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Development and validation of a single analytical method for the determination of tryptophan, and its kynurenine metabolites in rat plasma

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ABSTRACT

It is highly beneficial to monitor the activity of the kynurenine pathway in a large series of samples with high accuracy and reliability in a single experimental protocol. We have developed a rapid specific solid-phase extraction (SPE)–liquid chromatography–electrospray ionization tandem mass spectrometry method for assaying tryptophan, kynurenine, kynurenic acid (KYNA), 3-hydroxyanthranilic acid (3OHAA), anthranilic acid and quinolinic acid (QA) in rat plasma. We also evaluated picolinic acid (PA) in this method, but it presented with unacceptable validation parameters. The assay involves pre-purification by SPE followed by chromatographic separation by C18 reversed phase chromatography. Mass spectrometric detection was performed using a mass spectrometer in positive and negative electrospray ionization; with a flow rate of 0.2 mL/min and an injection volume of 10 μL. Total run time including sample clean-up was 12 min. The assay method was found to be linear ($R^2 > 0.95$) and all the validation parameters were within acceptance range. The developed technique also demonstrated a significant elevation in plasma tryptophan, kynurenine, anthranilic acid and QA, and a significant decrease in KYNA, in rats subjected to post-weaning social isolation rearing, a putative animal model of relevance for depression and schizophrenia. This method can therefore be applied to measure metabolites of the kynurenine pathway in plasma accurately and precisely by LC–MS/MS, thereby helping to realize new opportunities in pharmacological and diagnostic research.

1. Introduction

Tryptophan is one of the 10 essential amino acids that is predominantly metabolized by mammalian brain and peripheral tissues, and plays an important role in protein synthesis and as a precursor of many biologically active substances [1]. Tryptophan metabolism via the kynurenine pathway is essential in several fundamental biological processes, including neuronal excitability, antioxidant status, cell growth and cell division in various cell types [2]. The kynurenine pathway also mediates interactions between immunological and neuronal functions, and this interrelationship has been implicated in the pathophysiology of a wide range of disorders, such as human immune-deficiency virus (HIV)-infection, Huntington's disease, malaria, major depression and schizophrenia [3]. After tryptophan is first catabolized into kynurenine by two haem-dependent enzymes, namely tryptophan 2,3-dioxygenase (TDO) in the liver and indoleamine-2,3-dioxygenase (IDO) in the central nervous system, lungs and placenta, a further catabolic process involves the kynurenine pathway [4], as illustrated in Fig. 1. TDO specifically metabolizes tryptophan, while IDO is responsible for the oxidative metabolism of tryptophan, serotonin and melatonin [3,5]. The latter sequence of enzymatic steps produces two free radical generators, 3-hydroxykynurenine and 3-hydroxyanthranilic acid (3OHAA) as well as 2 neuroactive compounds that act at inotropic glutamate receptors, namely quinolinic acid (QA) and kynurenic acid (KYNA) (reviewed in [3]). The concentration of QA is substantially higher in plasma than in the brain, but an increase in plasma levels leads to enhanced QA concentrations in brain tissue probably due to an increase in blood brain barrier transport [5]. Indeed, although tryptophan catabolism does occur in astrocytes and microglia, 60% of cerebral kynurenine is contributed from the periphery [6].

In order to study the clinical importance of tryptophan and its predominant metabolites (kynurenines), it is necessary to monitor the activity of as many metabolites in the kynurenine pathway as possible, in a large series of samples with high accuracy and reliability in a single experimental protocol. Several methods for detection and quantification of tryptophan and its metabolites have been described using high performance liquid chromatography (HPLC) with different detectors (reviewed in [1]). QA itself has been determined in biological fluid by gas chromatography, liquid chromatography, gas chromatography–mass spectrometry

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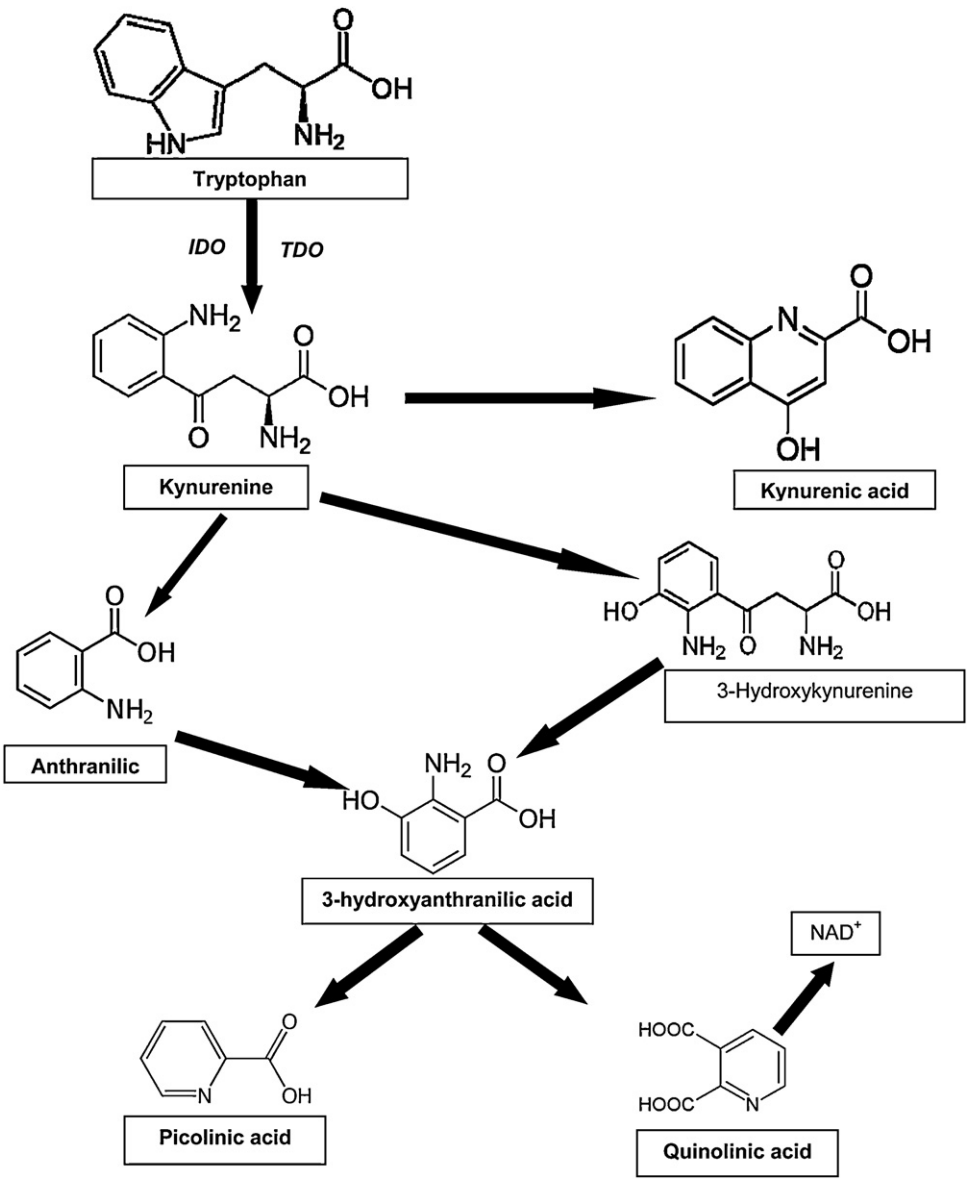

