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A simple LC–MS/MS method for determination of kynurenine and tryptophan concentrations in human plasma from HIV-infected patients

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Abstract

Background—Indoleamine 2,3-dioxygenase, catalyzing tryptophan (Trp) metabolism through the kynurenine (Kyn) metabolic pathway, plays important roles in immune suppression and the CNS. In this article, we report a simple, rapid and specific LC–MS/MS method for accurate determination of Kyn and Trp concentrations in human plasma from HIV-infected patients.

Results—The human plasma sample (100 μl) was mixed with Kyn-d4 and Trp-d5 internal standards and then precipitated with trifluoroacetic acid. The supernatant was directly analyzed by LC–MS/MS. The assay using surrogate matrix calibrators was validated for precision, accuracy, matrix effect, extraction efficiency and stability. Some assay validation issues for endogenous substance bioanalysis using an LC–MS/MS method are discussed.

Conclusion—A simple, specific and reproducible LC–MS/MS method has been developed and validated for measuring Kyn and Trp in human plasma samples.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalizes the first and rate-limiting step of tryptophan (Trp) metabolism through the kynurenine (Kyn) pathway [1]. IDO plays important roles in the CNS and the immune system [1–3]. The activation of IDO mediated by proinflammatory cytokines, such as interferon-γ, could trigger immune suppression and tolerance [1,4]. In HIV-infected patients, the activation of IDO has been reported *in vitro* and *in vivo* [2–6]. The activation of IDO has also been found in some cancer cells, which results in the cancer cells escaping from immune surveillance [1]. Therefore, IDO inhibition by chemicals has become a new AIDS treatment and cancer therapeutic target. At present,

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: WWW.FUTURE-SCIENCE.COM/DOI/SUPPL/10.4155/BIO.13.74

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

the IDO specific inhibitor, 1-methyl-D-Trp, is in clinical trials for treating advanced malignancies and other medical conditions [7,8].

Although IDO has been proven to be linked to immune suppression, the detailed inhibitory mechanisms of T-cell functions by IDO activation have not been well understood. Since IDO converts Trp to Kyn, measuring the Kyn/Trp ratio in blood plasma is currently used to monitor IDO activity *in vivo* [4,9]. To further study the mechanisms of IDO induced immunosuppression in HIV infection and the influence of prevalent co-infections as well as antiretroviral treatments on IDO activity, a high-throughput bioanalytical method for accurate determination of Kyn and Trp concentrations in human plasma is necessary.

Several HPLC methods with UV detection or coulometric detection have been developed to determine Kyn and Trp concentration in plasma [10–14]. However, HPLC UV detection usually lacks selectivity and has a relatively long run time. In recent years, LC–MS/MS methods have also been reported for analyzing Trp and Kyn in plasma samples [15–19], however, the detailed assay validations are not provided.

In this article, we report a simple, rapid and reliable LC–MS/MS method for accurate determination of Kyn and Trp concentrations in human plasma. Two stable isotope-labeled internal standards (SIL-ISs), Trp-d5 and Kyn-d4, were used to reduce assay variation. Calibrators using surrogate matricies such as water, phosphate buffered saline (PBS), 1% bovine serum albumin (BSA) and charcoal stripped human plasma were also compared. Some assay validation issues such as matrix effect, extraction recovery and **LOQ** for endogenous substance bioanalysis using LC–MS/MS are discussed.

Materials & methods

Materials

Trp and Kyn reference compounds were purchased from Sigma-Aldrich (MO, USA). Trpd5 and Trp-d8 were obtained from CDN Isotopes (Quebec, Canada) and Kyn-d4 was obtained from Buchem BV (Apeldoorn, The Netherlands). Acetonitrile, methanol, trifluoroacetic acid (TFA, 99%) and other solvents or reagents were HPLC- or analytical-grade. Healthy human plasma samples with acid citrate dextrose anticoagulant were obtained from Bioreclamation, LLC (NY, USA). HIV-infected human plasma samples were obtained from UCSF AIDS specimen bank and AIDS and Cancer Specimen Resource. BSA was purchased from Fisher Scientific, Inc.

Standard solutions

Trp and Kyn stock solutions were prepared at 1 mg/ml in 50% acetonitrile and stored at 4°C in brown bottles. Trp and Kyn calibrators were generated using combined Trp and Kyn stock solutions that were serially diluted with distilled water. The IS stock solutions of Trp-d5 and Kyn-d4 were prepared at 1 mg/ml in 50% acetonitrile. The IS working solution containing 3.5 μg/ml of Trp-d5 and 1.1 μg/ml of Kyn-d4 was prepared by dilution of IS stock solution with water and stored at 4°C.

PBS solution was prepared with 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 1 l of distilled water, and its pH was adjusted to 7.4 with HCl. Charcoal stripped human plasma was prepared according to a previous paper [19]; that is, 20 ml of plasma was added with 1.2 g of charcoal activated powder (Fisher Scientific) and rotated for 2 h, then centrifuged at 14,000 g for 10 min to obtain the supernatant. The ‘stripped’ human plasma was confirmed by LC–MS/MS to be free of Trp and Kyn.

Human plasma sample preparation

100 μl of human plasma sample, calibrator sample or QC sample was aliquoted into a test tube and 100 μl of IS working solution was added containing Trp-d5 (3.5 μg/ml) and Kyn-d4 (1.1 μg/ml) and mixed for 30 s. Then, the tube was spiked with 20 μl of TFA and vortexed for 1 min, followed by centrifuging at 3000 rpm for 10 min. The supernatant was transferred to an autosampler vial and 5 μl was injected into the LC–MS/MS system.

