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Short Communication

Analytical and clinical validation of an LC–MS/MS method for urine leukotriene E_4: A marker of systemic mastocytosis

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ABSTRACT

Objectives: Systemic mastocytosis (SM) is a disorder characterized by the excessive accumulation of clonally derived mast cells in various tissues. When triggered, mast cells release large amounts of histamine, prostaglandins and leukotrienes. Leukotriene E4 (LTE_4) is the primary stable metabolite of total cysteinyl leukotrienes. We hypothesized that secretion of LTE_4 would be increased in SM and could be used alone or in combination with current urinary biomarkers to optimize screening for SM.

Design and methods: LTE_4 was measured by liquid chromatography followed by tandem mass spectrometry (LC–MS/MS). Analytical assay validation was performed using residual urine specimens. LTE_4 results were normalized to urine creatinine for clinical use. Reference interval was established using a healthy volunteer cohort. Clinical sensitivity and specificity for SM detection were determined by measuring urinary biomarkers (LTE_4, N-methyl histamine [NMH] and 11β-prostaglandin $F_{2\alpha}$ [BPG]) in a cohort of 409 patients referred to allergy specialists, 66 (16%) of which were diagnosed with SM.

Results: Urinary LTE_4 measurement was accurate, precise and linear across a range of 31–3020 pg/mL. The 95th percentile of the reference interval population was <104 pg/mg creatinine. Median urine LTE_4 concentrations were significantly higher among patients with SM (97 pg/mg cr. vs. 50 pg/mg cr.; $p < 0.01$). Elevated urinary LTE_4 was 48% sensitive and 84% specific for SM. Clinical sensitivity was 53% for BPG (>1000 ng/mL) and 71% for NMH (>200 ng/mL). Incorporating all three urinary metabolites improved the SM diagnostic sensitivity to 97%, with minimal change in specificity.

Conclusions: We have developed a sensitive and precise LC–MS/MS assay for quantitation of LTE_4 in urine. Incorporating LTE_4 into a panel including BPG and NMH provides a much-needed screening tool for a complicated disease with non-specific symptoms and invasive confirmatory testing.

1. Introduction

Systemic mastocytosis (SM) is a disorder characterized by the excessive accumulation of clonally derived mast cells in various tissues. When triggered, mast cells release large amounts of tryptase, histamine, prostaglandins and leukotrienes. This release of signal molecules causes intermittent "spells" with varying symptoms that may include itching, flushing, lightheadedness, tachycardia, gastrointestinal distress, or even loss of consciousness. Diagnostic criteria established by the World Health Organization (WHO) recommend a bone marrow biopsy, specialized cytology studies or genetic testing [1].

Urine concentrations of N-methyl histamine (NMH) and 11-beta prostaglandin $F_{2\alpha}$ (BPG), which are metabolites of mast cell derived histamine and prostaglandin, have been used to aid in screening and reduce unnecessary biopsies [2–4]. Cysteinyl leukotrienes are another class of mast cell secretory molecules and potent inflammatory mediators. Leukotriene E_4 (LTE_4) is the primary stable metabolite of total cysteinyl leukotrienes [5]. Concentrations of LTE_4 are low in circulation but accumulate in the urine. We hypothesized that urinary LTE_4 could be used alone or in combination with NMH and BPG to optimize screening for SM. Here we describe a novel (LC–MS/MS) assay to accurately and precisely quantitate LTE_4 in urine and outline its clinical utility in SM screening.

2. Patients and methods

2.1. Study populations

All patient data were accessed in compliance with the Institutional Review Board. A normal reference population of 128 apparently healthy donors (64 men and 64 women) with a mean age of 43 (SD 14) years was recruited. Patients were self-reported as not having allergic disease or immunologic disorders and had not taken any antihistamines, non-

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steroidal anti-inflammatories, cyclooxygenase inhibitors, or 5-lipoxygenase inhibitors within 2 weeks.

Clinical performance was determined among a retrospective cohort of consecutive patients with clinically ordered 24-hour urine NMH measurement. Patients <18 years of age, solid organ transplant recipients or currently pregnant were excluded. A total of 409 patients were enrolled between April 26th, 2012 and March 21st, 2013. An allergy specialist evaluated all patients and final diagnoses were adjudicated by a chart review. The WHO criteria for the diagnosis of SM include the presence of the major criterion (the presence of multifocal dense infiltrates of mast cells (MC) in tryptase-stained biopsy sections of the bone marrow or of another extracutaneous organ) plus one minor criterion (more than 25% of MC show abnormal morphology; the presence of KIT Asp816Val mutation, abnormal mast cell phenotype indicated by the presence of CD25 on MC; serum total tryptase >20 ng/mL). Alternatively, the presence of three minor criteria will satisfy the requirements for the diagnosis [6]. Detailed diagnoses and medication usage are included in Supplemental Table 1.

2.2. LTE_4 method

D_5-labeled internal standard (D_5-LTE_4) was added to waste urine specimens, controls, and standards, followed by the addition of acetonitrile (ACN) to precipitate any excess salts. Precipitate was removed using positive pressure filtration through a 0.2 micron 96 well PTFE filter plate prior to injection of 45 μL via a CTC Pal autosampler and onto a Turboflow MAX mixed-mode anion exchange column (0.5 × 50 mm, Thermo Fisher Scientific). Following elution with Methanol(MeOH)/ammonium hydroxide, LTE_4 was further chromatographically separated on a C8 2.5-μm analytical column (Waters Xbridge, 2.1 × 50 mm, 60 °C) using a H_2O/MeOH mobile phase with ammonium hydroxide as the modifier. LTE_4 was monitored in negative MRM mode (AB Sciex API 5000 MS/MS). Detailed parameters are listed in Supplemental Table 2. The total analysis time was 10.3 min. All LTE_4 concentrations were normalized to creatinine (enzymatic method, Roche Diagnostics). SI unit conversions are 0.25 pg LTE_4/mg creatinine = 1.0 pmol LTE_4/mmol creatinine, 0.90 ng NMH/mg creatinine = 1.0 nmol/mmol creatinine, and 2.82 ng BPG/24 h = 1.0 pmol BPG/24 h.