Fig. 1. A simplified diagram of the kynurenine pathway, indicating the principle enzymes, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), and the subsequent formation of kynurenine and its metabolites from tryptophan [3].

and high-voltage electrophoreses (reviewed in [5]). However, to the best of our knowledge it has never been determined by SPE–LC–MS/MS, neither have all the metabolites of the kynurenine pathway been determined together in a single run. Because multi-step detection and separation methods are required to measure all the metabolites in the above-mentioned HPLC techniques [1], these methods are time consuming, employ gradient elution and require a large sample volume.

Polar compounds like tryptophan and its metabolites generally have lower electrospray ionization (ESI) responses than less polar compounds, probably due to their lower surface activity during electrospray droplet formation. Another aspect is that biological matrixes can dramatically affect ionization and severely limit detection and thus challenge the quantitation [7]. Regarding sample preparation, the main concern with adding an acid to enable protein precipitation is that indole derivatives are sensitive to acidic conditions [7].

This study therefore developed and validated a high-throughput, sensitive and specific SPE with LC–MS/MS method that enables simultaneous extraction, concentration, separation and mass selective detection of tryptophan and its metabolites in rat plasma, including kynurenine, KYNA, 3OHAA, anthranilic acid and QA. In order to establish the practical application of this method under typical experimental conditions, we analyzed plasma tryptophan and kynurenine metabolites in post-weaning social isolation reared (SIR) rats, a widely used pathophysiological model of relevance for depression and schizophrenia [8–10]. This method will allow investigators to determine the level of kynurenine metabolites in plasma that is both accurate and precise and as such will have important application in biological research where a disturbance in kynurenine metabolism is relevant.

2. Materials and methods

2.1. Chemicals and reagents

Tryptophan, kynurenine, KYNA, anthranilic acid, 3-OHAA, QA, picolinic acid (PA), the internal standard (IS) ethyl-4-hydroxy-2-quinolinecarboxylate (ethyl-4-OH-2-quin) and activated charcoal (DARCO®) were obtained from Sigma–Aldrich (Johannesburg,

South Africa). Solvents used as eluents were HPLC grade deionized water and HPLC grade methanol (MeOH) (Burdick and Jackson Laboratory Co.); formic acid (SAARCHEM) and ammonium formate (Agilent Technologies). All chemicals and reagents were of the highest purity and were stored at appropriate temperatures and conditions.

2.2. Liquid chromatography/tandem mass spectrometry

2.2.1. Chromatographic system

The HPLC system consisted of an Agilent 1100 series HPLC with a binary gradient pump, auto sampler and vacuum degasser coupled to an Applied Bio systems API 2000 triple quadrupole mass spectrometer and Analyst 1.4 data acquisition and analysis software (AB Sciex SA (Pty) Ltd, Johannesburg). Analysis was carried out with a Restek C18 Aqueous column of 100 × 2.1 mm dimensions (Restek, Bellefonte, PA). The temperature of the column was maintained at 25 °C. Detection was performed with API 2000 triple quadrupole mass spectrometer equipped with a Turbo Ion source operated in positive and negative ESI mode.

2.2.2. Optimisation of the MS/MS detection parameters

The mass spectrometer was individually tuned for each of the 7 analytes to achieve the optimal detection parameters. Each analyte was dissolved at 1 mg in 50 mL MeOH/water (1:49, v/v) for positive and negative optimization mode. The dissolved analytes were directly infused into the ion source using a Harvard syringe pump (Harvard Apparatus, Hollinston, MA, USA) at a flow rate for 0.2 mL/min. Multiple reaction monitoring (MRM) tuning was carried out to optimize fragmentation conditions and to identify the best precursor/product transition for quantitation. Analyst Software's "Quantitation Optimization" was finally employed to fine tune the optimal mass spectrometer under chromatographic flow conditions.

2.2.3. Mobile phases

A mobile phase gradient pumped at 0.2 mL/min was used to elute the analytes from the column. Mobile phase A consisted of ammonium formate in water (0.05%, v/v, pH adjusted to 5.5 with acetic acid). Mobile phase B consisted of 100% acetonitrile. The elution started at 5% B increasing linearly to 80% B at 5 min, holding at 80% until 8 min and returning to 5% B at 8.2 min and re-equilibrating to 12 min. The retention times of the IS were 8.11 min in positive mode and 6.51 min in negative mode, respectively.