LC–MS/MS

The LC–MS/MS system consisted of a Shimadzu LC-10 AD pump, a Waters intelligent Sample Processor 717 Plus autosampler and a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer. The mass spectrometer was set to ESI in positive SRM mode. The precursor/product transitions (*m/z*) were at *m/z* 204.9 > 187.9 for Trp, *m/z* 209.0 > 192.1 for Kyn, *m/z* 210.0 > 193.0 for Trp-d5, *m/z* 213.3 > 195.0 for Trp-d8 and *m/z* 213.0 > 196.0 for Kyn-d4. The cone voltage and collision energy were 35 V and 20 eV, for both Trp and Trp-d5, respectively. The cone voltage and collision energy were 35 V and 10 eV, respectively, for both Kyn and Kyn-d4. The source block and desolvation temperatures were 100 and 400°C, respectively.

The HPLC conditions were as follows: the column was a Synergi Polar RP column (75 × 4.6 mm) from Phenomenex (CA, USA) and the mobile phase was composed of 2% acetonitrile, 5.2% methanol and 0.1% formic acid. The flow rate was set at 1.0 ml/min and the run time for each sample was 4.5 min. The autosampler was set at 4°C. Data processing was performed using Masslynx 4.1 software.

For testing the **extraction efficiencies** of Trp and Kyn, an HPLC–UV detection was also carried out. The column was a Synergi Polar RP column (150 × 4.6 mm) from Phenomenex and the mobile phase was the same as above. The flow rate was set at 1.0 ml/min. The UV detection wavelength was set at 254 nm.

Method validation

The calibrators were generated using a surrogate matrix for Trp at 1250, 2500, 5000, 10,000 and 20,000 ng/ml, and for Kyn at 62.5, 125, 250, 500 and 1000 ng/ml from standard working solutions in water. Each concentration calibrator was duplicated. The standard curve was calculated by 1/× weighted least squares linear regression of standard curve calibrator concentrations and the peak area ratios of analyte to IS.

Assay precision & accuracy—The assay precision of pure QC samples, charcoal-stripped human plasma QC samples and nontreated human plasma QC samples were evaluated using one intra-day and three interday analytical runs. The low, medium and high concentrations of pure QC samples or stripped human plasma QC samples were generated at 5000, 10,000 and 15,000 ng/ml for Trp, and at 150, 300 and 600 ng/ml for Kyn. The nontreated human plasma QC samples were generated from pooled healthy human plasma, with an aliquot serving as a medium human plasma QC, an aliquot spiked with Trp (5000 ng/ml) and Kyn (300 ng/ml) serving as a high human plasma QC, and an aliquot diluted with water at a 1/1 (v/v) ratio serving as a low human plasma QC. The calibrators and various QC samples were processed with TFA protein precipitation in the same way as described above, then analyzed by LC–MS/MS.

The assay accuracy for the nontreated plasma was assessed by **spiked recovery** as follows:

$$\text{Spiked recovery} = 100 \times (\text{measured } C_{\text{spiked}} - C_{\text{nonspiked}})/C_{\text{spiked}} \quad \text{Equation 1}$$

Extraction efficiency—The extraction efficiencies of Kyn and Trp from human plasma samples were evaluated using surrogate analytes; that is, Trp-d5 and Kynd4 were spiked into human plasma, and the extraction recoveries of Trp-d5 and Kyn-d4 were determined following a conventional exogenous drug recovery test method. Briefly, Trp-d5 and Kyn-d4 were spiked into human plasma at low (Trp-d5, 5000 ng/ml and Kyn-d4, 150 ng/ml) and high (Trp-d5, 15,000 ng/ml and Kyn-d4, 600 ng/ml) concentration levels, then mixed for 1 min. To these samples, 0.1 ml of water was added and mixed, followed by protein precipitation with 20 μl of TFA. Then, 130 μl of the supernatants were transferred to autosampler vials and 20 μl of 10 μg/ml Trp-d8 (as IS) was added and mixed well to each before injection into the LC–MS/MS system. The nonextracted control samples were human plasma samples without added Trp-d5 and Kyn-d4, which were processed in the same way as above. After TFA protein precipitation, 130 μl of the supernatants were transferred to autosampler vials, and Trp-d5 and Kyn-d4 at low and high levels, and the same concentration of Trp-d8, were added. The total volumes of the extracted and nonextracted samples were kept the same. The extraction efficiency (%) was calculated as: 100 × volume factor × (peak area ratio [Trp-d5 or Kyn-d4 vs Trp-d8] of extraction sample/peak area ratio of nonextraction sample). The volume factor was calculated as approximately 200 μl (total supernatant volume)/130 μl (transferred volume).

In addition, the extraction efficiency was also evaluated with HPLC–UV detection. The extraction samples were prepared using stripped human plasma spiked with Trp-d5/Kyn-d4 or Trp/Kyn, then 100 μl of water was added, mixed for 1 min and followed by adding TFA (20 μl) for protein precipitation as above, while the control samples were prepared using water instead of the stripped plasma spiked to the same concentration of Trp-d5/Kyn-d4 or Trp/Kyn and processed in the same way as the extracted samples. Then 10 μl of extracted or control samples were injected into the HPLC–UV detection system at 254 nm with the same mobile phase but a longer Polar RP column (150 × 4.6 mm). The extraction efficiency (%) was calculated as: 100 × (peak height of extracted sample/peak height of control sample).