2.3. NMH and BPG analytical methods

NMH was isolated from urine specimens by solid phase extraction and measured by LC–MS/MS using a stable isotope labeled internal D_3-N-methyl histamine. BPG was measured by competitive ELISA (Cayman Chemicals, Ann Arbor, MI). The method uses an acetylcholinesterase-linked BPG tracer, which competes with patient BPG for a limited number of rabbit-antibody binding sites. The unbound BPG is then washed away, and the remaining BPG tracer signal is inversely proportional to the concentration of BPG in the urine sample.

2.4. Method validation

Accuracy of the clinical LC–MS/MS method for LTE_4 was determined by spiking pooled urine with known amounts of purified LTE_4 between 100 to 2000 pg/mL. Average recovery was 111% (range 99–120%). Assay precision was determined by repeat analysis of three urine pools with average LTE_4 concentrations of 44, 445 and 1378 pg/mL over 20 days. Analytical sensitivity was determined by repeat injection (n = 20) of a charcoal stripped urine pool and a mobile phase blank. Linearity was assessed by mixing urine specimens with high and low LTE_4 concentrations. Three mixing studies were performed on three different days for a total of nine unique experiments (Fig. 1). Specimen stability was determined by measuring 10 specimens immediately after collection and at 1, 3, 7, 14, 30, and 90 days stored ambient, refrigerated, or frozen (−20 °C). Analytical specificity was assessed by spiking a urine sample with 11-trans LTE_4.

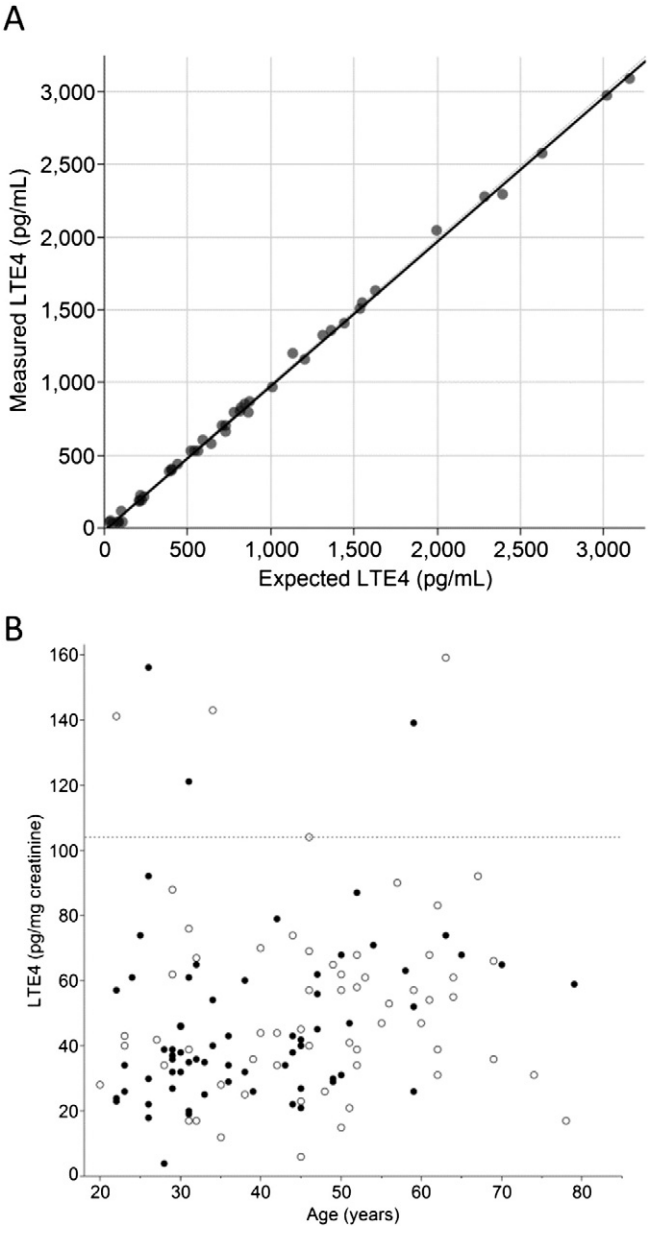

Fig. 1. Analytical performance of a novel liquid chromatography tandem mass spectrometry (LC–MS/MS) method for urinary leukotriene E_4 measurement. A) Linearity was demonstrated by mixing urine samples with high and low concentrations of LTE_4 (slope 0.9929; R^2 0.9985). B) LTE_4 values were measured in 128 healthy donors (67 males, closed circles; 61 females, open circles); the 95th percentile value was 104 pg/mg cr. (dotted line).

2.5. Statistical analyses

Statistical analyses were performed using JMP software (SAS Inc.; Cary, NC). Relationships of the appropriate percentiles with age and sex were evaluated using quantile regression by minimizing an asymmetrically weighted sum of absolute errors; 95th percentile was established using a smoothed empirical likelihood quantile regression. Receiver operator characteristic (ROC) curve analysis was used to identify the optimal diagnostic cutoff and the discrimination c-statistic for each biomarker. Linearity was assessed by linear regression and Passing–Bablok regression. Significant relationships were defined as a p-value <0.05.