2.3. Preparation of solutions

For the SPE the following solutions were prepared: 1% NH_4OH in water (v/v): methanol (50:50, v/v) and 1% formic acid in H_2O (v/v), and stored at 4 °C. We prepared stock solutions in deionized water. A stock solution of tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA, QA and PA was prepared by dissolving 1 mg of each analyte in 50 mL MeOH/water (1:49, v/v). IS stock solution was prepared by dissolving 1 mg of ethyl-4-OH-2-quin in 50 mL MeOH/water (1:49, v/v), final concentration of 92.08 μM. Both stock solutions were stored at 4 °C. Spiked plasma calibrants ($n = 5$) were prepared by serial dilution of the stock solution of the kynurenine metabolites to the following concentrations: tryptophan (1.126, 0.563, 0.282, 0.141 and 0.070 μM), kynurenine (1.105, 0.552, 0.276, 0.138, and 0.069 μM), KYNA (1.121, 0.560, 0.280, 0.140, and 0.070 μM), 3OHAA (1.345, 0.673, 0.336, 0.168, and 0.084 μM), anthranilic acid (1.502, 0.751, 0.376, 0.188, and 0.094 μM), QA (1.544, 0.772, 0.386, 0.193 and 0.096 μM), and PA (2.014, 1.007, 0.504, 0.252 and 0.126 μM). Five quality control (QC) standards representing the limit of quantification (LOQ) were prepared by spiking plasma, from the stock solution at low, medium and high concentrations of the linearity range, as follows: tryptophan (0.282, 0.141, 0.070, 0.035 and 0.018 μM), kynurenine (0.276, 0.138, 0.069, 0.035 and 0.017 μM), KYNA (0.280, 0.140, 0.070, 0.035 and 0.017 μM), 3OHAA (0.336, 0.168, 0.084, 0.042 and 0.021 μM), anthranilic acid (0.376, 0.188, 0.094, 0.047 and 0.023 μM), QA (0.386, 0.193, 0.096, 0.048 and 0.024 μM) and PA (0.503, 0.252, 0.126, 0.063 and 0.032 μM). All blood samples were obtained from healthy, drug naïve, male Sprague–Dawley rats in 4 mL vacutainer tubes (SGVac) containing K_2EDTA solution as anticoagulant, centrifuged at 1500 × g for 10 min at 4 °C and the plasma stored at −80 °C until the day of analysis. On the day of analysis the plasma samples were thawed on ice, centrifuged at 14,000 × g for 10 min at 4 °C and the supernatant used. Ethics approval was obtained from the Animal Ethics Committee of the North-West University (Ethics approval number NWU-0035-08-S5), and all animals were handled according to the code of ethics in research, training and testing of drugs as laid down by this committee.

2.4. Tryptophan metabolite-free plasma preparation

Pooled drug-free rat plasma was purified using activated charcoal, based on a previous method [11], to remove endogenous tryptophan metabolites. The charcoal purified plasma was used as biological matrix in this study. Briefly, 5 mL of the plasma was mixed with 280 mg of activated charcoal and the mixture was shaken moderately on an orbital shaker for about 2 h, at room temperature. After centrifugation at 14,000 × g for 15 min at 4 °C, the supernatant of purified plasma was transferred to clean tubes and used immediately.

2.5. Sample preparation

Aliquots of 800 μl plasma samples, calibrants or QC's were mixed with 50 μL of the IS and vortexed for 10 s. Plasma, calibrants and QC's were then prepared by means of SPE utilizing 3 cc Waters HLB cartridges (Waters, Milford, MA, part no. WAT094226), placed on a vacuum elution manifold. After conditioning the cartridges with MeOH (2 mL), followed by 1% formic acid (2 mL), the sample was passed onto the extraction cartridge using 1% formic acid (800 μL). The loaded cartridges were then washed with 1% formic acid (2 mL), taking care that the cartridges did not run dry at any stage. The cartridges were then dried for 5–10 min by applying low vacuum (±20 kPa). Elution of the absorbed analytes was performed by 1% NH_4OH in water: methanol (2 mL). The eluent was gently evaporated under a gentle stream of nitrogen in a heater block at 40 °C. The dried residue was reconstituted with 150 μL of 1% NH_4OH in water: methanol, vortexed for 10 s, transferred to auto sampler vials and 10 μL of the aliquots were injected on the LC–MS/MS system.

2.6. Method validation

The method for the assay in plasma was validated according to the FDA guidelines [12] to meet the general requirements of ISO 17025 (2005) and SANS 17025 [13,14], as discussed below.

2.6.1. Detection and quantification limits (sensitivity)

The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the minimum concentration where the signal was at least 3 times and 10 times greater than the average background noise of an un-spiked blank (only containing IS), at the retention time of each analyte, respectively.

2.6.2. Linearity

Calibrations were performed using standards prepared as described in Section 2.3. Five replicates were used to establish the

linear calibration equation ($y = mx + c$) and analyzed using the ratio of analyte peak area over IS peak area after quantitative integration by Analyst 1.4.2 software. Linearity was measured as the coefficient of determination (R^2) measured from the 5 calibration replicates. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LOQ, which was set at ±20% [12–14].

2.6.3. Precision and accuracy

Each level of the calibration curve was measured daily before sample analysis. Precision was assessed from the relative standard deviation from the mean (RSD) and calculated separately for each concentration level of the calibration curve. Similar to precision, accuracy was calculated using each concentration level of the calibration curve and reported as a percentage of the actual known concentration. Five different calibration curves were analyzed each day and on different days to establish the intra- and inter-day precision and accuracy, respectively. The criteria for acceptability of the data included accuracy within ±15% deviation (SD) and the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) [12].