Mass ionization suppression/enhancement—The matrix ionization suppression or enhancement was evaluated using surrogate analytes; that is, evaluation of the matrix factors (MFs) of Trp-d5 and Kyn-d4 [20]. Six lots of human plasma samples (0.1 ml) were spiked with 0.1-ml water and precipitated with 20 μl of TFA, then the supernatants (130 μl) were transferred to autosampler vials and added with Trp-d5/Kyn-d4 (as the surrogate analyte) and Trp-d8 (20 μl × 10 μg/ml as IS) and analyzed by LC–MS/MS. The control was water instead of human plasma and was processed in the same way. In addition, water without adding TFA but with the same Trp-d5/Kyn-d4 and Trp-d8 was also prepared and analyzed, to test the TFA effect on ionization suppression.

The MFs of Trp/Kyn were also evaluated using stripped human plasma. Brief ly, the stripped human plasma (100 μl) was added with 100 μl of water and mixed for 30 s. Then, 20 μl of TFA was added for protein precipitation; the supernatant (130 μl) was transferred to an autosampler vial and added with Trp/Kyn and IS (Trp-d5/Kyn-d4) mixed well and injected into the LC–MS/MS system. The control sample was water with or without TFA and added with the same Trp/Kyn and IS.

The MF was calculated as the peak area of the analyte or surrogate analyte in the presence of biological matrix compared with that in the absence of biological matrix (in pure solution without TFA). The IS normalized MF = peak area/IS ratio in presence of matrix versus peak area/IS ratio in absence of matrix [20].

The sample process efficiency [21,22], which includes extraction efficiency and mass ionization suppression/enhancement, was assessed by comparing the peak area of Trp-d5 or

Kyn-d4 spiked into pre-extraction samples to that spiked into pure solution (water) samples and processed in the same way.

Matrix variation—Matrix variation [23] or matrix accuracy [24], defined as variation in different lots of matrix on assay accuracy, was evaluated using six different lots of healthy human plasma spiked with the analytes and assessing the spiked recovery.

Dilution linearity—Six lots of human plasma samples were diluted two- or four-times with water, then analyzed by LC–MS/MS to test the dilution linearity or dilution accuracy.

Stability tests—The bench-top stability of human plasma samples was evaluated using pooled human plasma stored at room temperature for up to 24 h or 56°C for 30 min. The human plasma sample freeze–thaw stability was evaluated using a pooled human plasma sample repeatedly frozen at −20°C, then thawed to room temperature for five cycles. Processed sample stability was evaluated by injecting processed QC samples immediately, as well as processed samples stored at 4°C for different time periods along with fresh prepared standard curve samples.

Results & discussion

Method development

Trp is an essential amino acid and presents a high concentration (in the order of 10 μg/ml) in human plasma, while the Kyn plasma level is at an approximately 40-times lower level [9,10]. Both analytes in human plasma can be easily detected by LC–MS/MS. To measure Trp and Kyn simultaneously, a higher collision energy value for Trp was set in order to lower its sensitivity so that there is no detection quenching. Since both Kyn and Trp are polar compounds, a Polar RP column with a low percentage of organic solvent was used as a mobile phase for achieving appropriate retention times. To extract Kyn and Trp efficiently from the plasma matrix, a protein precipitation with TFA was chosen, which shows a high extraction recovery for both analytes. TFA is compatible with MS and has less mass ionization suppression than the nonvolatile trichloroacetic acid, which was used in previous methods for Trp/Kyn plasma sample preparation [12,13,18]. Figure 1 shows the LC–MS/MS chromatograms of Kyn, Trp in plasma and reference standards.

Comparison of different calibrators on Kyn & Trp plasma assay

One challenge in the development and validation of a quantitative method for analyzing an endogenous substance or biomarker is in choosing the calibrator, especially when the ‘control’ matrix sample contains a high level of the analyte [25,26]. Currently, three major ways are used. The first is to use the authentic matrix and authentic analyte; the second is to use a surrogate matrix and the authentic analyte; the third is to use the authentic matrix and a surrogate analyte [27,28]. Since both Trp and Kyn present high levels in human plasma, using an authentic matrix standard addition method is impractical. The third way, using the authentic matrix and a surrogate analyte requires two SIL-IS, which is difficult to obtain in most cases. The analyte response ratio (or response factor) of the authentic analyte and surrogate analyte may not always be consistent between different runs. The second way is most often used for endogenous compound bioanalysis. However, the differential extraction recovery and mass ionization suppression issues between the true matrix and a surrogate matrix must be overcome. To test the effects of surrogate calibrators on the Kyn and Trp assay, we prepared the calibrators in H_2O, PBS, 1% BSA or charcoal-stripped human plasma and ran them under the same conditions. As Figure 2 shows, all these calibrators are equivalent with similar slopes and intercepts. Kyn and Trp measured values were not significantly affected by using the different calibrators (data not shown). These results

support equivalence between the calibrator prepared just in pure H_2O and the matrix-matched calibrator. The main reasons for similar standard curves obtained from these various matrices are a high extraction recovery, minimal ionization suppression and the matrix effects compensated by SIL-IS.

Method validation

At present, there is no consensus guideline for endogenous substance assay validation [25,29]. There are several challenges for an endogenous substance bioanalysis with LC–MS/MS methods when a 'blank' matrix sample is not available [25,26]. The following are our adapted validation methods for the Kyn/Trp assay.

