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Mass spectrometric measurement of urinary kynurenine-to-tryptophan ratio in children with and without urinary tract infection

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ABSTRACT

Background: Indoleamine-2,3-dioxygenase (IDO) catalyzes the first step of tryptophan (Trp) catabolism, yielding kynurenine (Kyn) metabolites. The kynurenine-to-tryptophan (K/T) ratio is used as a surrogate for biological IDO enzyme activity. IDO expression is increased during *Escherichia coli* urinary tract infection (UTI). Thus, our objective was to develop a method for measurement of Kyn/Trp ratio in human blood and urine and evaluate its use as a biomarker of UTI.

Methods: A mass spectrometric method was developed to measure Trp and Kyn in serum and urine specimens. The method was applied to clinical urine specimens from symptomatic pediatric patients with laboratory-confirmed UTI or other acute conditions and from healthy controls.

Results: The liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was linear to 500 μmol/L for both Trp and Kyn. Imprecision ranged from 5 to 15% for Trp and 6–20% for Kyn. Analytical recoveries of Trp and Kyn ranged from 96 to 119% in serum and 90–97% in urine. No correlation was found between the K/T ratio and circulating IDO mass ($r = 0.110$) in serum. Urinary Kyn and Trp in the pediatric test cohort demonstrated elevations in the K/T ratio in symptomatic patients with UTI (median 13.08) and without UTI (median 14.38) compared to healthy controls (median 4.93; $p < 0.001$ for both comparisons). No significant difference in K/T ratio was noted between symptomatic patients with and without UTI ($p = 0.84$).

Conclusions: Measurement of Trp and Kyn by LC-MS/MS is accurate and precise in serum and urine specimens. While urinary K/T ratio is not a specific biomarker for UTI, it may represent a general indicator of a systemic inflammatory process.

1. Introduction

Indoleamine 2,3-dioxygenase (IDO) is a mammalian enzyme that catalyzes the enzymatic conversion of tryptophan to its first stable metabolite, L-kynurenine. The best recognized physiologic role of IDO is in regulatory T lymphocytes, where consumption of tryptophan limits local proliferation of effector T cells in the settings of maternal-fetal tolerance, autoimmunity, and tumorigenesis [1,2]. Expression of IDO in many cell types is low in the basal state but is upregulated in a variety of inflammatory conditions, and several groups have investigated the clinical utility of measuring IDO activity as a marker of disease burden [3–7]. This has typically been accomplished by measurement of kynurenine and tryptophan in serum samples, with the so-called "K/T ratio" serving as a surrogate for IDO enzymatic activity [8,9]. Mass spectrometry (MS) has been used to define the urine metabolome, first in a diabetic murine model and subsequently applied clinically in the search for bladder cancer markers [10–12]. Measurement of urine kynurenine has been reported previously with high-performance liquid chromatography (HPLC) [13]. Here, we sought to establish methods for measurement of urine kynurenine and tryptophan utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The diagnosis of urinary tract infection (UTI) in children remains challenging, as current methodologies provide suboptimal sensitivity and specificity, and clinical presentations may feature only nonspecific

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symptoms (e.g., fever), especially in preverbal children [14]. Infections of the bladder (cystitis) and kidney (pyelonephritis) are caused primarily by uropathogenic strains of *Escherichia coli* (UPEC). Current guidelines for UTI diagnosis require thoughtful interpretation of the dipstick urinalysis in conjunction with clinical features and the results of urine culture [14]. To reduce contamination, appropriate urine cultures in young children are typically obtained via an invasive procedure, either urethral catheterization or suprapubic aspiration. Finally, urine culture and antimicrobial susceptibility results may require 24–48 h of incubation, making timely treatment decisions difficult.

These clinical conundrums have prompted calls for research to identify new UTI biomarkers, ideally in samples collected noninvasively [14–18]. Recent efforts include evaluations of urinary small peptides for rapid diagnosis in the clinical setting, which have not yet proven successful [19,20]. Preclinical studies have demonstrated that UPEC induce local IDO expression in the urinary bladder during acute cystitis in vivo [21,22]. We therefore aimed to develop mass spectrometric methods to measure K/T ratio in human urine as well as serum, and to evaluate the potential utility of the urinary K/T ratio in the diagnosis of UTI in young children.

2. Materials and methods

2.1. Materials and hardware

Tryptophan, kynurenine, acetonitrile, methanol, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Tryptophan (indole-D5) and kynurenine (ring-D4, 3,3-D2) were obtained from Cambridge Isotope Laboratories (Andover, MA). Human indoleamine-2,3-dioxygenase (IDO) ELISA was purchased from Kamiya Biomedical (Seattle, WA). Metabolite assays were performed using an AB-Sciex API 3200 tandem mass spectrometer (Foster City, CA) equipped with an electrospray ion source coupled to an Agilent 1200 HPLC system (Santa Clara, CA) in positive ionization mode.