2.6.4. Recovery

The recovery of each analyte were determined using 5 spiked MeOH/water (1:49, v/v) and equivalent spiked plasma standards, as described in Section 2.3, as the calibrant concentrations for each analyte, prepared from the same stock solutions of the kynurenine metabolites and IS. The plasma samples were processed according to the preparation protocol using SPE and each spiked plasma sample was analyzed by LC–MS/MS in triplicate. The MeOH/water standards were injected in triplicate without any further workup. The percentage difference between the measured plasma concentrations compared to unprocessed water based calibrants was used to calculate the analyte recoveries. The assay was repeated 3 times.

2.6.5. Stability

Stability was determined by injecting the 5 plasma calibrators in triplicate, as described in Section 2.3, directly after sample preparation. The 1st set was assayed immediately and served as reference point, after reinjection of the same calibrators again at 3 h, 6 h, 9 h, 12 h, 24 h and 48 h, while constantly keeping the calibrators at room temperature in the auto-sampler tray. % Stability for each analyte is given as the mean % stability of the 5 calibrators ± SD at the given hour.

2.7. Application study in a pathological animal model

To establish the practical application of this method under typical experimental conditions, we used post-weaning SIR in rodents, a widely used pathophysiological model of relevance for depression and schizophrenia [16,17]. This animal model has been established in our laboratory [18,19].

Male Sprague–Dawley rats (160–190 g; Animal Research Centre, North West University) were randomly allocated to groups of 5 rats/group. At weaning (post-natal day 21) the animals were randomized to SIR (1 animal/cage) or socially reared (2–3 rats/cage) for 8 weeks (day 77). The rats were reared under identical conditions: cages ($230(h) \times 380(w) \times 380(l)$ mm) with sawdust [19,20], temperature (21 ± 0.5 °C), humidity (50 ± 10%), free access to food and water, white light (350–400 lux), and a 12 h light/dark cycle. SIR and socially reared animals experienced minimal handling and no environmental enrichment. Sawdust was changed weekly. The study protocol was approved under ethics approval number NWU-0035-08-S5 by the North West University ethical committee.

After the respective 8 weeks of SIR or social rearing, the two groups of animals were sacrificed and trunk blood collected for tryptophan metabolite analysis. Trunk blood was collected in pre-chilled, 4 ml vacutainer tubes (SGVac) containing K_2EDTA solution as anticoagulant, and stored at −80 °C until the day of analysis. On the day of analysis the plasma samples were thawed on ice, centrifuged for $1500 \times g$ for 10 min at 4 °C. Samples were pre-purified by SPE, as described above, followed by quantification of tryptophan and kynurenine metabolites using the above-described tandem LC–MS/MS procedure. Data were analyzed comparing the mean ± SD of the socially reared and the SIR rats using a two-tailed Student's *t*-test for each analyte as indicated in Table 5.

3. Results and discussion

3.1. Optimization of MS/MS detection parameters

The highest sensitivity, by a factor of at least three, were positive ionization for tryptophan, kynurenine, KYNA, anthranilic acid and 3OHAA, while negative ionization generated the highest sensitivity of QA and PA. Method optimization used the three most intense precursor/product transitions for each of the analytes. The MRM transition yielding the highest intensity was used to further fine-tune the method. The collision energy voltage, fragmentation voltage and capillary voltage were adjusted to provide the highest sensitivity. In positive ESI mode, tryptophan, kynurenine, KYNA, 3OHAA and anthranilic acid were analyzed with multiple reaction monitoring (MRM) scan with ion pairs as quantifier and qualifier, respectively (see Table 2), with ion spray voltage (ISV) at 5500 V, source temperature (TEM) at 300 °C, curtain gas (CUR) at 10 psi, nebulizer gas (GS1) at 20 psi, heater gas (GS2) at 15 psi and collision gas (CAD) at 5 psi. In negative ESI mode, QA and PA were analyzed with ion pairs (see Table 1), with ion spray voltage at −4500 V, TEM at 300 °C, CUR at 10 psi, GS1 at 20 psi, GS2 at 15 psi and CAD at 6 psi. This LC–MS/MS method enables the measurement of kynurenine metabolites with the following retention times in positive mode: tryptophan (6.61 min), kynurenine (5.60 min), KYNA (6.25 min), anthranilic acid (3.51 min), 3OHAA (3.15 min), IS (8.11 min) and negative mode: QA (2.05 min), PA (2.99 min), IS (6.51 min). Typical chromatographic profiles of a calibration sample in positive and negative mode are shown in Fig. 2A and B, respectively.

3.2. Method validation

3.2.1. Detection and quantification limits (sensitivity)

LOD was 0.0176 μM for tryptophan, 0.0173 μM for kynurenine, 0.0175 μM for KYNA, 0.0235 μM for anthranilic acid, 0.0420 μM for 3OHAA, 0.0482 μM for QA and 1.007 μM for PA. Respectively, the LOQ's were 0.0352 μM, 0.035 μM, 0.035 μM, 0.047 μM, 0.084 μM, 0.096 μM and 2.014 μM, with an assay variance of less than 15%. These results indicate that PA had the highest LOD and LOQ, compared to all the other analytes.

3.2.2. Linearity

The calibration curves constructed was evaluated by its correlation coefficient. Linearity was excellent over the respective calibration ranges, as mentioned in Section 2.3, with corresponding correlation coefficients (R^2) constantly >0.99 for tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA and QA, and $R^2 > 0.94$ for PA. The regression line equation for each analyte was as follows: tryptophan ($y = 0.1181x + 0.0173$), kynurenine ($y = 0.2851x + 0.0882$), KYNA ($y = 0.1493x + 0.0167$), anthraniic acid ($y = 0.0956x - 0.0015$), 3OHAA ($y = 0.1098x + 0.0357$), QA ($y = 0.3474x + 0.107$) and PA ($y = 149.32x + 10$). Calibration curves were reproducible between days with $R^2 > 0.99$ and $R^2 > 0.94$ for PA.