Selectivity—The assay selectivity was assessed by confirming that the detected Trp and Kyn peaks in human plasma were the same as the reference standards by using two transitions (two product ions) in SRM mode. As shown in Supplementary Table 1, the peak area ratios of two product ion transitions in different plasma samples from HIV patients were the same as that of the standards, which supports the idea that the detected Trp and Kyn peaks under the current conditions were indeed Trp and Kyn. No biological matrix interference peaks were observed in different runs in the analysis of various clinical samples.

Linearity—Good linearities of the Trp concentrations from 1250 to 20,000 ng/ml and Kyn concentrations from 62.5 to 1000 ng/ml versus the analyte to IS peak area ratios were obtained. The regression coefficients were more than 0.99 with a relatively small intercept (the line almost crossing at zero). Although the curves linear range can be extended to 312.5 ng/ml for Trp and 15.6 ng/ml for Kyn, the current ranges are sufficient to cover the Trp and Kyn concentrations in HIV-infected human plasma samples.

Assay precision & accuracy—The intra-day and inter-day accuracy and precision for the spiked pure QC samples, stripped human plasma QC and nontreated human plasma QC samples are summarized in Table 1. The CVs of inter- and intra-day pure water QC, stripped QC, as well as nontreated human plasma QC samples, were less than 11%. These results indicate that the established method has good precision for the measurement of Kyn and Trp in human plasma samples.

The mean accuracies of Trp and Kyn spiked to water and stripped human plasma at low, medium and high levels were 93–108%. The assay accuracy for the nontreated human plasma was assessed by spiked recovery using the Equation 1 calculation. The intra-day and inter-day spiked recoveries for Trp spiked at 5000 ng/ml and Kyn spiked at 300 ng/ml were 89–101%.

LOQ—The LOD for Trp and Kyn were estimated at approximately 50 and 5 ng/ml, respectively, based on a S/N ≥ 3. The LOQ if defined as the lowest standard curve concentration, were 1250 ng/ml for Trp and 62.5 ng/ml for Kyn. Both CV and relative error using stripped human plasma or water spiked samples at this lowest standard curve concentration were <20% (Supplementary Table 2). However, the accuracy and precision for the spiked surrogate matrix samples may not completely represent the incurred human plasma samples. Obtaining the true LOQ for an endogenous compound assay is challenging as the accuracy is not easy to assess, especially when the concentration approaches the LOQ level [30]. To assess the actual LOQ for the human plasma Trp/Kyn assay, an eighttimes dilution of one lot of human plasma samples still shows a good dilution linearity in which the measured Kyn concentration is as low as 25 ng/ml and the CV < 20% (Supplementary Table 2). This also suggests the true LOQ of Kyn to be ≤25 ng/ml. However, it should be

pointed out the LOQ may be a less useful parameter when the human plasma sample ranges are far higher than the LOQ values [26]. Few human plasma samples were less than 1000 ng/ml for Trp and 100 ng/ml for Kyn.

Extraction efficiency—Since the true values of Trp and Kyn in human plasma samples are unknown, the extraction efficiency, therefore, cannot be obtained directly. The extraction recovery of this method was evaluated using surrogate analytes because the SIL-IS has the same physiochemical properties as the analyte and should have the same or a very similar extraction recovery, if mixed well with the analytes in the matrix before extraction. The extraction efficiencies of Trp-d5 and Kyn-d4 spiked into nontreated human plasma or Trp/Kyn spiked into stripped human plasma were >90% (Table 2). Similar extraction efficiencies for Trp and Kyn were also confirmed using HPLC–UV detection. UV-detection has no mass ionization suppression/enhancement problem, which may affect the extraction recovery assessment.

Mass ionization suppression/enhancement—The matrix ionization suppression or enhancement of Trp and Kyn were assessed by measuring the MFs of surrogate analytes (Trp-d5 and Kyn-d4). Both MFs of Trp-d5 and Kyn-d4 at low and high concentrations from different lots of human plasma were 0.86–1.07 (Table 3), which indicates there is no significant mass ionization suppression or enhancement.

In addition, the ionization suppression/ enhancement of Trp and Kyn was also evaluated using stripped human plasma by measuring the MF, as well as IS normalized MF. As Supplementary Table 3 shows, both MF and IS normalized MF were close to 1.0, which also supports that there is no significant ionization suppression/enhancement for the Trp and Kyn assay. However, a significant ionization suppression (~50% inhibition) of Kyn and Kyn-d4 by TFA was observed in some column lots, which may be due to those lots having less separation of Kyn with TFA. No significant ionization suppression by the biological matrix was observed, because the mass response of human plasma postextraction spiked samples was similar to that of water/TFA spiked samples. Although there is ionization suppression of Kyn and Kyn-d4 in some column lots, the IS normalized MF for Kyn was close to 1.0 (data not shown), which suggests the Kyn-d4 IS can compensate for the ionization matrix effect. The accuracy and precision for the Kyn assay were not significantly affected in different lots of columns.

The process efficiency [21,22], which covers both extraction recovery and mass detection ionization, were assessed by comparing the peak areas of Trp-d5 or Kyn-d4 spiked into pre-extraction human plasma samples to that spiked into pure solution (water) samples and processed in the same way. Both Trp-d5 and Kyn-d4 process efficiencies were >95%. The process efficiency is easy to assess monitoring the IS (Trp-d5 and Kyn-d4) peak area from surrogate matrix samples and from authentic matrix samples.

