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Quantification of Lipid Mediator Metabolites in Human Urine from Asthma Patients by Electrospray Ionization Mass Spectrometry: Controlling Matrix Effects

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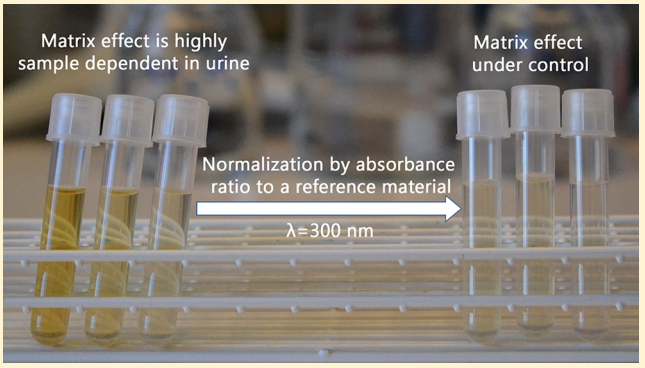
Supporting Information

ABSTRACT: Eicosanoids (e.g., prostaglandins and leukotrienes) are inflammatory signaling molecules that are metabolized and excreted in urine. The quantification of eicosanoid metabolites in human urine has been demonstrated to provide insight into the inflammatory and oxidative stress status of the individual. However, urine is a complex matrix that can exhibit profound matrix effects for quantification via liquid chromatography coupled to mass spectrometry (LC-MS/MS). This phenomenon can lead to impairment and biasing of results, because the sample background is dependent on the fluid intake and water−salt balance. Herein we describe an analytical methodology to address these limitations via the normalization of extracted urine volume by the ratio of absorbance at 300 nm to an optimized reference material. The platform is composed of 4 LC-MS/MS methods that collectively quantify 26 lipid mediators and their metabolites, with on-column limits of detection between 0.55 and 15 fmol. Prior to optimization, internal standards exhibited strong matrix effects with up to 50% loss of signal. Notably, the accuracy of exact deuterated structural analogues was found to vary based upon the number of incorporated deteurium. The platform was used to analyze urine from 16 atopic asthmatics under allergen provocation, showing increases in metabolites of prostaglandin D_2, cysteinyl leukotrienes, and isoprostanes following the challenge. This method presents a functional and reproducible approach to addressing urine-specific matrix effects that can be readily formatted for quantifying large numbers of samples.



Eicosanoids are lipid mediators that can act as autocrine and paracrine hormones.[1] They are produced following oxidation of arachidonic acid (AA) via three enzyme-mediated pathways: cyclooxygenase (COX; to form prostaglandins, thromboxane, and prostacyclin), lipoxygenase (LOX; to form leukotrienes [LTs] and hydroxyeicosatetraenoic acids [HETEs]), and cytochrome P450 (CYP; to form the regio- and stereospecific epoxides as well as 20-HETE).[2] The key role of eicosanoids in inflammatory processes has been extensively described.[3,4] In addition, oxidative stress can lead to autoxidation of AA via nonenzymatic pathways to produce prostaglandin (PG) isomers collectively known as isoprostanes.[5] The production of this plethora of compounds has been studied in order to assess their importance in multiple diseases.[3,4,6] In respiratory diseases, eicosanoids have been examined in multiple matrices including bronchoalveolar lavage fluid (BALF), induced sputum, exhaled breath condensate, plasma/serum, and urine.[2,7] While potentially insightful into pathophysiological processes, the collection of many of these matrices is invasive. COX- and LOX-derived eicosanoids possess fundamental roles in the pathology of asthma, including involvement in hyperresponsiveness (e.g., prostaglandin D_2 [PGD_2],[8] cysteinyl leukotrienes [cysLTs][9]), and bronchodilation (PGE_2).[10] For example, antileukotriene drugs (e.g., montelukast, zileuton) are established therapeutics for the treatment of asthma.[11] Eoxins (EX) are structural isomers of the cysLTs, whose biosynthesis is initiated via the 15-LOX pathway.[12]