Table 1
Atmospheric pressure electron ionisation positive and negative ion mode, multiple reactions monitoring (MRM) scan with the following ion pairs as quantifier and qualifier, respectively.

	MRM_1	MRM_2	DP	FP	EP	CEP	CE	CXP
Positive ionisation								
Tryptophan	204.94/188.10	204.94/117.80	26	350	9.5	14	15	8
Kynurenine	208.97/192	208.97/93.8	21	370	10	14	13	8
Anthranilic acid	137.86/119.9	137.86/91.9	16	350	9.5	14	29	10
3OHAA	153.82/135.8	153.82/79.8	16	350	8.5	12	17	18
KYNA	189.92/143.7	189.92/89	26	360	4	12	27	20
Negative ionisation								
QA	165.88/121.9	165.88/77.8	−11	−160	−5.5	−6	−20	−14
PA	121.83/77.8	121.83/93.90	−26	−260	−9.0	−8	−10	−8

DP, declustering potential; FP, focusing potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential, all in Volt. 3-Hydroxyanthranilic acid (3OHAA), kynurenic acid (KYNA), quinolinic acid (QA) and picolinic acid (PA).

3.2.3. Precision and accuracy

Precision and accuracy of plasma samples at low, medium and high concentrations are provided in Table 2. Throughout these concentration ranges, the mean intra-assay precision was below 4%, and below 10% for the inter-assay precision. The accuracy for all three concentration levels for tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA, and QA was between 86.1% and 100.1%. However, PA's accuracy was between 75.0% and 80.4% at the highest concentration and the medium and low concentrations were below the LOQ for PA.

3.2.4. Recovery

The mean absolute recovery for each analyte measured at low, medium and high concentrations were constant with a mean recovery indicated in Table 3. PA had the worst recovery, compared to all the other analytes and we were unable to determine PA's recovery at the medium and low concentrations.

3.2.5. Stability

Stability studies indicated that tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA and QA were stable when tested at room temperature in the auto-sampler tray at 3 h, 6 h, 9 h, 12 h, 24 h and 48 h, however, PA were not stable over 48 h, shown in Table 4. The average decrease for all five plasma calibrant concentrations measured was acceptable and below 15% for a time period of 48 h [12,13]. However, for PA the percentage decrease exceeded 15% after 12 h, indicating that PA was only stable for 12 h after sample workup if maintained at room temperature.

3.3. Method development and optimization

The determination of tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA, and QA in plasma by the developed LC–MS/MS method required an SPE step in order to eliminate the interference compounds and to concentrate the analytes.

Table 2
Precision and accuracy of 7 kynurenine metabolites in rat plasma.

Analyte concentration (μM)	Intra day ($n=5$)			Inter-day ($n=5$)		
	Measured concentration (μM) (mean ± SD)	Precision (% CV)	Accuracy (%)	Measured concentration (μM) (mean ± SD)	Precision (% CV)	Accuracy (%)
Tryptophan						
1.126	1.120 ± 0.08	3.70	99.47	1.103 ± 0.07	7.00	97.96
0.282	0.230 ± 0.06	2.70	86.15	0.281 ± 0.02	4.80	99.65
0.070	0.069 ± 0.01	1.60	98.57	0.065 ± 0.01	6.10	92.86
Kynurenine						
1.105	1.104 ± 0.05	1.60	99.91	1.102 ± 0.05	5.60	99.73
0.276	0.275 ± 0.11	2.90	99.64	0.271 ± 0.04	2.30	98.19
0.069	0.068 ± 0.06	1.60	98.55	0.061 ± 0.01	8.60	88.41
KYNA						
1.121	1.190 ± 0.3	3.40	106.16	1.120 ± 0.11	9.90	99.91
0.280	0.280 ± 0.07	4.29	100.00	0.270 ± 0.05	8.60	96.43
0.070	0.069 ± 0.03	2.10	98.57	0.069 ± 0.03		98.57
Anthranilic acid						
1.502	1.501 ± 0.09	3.32	99.93	1.503 ± 0.05	9.30	100.07
0.376	0.375 ± 0.04	3.40	99.73	0.371 ± 0.02	7.50	98.67
0.094	0.091 ± 0.01	1.00	96.81	0.089 ± 0.01	5.30	94.68
3OHAA						
1.345	1.301 ± 0.04	4.60	96.73	1.341 ± 0.08	3.60	99.70
0.336	0.331 ± 0.01	2.29	98.51	0.334 ± 0.05	4.70	99.41
0.084	0.080 ± 0.01	3.40	95.24	0.081 ± 0.03	3.40	96.43
QA						
1.544	1.541 ± 0.07	2.46	99.81	1.539 ± 0.06	6.80	99.68
0.386	0.382 ± 0.03	2.50	98.96	0.379 ± 0.02	5.40	98.19
0.096	0.092 ± 0.02	3.40	95.83	0.091 ± 0.01	8.50	94.79
PA						
2.014	1.511 ± 0.05	3.90	75.03	1.620 ± 0.12	9.80	80.44
0.503	Below LOQ			Below LOQ		
0.126	Below LOQ			Below LOQ		

3OHAA, 3-hydroxyanthranilic acid; KYNA, kynurenic acid; QA, quinolinic acid; PA, picolinic acid.