Matrix variation—Matrix variation [23], can also be called a total or overall matrix effect, which includes the extraction variation and mass ionization variation [31]. Evaluation of the overall matrix effect for an endogenous compound assay is very challenging, especially when the reference method is not available [31,32]. One way to test whether an assay has any (overall) matrix effect or not is to evaluate the spiked recovery. The spiked recovery test is spiking the analyte into pre-extraction samples, which is different from the MF test with spiking analyte into post-extraction samples [31]. As Table 4 shows, the spiked recoveries were 104–116% when adding 5000 ng/ml of Trp and 300 ng/ml of Kyn to six different lots of human plasma with various baseline Trp and Kyn levels. The CVs of measured Trp and Kyn concentrations from these lots were ≤5% (n = 5). These results indicate different lots of matrix do not affect the Trp/Kyn assay significantly.

Dilution linearity—Another way to assess the matrix effect is to test the dilution accuracy by diluting different lots of nontreated human plasma with pure water or buffer, which is also called a 'parallelism' test [25]. As Figure 3 shows, the flat profiles of both Trp and Kyn indicate good dilution linearity or dilution accuracy of six lots of nontreated human plasma, when diluted two- and four-times with water, which also supports the idea that there are no significant matrix effects for this assay.

Stability tests—The human plasma samples stored at room temperature were stable for at least 24 h. Heating plasma samples to 56°C for 30 min, which is often used to deactivate HIV prior to sample processing, also did not affect Trp and Kyn levels. Human plasma samples frozen to −20°C and thawed to room temperature for five cycles did not change the Trp and Kyn concentrations. A slow degradation of Trp to Kyn in processed samples stored at 4°C was observed. However, the Kyn formed from Trp was insignificant, if the processed samples were injected within 24 h. The processed calibrator and QC samples injected immediately after processing or injected 24 h later in a 4°C autosampler show no significant differences. Nontreated human plasma samples stored in −20°C freezers for 12 months were found to be stable for both Trp and Kyn. The stock solutions of Trp and Kyn were stable at room temperature at least for 6 h and at 4°C for 1 year.

Application of method to clinical human plasma sample analysis

This method has been successfully applied to analyze Trp and Kyn concentrations in human plasma samples from HIV-infected patients. The CVs of the spiked pure water QC samples at low, medium and high concentrations were <7.9% (n = 6). The mean accuracies of low, medium and high QC samples were from 98–103%. The CVs of pooled human plasma QC samples at low, medium and high levels were less than 9% (n = 16). These results indicate that this method has a good reproducibility when analyzing for Trp/Kyn in human plasma samples.

Conclusion

A simple, specific and reproducible LC–MS/MS method has been developed and validated for measuring Kyn and Trp in human plasma samples. Assay validation issues such as matrix effect, extraction recovery, LOQ and endogenous substance bioanalysis using LC–MS/MS have also been discussed.

Future perspective

In recent years, there have been many discussions about endogenous compound or biomarker bioanalysis by LC–MS/MS. However, there is no consensus validation guideline on it. An endogenous analyte assay has some specific issues that are not addressed in the current bioanalytical method validation guidance [101,102], which is mainly used for xenobiotic drug assays. In this article, we use the Kyn/Trp LC–MS/MS assay as an example to report our approach to the endogenous compound bioanalysis issues such as matrix effect, extraction recovery, LOQ and calibrators. We hope it can stimulate additional discussions in the bioanalysis community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Financial & competing interests disclosure

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Key Terms

LOQ	Lowest concentration in a sample that can be quantitatively determined with predefined precision and accuracy
Extraction efficiency	Percentage of analyte that is extracted from a sample matrix during the sample extraction process. The analyte in sample matrix can be a blank matrix sample fortified with the analyte or the analyte incurred in the sample
Spiked recovery	Ratio of a measured value for spiked sample minus the measured value for nonspiked sample to the spiked value. It is often used as a measure of accuracy for an assay
Matrix variation	Effect of different sources (or lots) of matrix on an assay

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Executive summary

Background

- Human plasma kynurenine (Kyn) and tryptophan (Trp) concentration ratio is currently used as a biomarker to monitor Indoleamine 2,3-dioxygenase activities in HIV-infected patients.

Results

- A simple, rapid and specific LC–MS/MS method for accurate determination of Kyn and Trp concentrations in human plasma from HIV-infected patients was developed and validated.
- Calibrators using surrogate matricies such as water, phosphate buffered saline, 1% bovine serum albumin or stripped human plasma were compared and no significant differences were found for human plasma Trp and Kyn assay.
- The extraction efficiency and mass ionization suppression/enhancement were evaluated using stable isotope-labeled internal standard surrogate analytes method.
- Matrix variation or matrix accuracy defined by different lots of matrix on the assay accuracy was evaluated using six lots of human plasma to assess spiking recovery.
- Dilution linearity was assessed from analysis of six lots of human plasma diluted two- and four-times with water, and results also support a conclusion that there is no significant matrix effect in the analysis for Trp and Kyn.
- Some assay validation issues such as LOQ, matrix effect, and extraction recovery for endogenous substances using LC–MS/MS methods are discussed.

