparison, the signal height decreased with 30% for kynuerine. The main concern with adding an acid in this case is that indole derivatives are sensitive to acidic con-

dition. Another drawback with adding acid is that if still present in ESI it may case suppression of signal. Addition of antioxidants and decreasing of temperature can protect indole derivatives against degradation in an acidic medium in various biological samples [19–21]. However, a fast sample preparation, cooling of the sample and protection from light was used in this method to give satisfactory decreased degradation of tryptophan (the only indole derivative included in this study). Further, no trend was noticed in three consecutive injections of plasma samples (integrated areas 541 950, 544 860 and 568 239).

A different sample pretreatment may be of value to decrease the matrix effect even more and allow external calibration to be used for quantitation. However, the chemical structure (polarity) of analytes has probably a larger impact on the ESI response suppression than the sample preparation methods [16].

3.2. Chromatography and mass spectrometry

Tryptophan, kynurenine and kynurenic acid contain one or more acidic groups. The addition of 2.1% formic acid in the mobile phases will decrease the pH to 2 and protonate the acidic groups, which results in higher capacity factor and separation of these substances on the column. TCA acts as ion pairing agent and promotes pre-concentration of kynurenine and tryptophan, due to the double and single basic groups, respectively. The pre-concentration and separation of the analytes are also facilitated by absent of organic solvent in the mobile phase A. The packing material, ODS-AQ, was chosen because it can be used with 100% water as a mobile phase without experiencing stationary phase collapse [22].

The use of a flow direction valve and a tee (see Fig. 1) gave a decrease in the dead volume, which influences the capillary-column performance. Diversion of salts in the samples from the ion source was effective in protecting the interface, reducing the accumulation of salts on the tip as well as the curtain plate and the orifice.

The matrix effect on the signal intensity can be largely accented for by use of stable isotope labelled analytes as internal standards [23]. The standard addition method was used because of lack of proper internal standards. However standard addition method can not compensate for late co-eluting endogenous species from previous injections, therefore using of a gradient method help to get rid of these interference. A gradient method takes longer time to run than a corresponding isocratic method, because of the need for column equilibration after each run.

A chromatogram obtained by injection of 7 μl of plasma is shown in Fig. 3. The analytes are well separated and no interfering peak was detected. The relative standard deviation in retention times was less than 1% (within-day).

The injection of a reagent blank after the highest level of spiked plasma also showed no carry-over.

3.3. Concentration analysis

The results of the total concentration measurements in plasma sample are listed in Table 1. The listed concentrations are mean values of two assays over the course of two consecutive days. The obtained results for the plasma sample were also found to be well within ranges previously reported for concentrations in human serum [2,4,24,25].

The relative standard deviations were between 0.4 and 2.2% ($n=18$) which include sample preparation and detection. Limit of detection (LOD=3:1 S/N) and limit of quantitation (LOQ=10:1 S/N) were ~3 and ~11 nM, respectively, for all analytes. The calculated LOD and LOQ values for tryptophan are based on the detection of the most abundant isotope of tryptophan since at low amount it would be more realistic to use this isotope for quantitation.

4. Conclusion

Mass spectrometry has the sensitivity and selectivity that enables quantitation of tryptophan, kynurenine and kynurenic acid in plasma. The method of standard addition was used because of matrix effects on the ESI response. Initial determinations of the analytes in human plasma have given results well within ranges previously reported

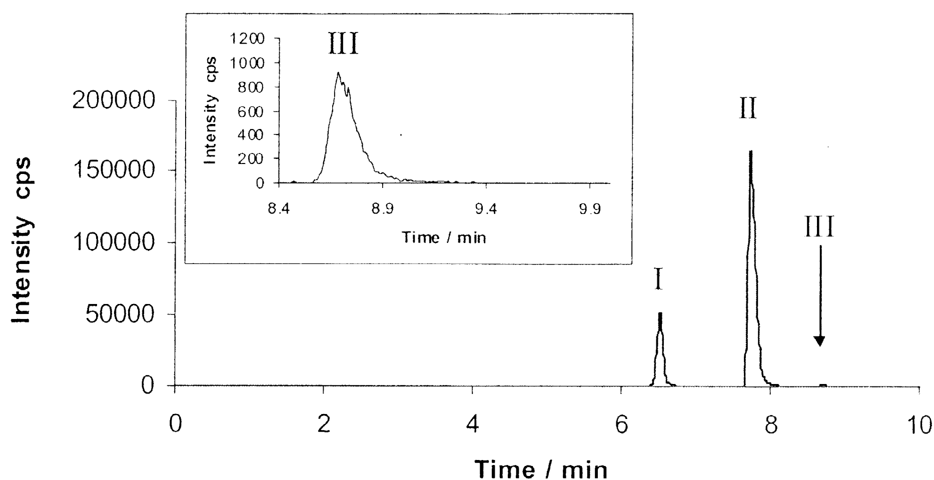

Fig. 3. Total ion chromatogram obtained by injection of 7 μl plasma. Column: 150×0.5 mm packed with 3 μm C_{18} particles (ODS AQ, YMC). Mobile phases: A=2.1% formic acid, B=2.1% formic acid, 40% acetonitrile and C=2.1% formic acid, 90% acetonitrile. Flow rate, 12 μl/min at ambient temperature. Gradient from 0 to 100% B in 5 min following by 3 min 100% B and 5 min 100% C. Peaks: I=kynurenine, II=tryptophan and III=kynurenic acid. Detection: MRM of kynurenine (*m/z* 209→192), tryptophan (*m/z* 206→189) and kynurenic acid (*m/z* 190→162).

Table 1
Concentration of tryptophan, kynurenine and kynurenic acid measured in human plasma, with precision, limit of detection (LOD) and limit of quantitation (LOQ)

Analyte	Found concentration	Day-to-day variation (RSD, %, n=18)	LOD (n*M*)	LOQ (n*M*)
Tryptophan	58 μ*M**	2.2	2.9**	9.5**
Kynurenine	1.5 μ*M*	0.4	3.4	11.2
Kynurenic acid	23 n*M*	0.8	3.4	11.5

*Calculated value based on detection of the second most abundant isotope of tryptophan.
**Calculated values for the most abundant isotope of tryptophan.

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