2.2. Analytical methods

Serum or urine was diluted 10-fold in mobile phase (80% acetonitrile/0.1% formic acid) containing 25 μmol/L D5-tryptophan and 2.5 μmol/L D6-kynurenine, then centrifuged at 13,000 × *g* for 5 min at room temperature. 1.0 μL of supernatant was injected into mobile phase at a flow rate of 350 μL/min, and ion current was monitored for 1.5 min. Precursor/product pairs monitored were 205/146, 210/150, 209/94, and 215/98 for tryptophan, D5-tryptophan, kynurenine, and D6-kynurenine, respectively. Declustering, entrance, collision cell entry, and collision cell exit potentials were 26.0/31.0 V, 7.0/6.5 V, 7.0/14.0 V, 4.0/4.0 V, respectively for tryptophan/kynurenine. Collision energies were 23 eV and 19 eV for tryptophan and kynurenine, respectively. Desolvation temperature was 350 °C, and dwell time was 100 msec. A seven-point, linear, unweighted calibration curve was employed for quantitation. Circulating immunoreactive IDO was determined in serum from patients with a broad range of K/T ratios using a two-site ELISA method performed according to manufacturer instructions. Samples were diluted 20-fold in PBS prior to analysis, and concentrations were determined in 2–4 replicates.

2.3. Study subjects

All activities involving human subjects received review and approval in advance from the Human Research Protection Office at Washington University. Analytic development was supported with residual serum and urine specimens submitted to the St. Louis Children's Hospital (SLCH) Core Laboratory for clinical indications. Serum and urine reference intervals were determined in specimens with negative serologic testing and normal urinalysis that were frozen at −20 °C until analysis. Prior studies have indicated that there is no significant change in either kynurenine or tryptophan concentration after prolonged specimen storage at freezing temperatures in serum and urine [23,24]. For the UTI test cohort, children < 2 years of age were enrolled from the Emergency Department (ED) and inpatient units at SLCH from March 2013 through June 2014. Written consent was obtained in all cases from the parent or legal guardian; enrolled children were not of appropriate age to provide assent. Children were selected for screening if there was clinical concern for UTI and if urine and blood sampling had been ordered for routine clinical purposes. Children were excluded from the study if they had a history of urinary tract instrumentation (surgery, cystoscopy, stenting), if there was no English-speaking caregiver, or if consent was not obtained. After full laboratory results and clinical follow-up were available, subjects were then categorized into one of two groups. Children with "Confirmed UTI" had urinalysis compatible with UTI (positive leukocyte esterase, nitrites, and/or pyuria by microscopy) plus a catheter-obtained urine culture yielding > 50,000 colony-forming units (CFU) of a typical uropathogen [14]. Enrolled patients not meeting these UTI criteria, and therefore having other final diagnoses, were termed "Symptomatic." Separately, we contemporaneously enrolled healthy control children < 2 years of age from the Same Day Surgery unit at SLCH. This "Healthy" cohort included children presenting for elective ambulatory surgery procedures, those receiving sedation in the ambulatory procedure center for a scheduled outpatient procedure, or those visiting SLCH outpatient areas who were having blood drawn for clinical testing.

2.4. Statistical analysis

Correlations of analytes with age, and of K/T ratio with measured IDO mass, were studied using Pearson's coefficient in SPSS version 25 (IBM Analytics, Armonk, NY). To compare the K/T ratios in healthy controls vs. UTI or symptomatic non-UTI groups, Mann-Whitney *U* test was performed using Prism version 7 for Windows (GraphPad, La Jolla, CA). Proportions of true positive (TP), false positive (FP), true negative (TN), and false negative (FN) test results were determined using urine culture as the gold standard for comparison. Sensitivity and specificity were calculated as follows: sensitivity = TP / (TP + FN) and specificity = TN / (TN + FP).

3. Results

3.1. Analytical performance of Trp and Kyn measurement by mass spectrometry

After correcting for endogenous concentrations, Trp and Kyn were measured in serially diluted serum and urine pools supplemented with Trp and Kyn to a starting concentration of 500 μmol/L. Measurements of both analytes were linear to at least 500 μmol/L (Fig. 1). Accuracy of both measurements was established by assessing recovery at two concentrations in both serum and urine. Mean recovery of Trp and Kyn ranged from 97 to 101% in serum and from 90 to 97% in urine (Table 1). Imprecision of Trp and Kyn was assessed at two concentrations in both sample matrices (Table 1) by performing 5 replicate measurements in 5 analytic runs over 5 d. At concentrations of 51 and 224 μmol/L in serum, imprecision in Trp measurement was 5.1% and 6.2%, respectively; in urine, imprecision was 15% and 9.2% at 85 and 1150 μmol/L, respectively. For Kyn measurement, imprecision was 14.4% and 6.7% in serum at 1.6 and 6.6 μmol/L, respectively, and 20.8% and 9.0% in urine at 3.2 and 18 μmol/L, respectively. Within-run imprecision (n = 10) at the lowest measured concentration of Trp and Kyn (0.5 μmol/L) was 12% and 3.1%, respectively (data not shown).

Endogenous Kyn and Trp concentrations were measured in residual serum samples ($n = 53$) from general pediatric patients (1–21 years of age). Non-parametric reference intervals were calculated as 14–97 μmol/L for Trp and < 3.5 μmol/L for Kyn (Fig. 2). The reference interval for K/T ratio was calculated as 1.6–8.0. No age-dependent

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