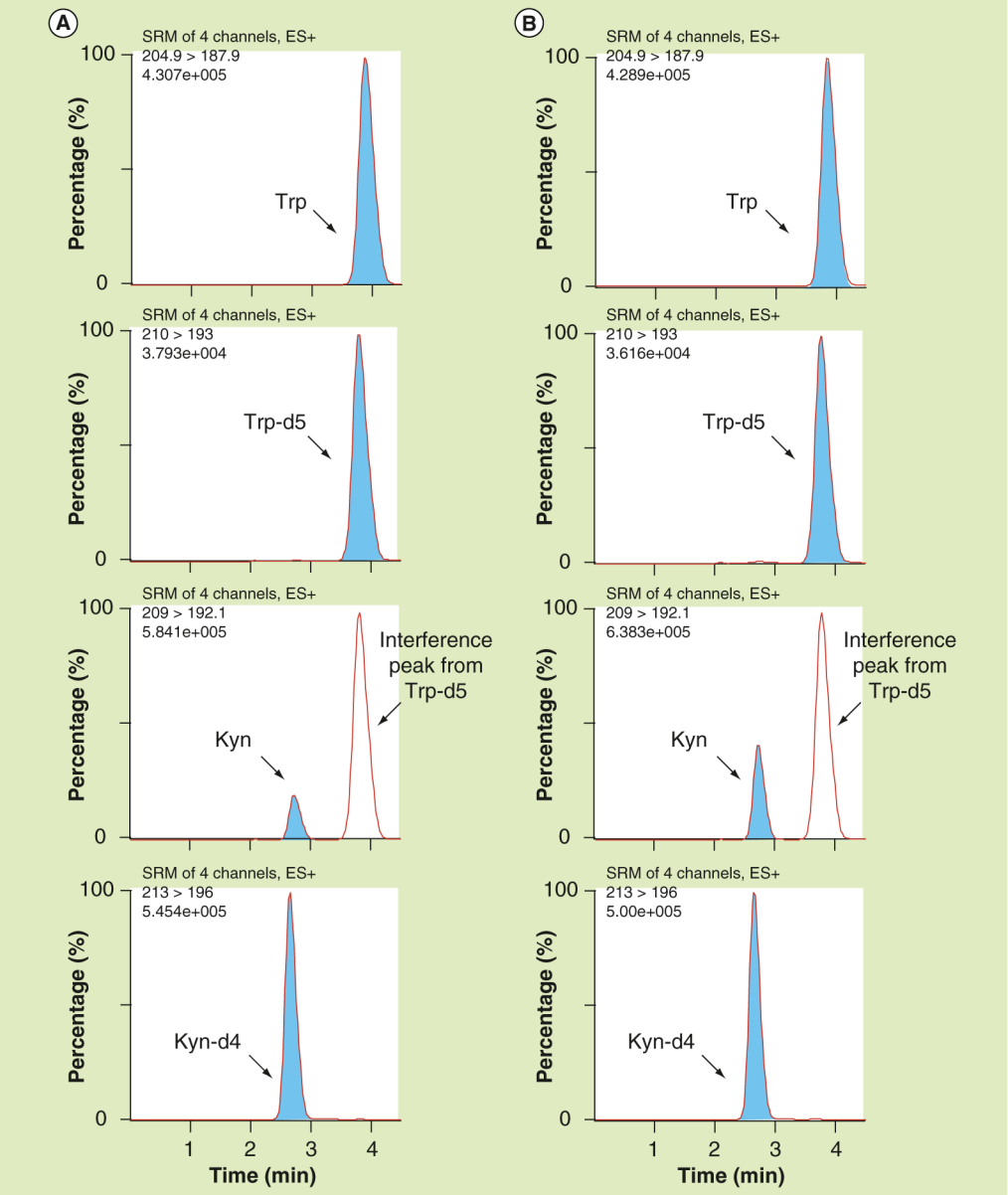

Figure 1. LC–MS/MS chromatograms for analysis of tryptophan and kynurenine in human plasma

(A) Human plasma and **(B)** standard.