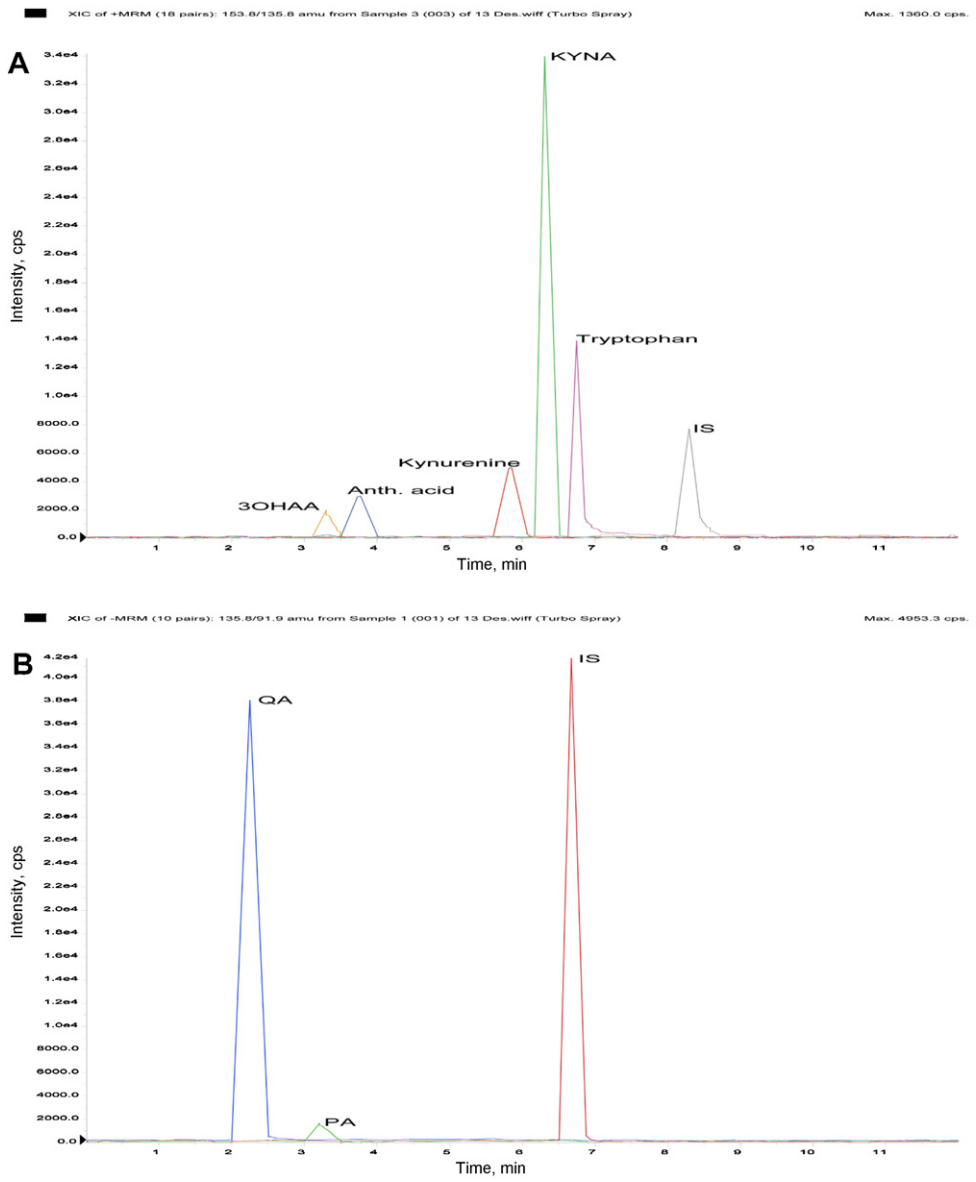

Fig. 2. Typical LC–MS/MS selected ion chromatograms in positive (A) and negative (B) electrospray ionization mode, obtained with the highest calibration concentration of each analyte, as mentioned in the text. In positive mode (A): 3-hydroxyanthranilic acid (3OHAA), anthranilic acid, kynurenic acid (KYNA), kynurenine, tryptophan and the internal standard (IS) ethyl 4-hydroxy-2-quinolinecarboxylate. In negative mode (B): quinolinic acid (QA), picolinic (PA), and the internal standard (IS) ethyl 4-hydroxy-2-quinolinecarboxylate.

However, PA was difficult to detect, possibly due to the fact that it binds to the column matrix and forms complexes with proteins [21,22], and we therefore found that PA in our plasma samples were below our LOQ and LOD with poor recovery and stability. PA is an endogenous metabolite of L-tryptophan that has been reported to possess a wide range of neuroprotective, immunological, and anti-proliferative effects within the body [reviewed in 23]. However the salient physiological function of this molecule is yet to be established and has not yet been determined with an LCMS method. We therefore believe that this method could be used in future studies with a more sensitive LCMSMS apparatus, in measuring PA.

Tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA, QA and PA are polar acids and soluble in alkalis and almost insoluble in organic solvents. These acids are negatively charged at the carboxylic acid group at high pH and positively charged at the pyridinyl nitrogen at low pH, while zwitterions (positively charged on nitrogen and negatively charged on the carboxylic acid at the same time) forms at medium pH values [22,24]. We therefore chose copolymer, reverse-phase, 3 cc Waters HLB cartridges, for the pre-purification procedure and the mobile phase was adjusted to pH 5.5 in order to obtain the analytes in the anionic form. An experimental design approach to find the optimum SPE procedure was employed and the goal was to select the best retention, wash and elution solvents and their volumes, in order to maximize the LC–MS/MS signal.

3.4. Charcoal purified plasma evaluation

Endogenous tryptophan metabolites in plasma have been a problem for validation of tryptophan metabolite analysis methods. Several methods fail to take into account the matrix effect of biological fluids, which is an important factor not only for the chromatographic separation and mass spectrometric signal, but

Table 3
Recoveries for tryptophan, kynurenine, KYNA, anthranilic acid, 3OHAA, QA and PA at low, medium and high concentrations.

Analyte	Concentration (μM)	% Recovery (mean ± SD)
Tryptophan	1.126	96 ± 1.07
	0.282	98.43 ± 0.21
	0.070	94.54 ± 1.2
Kynurenine	1.105	98.51 ± 0.57
	0.276	97.72 ± 0.65
	0.069	97.82 ± 0.57
KYNA	1.121	96.38 ± 0.07
	0.280	96.66 ± 0.17
	0.070	97.38 ± 0.37
Anthranilic acid	1.502	100.07 ± 0.33
	0.376	99.59 ± 0.93
	0.094	100.21 ± 0.89
3OHAA	1.345	97.50 ± 0.11
	0.336	96.47 ± 0.13
	0.084	95.21 ± 0.15
QA	1.544	97.38 ± 0.24
	0.386	97.20 ± 0.84
	0.096	97.66 ± 0.22
PA	2.014	37.07 ± 2.89
	0.503	Below LOQ
	0.126	Below LOQ

Each mean value is the result of triplicate analysis. KYNA, kynurenic acid; 3OHAA, 3-hydroxyanthranilic acid; QA, quinolinic acid; PA, picolinic acid.

also for the sample extraction process. Previous studies indicate that activated charcoal is an efficient absorbent of amphiphilic compounds such as tryptophan metabolites from the protein matrix [11,25]; in our study, activated charcoal was used to remove the endogenous tryptophan metabolites from pooled rat plasma. Our results demonstrate that activated charcoal efficiently removes the endogenous tryptophan metabolites from plasma. This tryptophan metabolite free plasma is a suitable blank matrix for the preparation of calibration standard and quality control samples for tryptophan metabolite analysis. Fig. 3 depicts a blank charcoal treated plasma sample in positive and negative ionization mode.

3.5. Analysis of the kynurenine pathway in a pathophysiological animal model

The developed technique was able to demonstrate a significant elevation in plasma tryptophan, kynurenine, anthranilic acid, and QA in post-weaning SIR rats versus their socially reared controls (see Table 5). A significant decrease in KYNA was also demonstrated (Table 5). However, no change with respect to 3OHAA levels was observed (Table 5). When considering the dynamics of the KYN pathway (see Fig. 1), an increase in QA (a glutamate NMDA receptor agonist) and a decrease in KYNA (an NMDA antagonist) is congruent with current theories that centre on glutamate dysfunction in mood disorders and schizophrenia, and has been proposed to underlie the behavioral and neurochemical changes observed in SIR animals and in patients with depression and/or schizophrenia [26,27]. In fact, post-weaning SIR is widely regarded as a useful and valid animal model of relevance for studying the neurobiology of schizophrenia [17,18]. We have earlier observed that SIR significantly alters frontal–cortical glutamate NMDA receptor density [18] that may originate through dysfunctional glutamatergic and/or redox signaling induced by altered tryptophan–kynurenine metabolism [18,20]. Our data thus emphasizes the validity of these findings and of the successful application of this LC–MS/MS method for biological research in animals and humans.

Table 4
% Stability (mean ± SD) of kynurenine analytes at room temperature after 3 h, 6 h, 9 h, 12 h, 24 h and 48 h.

Time:	3 h	6 h	9 h	12 h	24 h	48 h
Tryptophan						
% Stability	99.95 ± 0.14	99.53 ± 0.42	98.53 ± 0.60	97.85 ± 1.26	92.86 ± 0.83	89.86 ± 0.70
Kynurenine						
% Stability	98.36 ± 1.81	99.49 ± 0.70	97.81 ± 2.46	96.63 ± 1.92	91.33 ± 1.86	88.67 ± 2.59
KYNA						
% Stability	100.33 ± 3.03	99.67 ± 1.37	92.28 ± 1.02	95.86 ± 3.27	94.16 ± 5.36	90.96 ± 4.09
Anthranilic acid						
% Stability	101.16 ± 2.52	99.77 ± 2.18	99.98 ± 2.47	96.05 ± 4.63	94.27 ± 4.40	91.19 ± 2.11
3OHAA						
% Stability	100.05 ± 0.42	98.12 ± 2.59	95.61 ± 2.26	94.44 ± 6.44	91.64 ± 5.40	88.20 ± 4.55
QA						
% Stability	101.38 ± 2.19	100.34 ± 0.89	96.91 ± 2.85	96.07 ± 1.80	92.06 ± 1.05	87.35 ± 5.40
PA						
% Stability	99.36 ± 3.75	91.18 ± 4.83	86.66 ± 5.16	80.55 ± 3.87	75.52 ± 7.25	49.91 ± 1.46

Compared to 100% at $t = 0$, mean ± SD. 3OHAA, 3-hydroxyanthranilic acid; KYNA, kynurenic acid; QA, quinolinic acid; PA, picolinic acid.

Table 5
Plasma concentrations of tryptophan and kynurenine-related metabolites (mean ± SD) in SIR rats ($n = 5$) compared to their socially reared controls ($n = 5$).

	Socially reared analyte concentration (μM) (mean ± SD)	SIR analyte concentration (μM) (mean ± SD)	p Value
Tryptophan	31.61 ± 1.54	43.93 ± 2.56	0.003*
Kynurenine	2.16 ± 0.25	3.40 ± 0.19	0.004*
KYNA	0.07 ± 0.01	0.04 ± 0.01	0.023*
Anthranilic acid	0.05 ± 0.02	0.09 ± 0.01	0.022*
3OHAA	0.09 ± 0.01	0.09 ± 0.01	0.178
QA	0.07 ± 0.01	0.09 ± 0.004	0.037*

SIR, social isolation reared; 3OHAA, 3-hydroxyanthranilic acid; KYNA, kynurenic acid; QA, quinolinic acid; PA, picolinic acid.
* $p < 0.05$ (two-tailed Student's t-test).