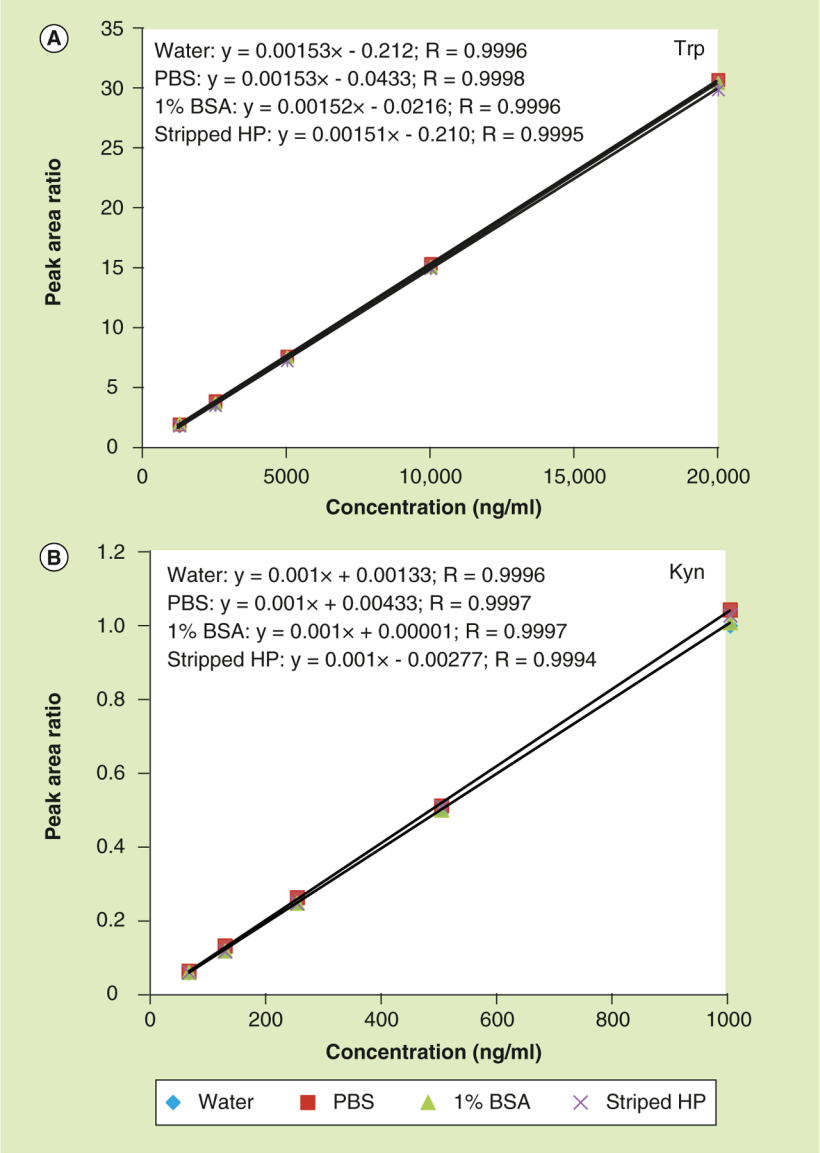

Figure 2. Comparison of different calibrators

The calibrators for **(A)** Trp and **(B)** Kyn assay were prepared in different matrices. Each data point was the mean of duplicate measurements.

BSA: Bovine serum albumin; HP: Human plasma; Kyn: Kynurenine; PBS: Phosphate buffered saline; Trp: Tryptophan.

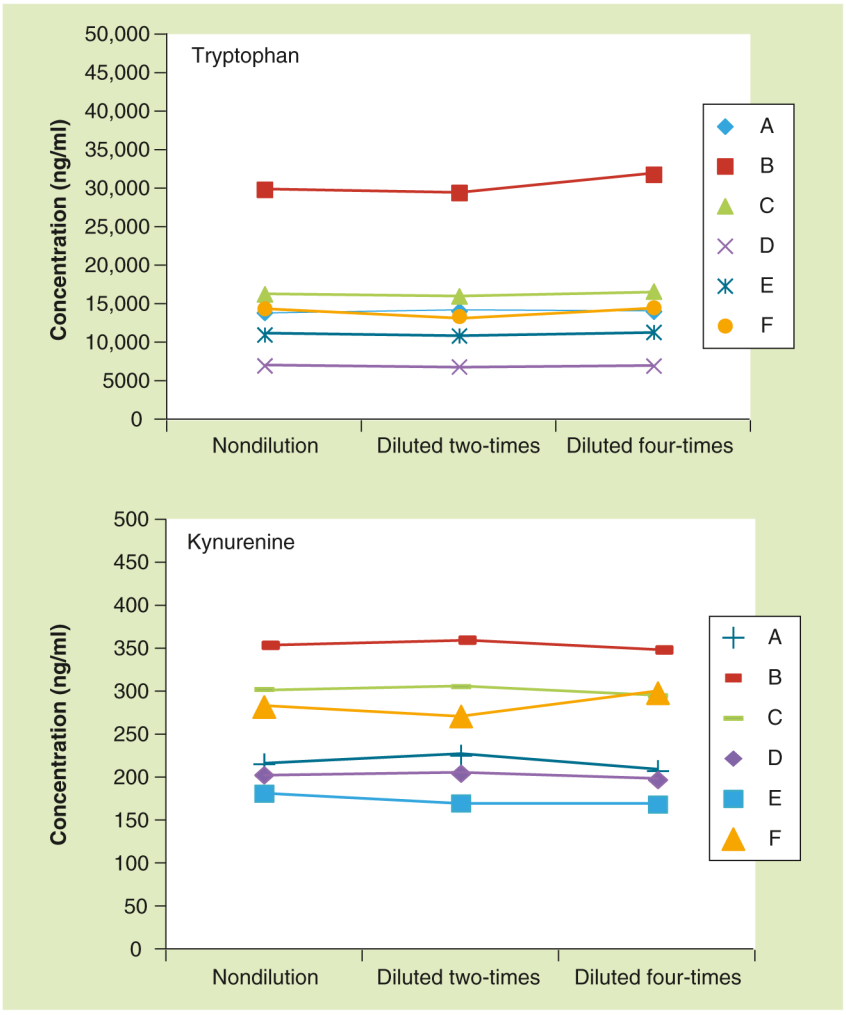

Figure 3. Dilution linearity tests for tryptophan and kynurenine assays

Six lots (A–F) of healthy human plasma were diluted zero-, two- and four-times with water. The data was the mean of triplicates. The analyte concentrations were multiplied with the dilution factors.

Table 1

Intra-day and inter-day precision and accuracy for analysis of tryptophan and kynurenine in human plasma.

QC type		Striped HP			Pure water			Nontreated HP		
QC level		H	M	L	H	M	L	H[†]	M	L[‡]
Tryptophan QC conc. (ng/ml)		***15,000***	***10,000***	***5000***	***15,000***	***10,000***	***5000***	***M + 5000***	***M***	***½ M***
Intra-day	n (1 × 6)	6	6	6	6	6	6	6	6	6
	Mean measured conc. (ng/ml)	15,433	10,467	5192	15,683	10,483	5127	14,733	10,287	5007
	SD	371	234	116	567	331	134	480	360	147
	CV (%)	2.4	2.2	2.2	3.6	3.2	2.6	3.3	3.5	2.9
	Accuracy	103	105	104	105	105	103	89		97
Inter-day	n (3 × 4)	12	12	12	12	12	12	12	12	12
	Mean measured conc. (ng/ml)	14,467	9692	4792	14,775	9625	4753	14,542	9513	4740
	SD	496	271	147	449	226	96	491	359	107
	CV (%)	3.4	2.8	3.1	3.0	2.3	2.0	3.4	3.8	2.3
	Accuracy	96	97	96	99	96	95	101		100
Kynurenine QC conc. (ng/ml)		***600***	***300***	***150***	***600***	***300***	***150***	***M + 300***	***M***	***½ M***
Intra-day	n (1 × 6)	6	6	6	6	6	6	6	6	6
	Mean measured conc. (ng/ml)	610	301	145	616	317	162	518	236	111
	SD	12	7.2	7.0	10	7.8	17	14	4.3	5.0
	CV (%)	1.9	2.4	4.9	1.7	2.5	11	2.8	1.8	4.6
	Accuracy	102	100	97	103	106	108	94		94
Inter-day	n (3 × 4)	12	12	12	12	12	12	12	12	12
	Mean measured conc. (ng/ml)	572	285	139	604	306	158	511	230	112
	SD	20.	6.6	5.2	15	14	11	27	7.8	3.5
	CV (%)	3.5	2.3	3.8	2.5	4.4	7.0	5.3	3.4	3.1
	Accuracy	95	95	93	101	102	105	94		97

[†]H nontreated HP = M HP spiked with 5000 ng/ml of tryptophan and 300 ng/ml of kynurenine.

[‡]L nontreated HP = M HP diluted two-times with water.

Accuracy of H nontreated HP = 100 × ([H C_{mean}−M C_{mean}]/C_{spiked}); Accuracy of L nontreated HP= 100 × L C_{mean} /(M C_{mean}/2).

H: High; HP: Human plasma; L: Low; M: Medium.

Table 2

Extraction efficiency for analysis of kynurenine and tryptophan in human plasma.

Analyte	Spiked conc. (ng/ml)	Matrix	Detection	Mean extraction efficiency (%; n = 3)
Trp	15,000	Striped HP	UV	90
	5000	Striped HP	UV	97
Trp-d5	15,000	Striped HP	UV	90
	5000	Striped HP	UV	95
	15,000	Nontreated HP	MS	94
	5000	Nontreated HP	MS	100
Kyn	600	Striped HP	UV	99
	150	Striped HP	UV	104
Kyn-d4	600	Striped HP	UV	92
	150	Striped HP	UV	96
	600	Nontreated HP	MS	98
	150	Nontreated HP	MS	109

HP: Human plasma; Kyn: Kynurenine; Trp: Tryptophan.

Table 3

Matrix factor[†] of kynurenine-d4 and tryptophan-d5 using nontreated human plasma.

Human plasma lot	Trp-d5		Kyn-d4	
	L[‡]	H[§]	L[‡]	H[§]
A	0.97	0.88	1.01	0.91
B	0.89	1.03	0.93	1.07
C	0.93	0.96	0.99	0.99
D	0.86	0.95	0.88	0.97
E	0.89	1.00	0.93	1.03
F	0.95	1.01	1.02	1.00
Average	0.92	0.97	0.96	0.99
SD	0.04	0.05	0.06	0.05
CV%	4.7	5.3	5.9	5.3

[†]Peak area of post-extraction spiked with Trp-d5/Kyn-d4 at L or H level compared with peak area of Trp-d5/Kyn-d4 spiked into pure water without trifluoroacetic acid.

[‡]Trp-d5, 5000 ng/ml, Kyn-d4, 150 ng/ml.

[§]Trp-d5, 15,000 ng/ml, Kyn-d4, 600 ng/ml.

H: High; Kyn: Kynurenine; L: Low; Trp: Tryptophan.

Table 4

Matrix variation on tryptophan and kynurenine assay.

Plasma lot	Parameters	Trp		Kyn	
		Baseline†	Spiked‡	Baseline†	Spiked‡
	Analyte spiked amount (ng)		500		30
	Analyte spiked concentration (ng/ml)		5000		300
A	Measured mean concentration (ng/ml; n = 5)	11220	16440	190	502
	CV (%)	3.5	1.8	4.3	1.6
	Spiked recovery (%)§		104		104
B	Measured mean concentration (ng/ml; n = 5)	23360	29080	276	603
	CV (%)	4.4	1.5	3.2	2.1
	Spiked recovery (%)§		114		109
C	Measured mean concentration (ng/ml; n = 5)	12600	18400	261	610
	CV (%)	2.7	2.9	2.4	2.2
	Spiked recovery (%)§		116		116
D	Measured mean concentration (ng/ml; n = 5)	6224	11560	191	522
	CV (%)	3.6	2.4	2.9	1.5
	Spiked recovery (%)§		107		110
E	Measured mean concentration (ng/ml; n = 5)	9330	14600	172	499
	CV (%)	2.2	2.2	5.0	0.9
	Spiked recovery (%)§		105		109
F	Measured mean concentration (ng/ml; n = 5)	11380	16600	244	574
	CV (%)	2.6	1.7	1.4	1.9
	Spiked recovery (%)§		104		110

†The baseline: take 80 μl of baseline HP and add with 20 μl of water.

‡The spiked: take 80 μl of baseline HP and add with 20 μl of Trp (25 μg/ml) and Kyn (1.5 μg/ml).

[§] Spiked recovery = 100 × (measured concentration spiked - concentration baseline)/concentration spiked.

HP: Human plasma; Kyn: Kynurenine; Trp: Tryptophan.