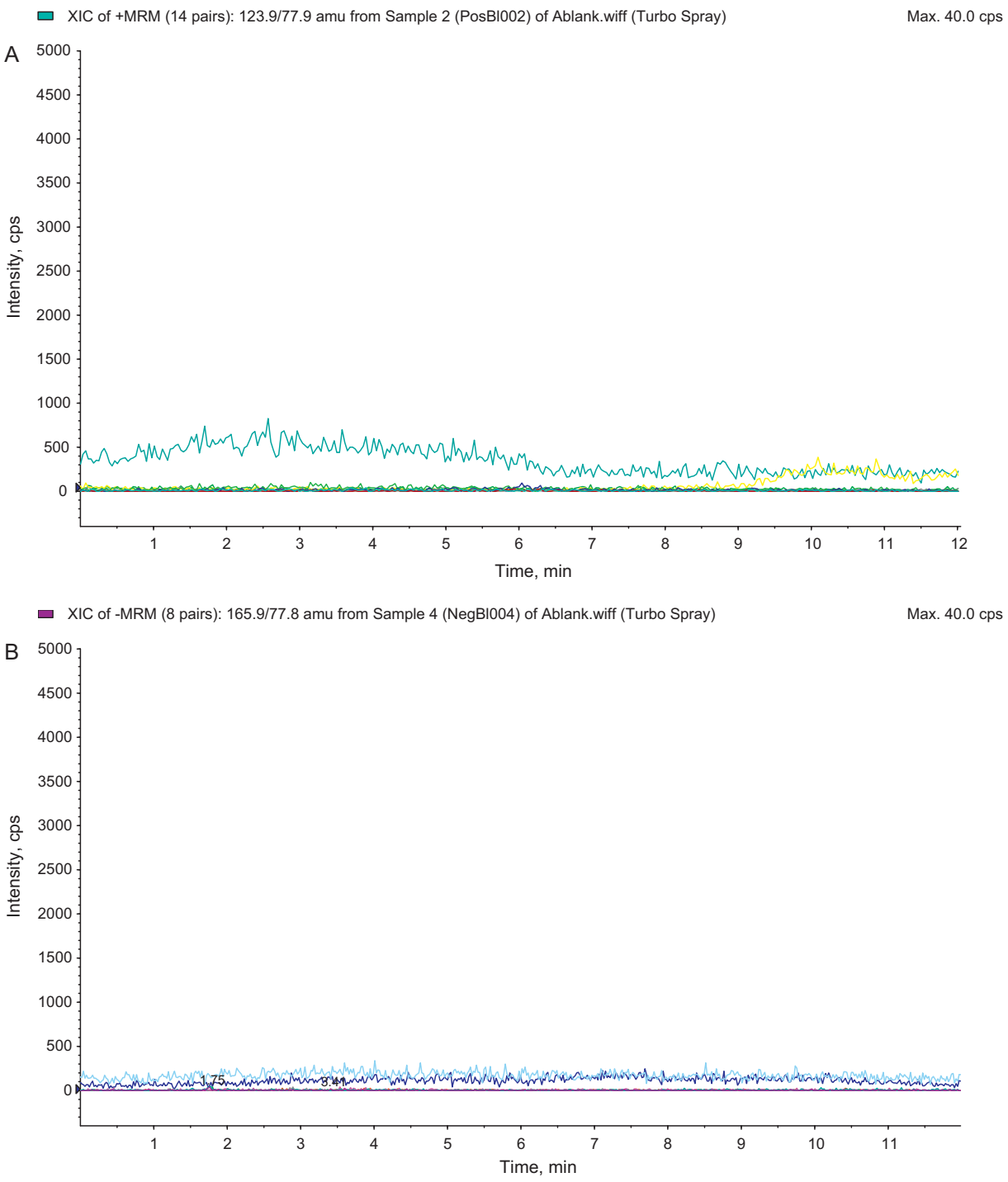

Fig. 3. Chromatograms of blank charcoal purified plasma after extraction in positive (A) and negative (B) electrospray ionization mode.

4. Conclusion

A rapid, specific solid-phase extraction (SPE)–liquid chromatography–electrospray ionization tandem mass spectrometry method has been developed for assaying tryptophan, kynurenine, KYNA, 3OHAA, anthranilic acid and QA in rat plasma. This method was validated for sensitivity, linearity, precision, accuracy, recovery and stability. LOD was 0.0176 μM for tryptophan, 0.0173 μM for kynurenine, 0.0175 μM for KYNA, 0.0235 μM for anthranilic acid, 0.0420 μM for 3OHAA and 0.0482 μM for QA and 1.007 μM for PA. Respectively, the LOQ's were 0.0352 μM, 0.035 μM, 0.035 μM, 0.047 μM, 0.084 μM, 0.096 μM and 2.014 μM. Linearity was excellent ($R > 0.94$) across all the calibration ranges for each analyte. Recovery for all analytes were > 94%, except PA, and percentage stability for all analytes were > 98% after 3 h, >91% after 6 h, >86% after 9 h, >80% after 12 h, >75% after 24 h and >49% after 48 h. PA was particularly challenging to quantify and validate in this method: all validation parameters of PA represented as not acceptable. This method could however be used in the future for the determination of PA, provided a more sensitive LCMSMS apparatus is used.

The method uses a simple mobile phase composition that is easy to prepare with little or no variation. The relatively rapid run time of

12 min (Fig. 2), and the low flow rate (0.2 mL/min), allows the analysis of a large number of samples in a single day yet using less mobile phase. It is thus a cost and time efficient technique that provides a useful alternative to existing methods of analysis. We also found that the developed technique demonstrated noteworthy changes in plasma tryptophan metabolism in rats subjected to SIR, with a significant decrease in KYNA and a significant increase in QA levels in SIR animals (Table 5). SIR is a putative animal model of relevance for depression and schizophrenia. The observed changes in tryptophan metabolism closely parallel current thinking regarding glutamate dysfunction in schizophrenia. This method therefore provides the opportunity for more detailed analysis of the kynurenine pathway in a number of pathological conditions in humans and animals, and therefore to yield new insight into disease mechanisms.

Conflict of interest

The authors have no conflict of interest to declare.

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