After release, eicosanoids are metabolized and excreted in urine[13−16] (Figure 1), which is a noninvasive matrix that reflects the metabolic status of the whole organism.[17,18]

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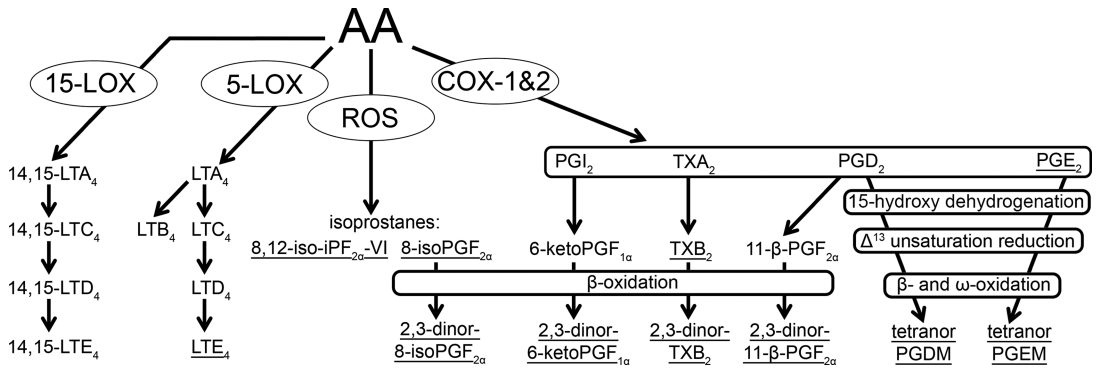

Figure 1. Schematic of eicosanoid metabolic cascade leading to urinary metabolites. Arachidonic acid (AA) can be metabolized via lipoxygenase (LOX), cyclooxygenase (COX), and oxidative stress (ROS) pathways, which eventually lead to urinary metabolites. Metabolites present in the current platform that have been previously reported in urine are underlined. It should be noted that this scheme is not intended to provide a comprehensive overview of eicosanoid metabolism and that many of these pathways can produce multiple compounds not displayed here (e.g., the primary 15-LOX product is 15[*S*]-hydroperoxyeicosatetraenoic acid [15-HPETE]).

Eicosanoids can be excreted as the free acids or in glucuronide conjugates.[19] Although eicosanoids are produced in numerous physiological and pathophysiological processes,[3,6] only a few compounds have been reported to be excreted in the urine of healthy individuals (Figure 1).[14,15,20,21] In the case of cysLTs, LTE_4 is the predominant species excreted in healthy and asthmatic individuals, but LTC_4 has been detected in patients deficient in γ-glutamyl transpeptidase.[22] Accordingly, it is beneficial to screen the complete biosynthetic pathway, not only the end point (LTE_4). In addition, there are further downstream products. For example, ω-oxidized metabolites of LTE_4 (e.g., 20-carboxy-LTE_4 and 18-carboxy-dinor-LTE_4) and LTB_4 (20-carboxy-LTB_4 and 18-carboxy-dinor-LTB_4) have been detected in urine.[13,23,24] While glucoronide conjugation can be of interest, levels need to be evaluated on a compound-specific basis with as little as <1% of ω-oxidized urinary metabolites of LTB_4 excreted as glucoronides[24] and no effect of gluoronidase treatment upon urinary levels of arachidonic, 20-HETE, and linoleic diols.[25] There are no known reports to date on the excretion of EXs in urine, making it of interest to screen this pathway. Both the lung and kidney are well-perfused systems and changes in the lung are reflected rapidly in the levels of excreted eicosanoids. Accordingly, even the fast events during a lung provocation can be reflected in the urinary eicosanoid output. The levels of mediators are low in resting inflammatory cells and increase during natural or provoked exacerbations, hence in circulation and finally in urine by excretion.

A distinct advantage of sampling urine is that it can be collected longitudinally and temporally multiple times without impacting the patient, providing not only a snapshot of the metabolite levels but also their rate of excretion. A challenge in analyzing urine is the inter- and intraindividual variability in concentration depending upon the sampling regimen. A number of different approaches have been proposed to account for this variability, including normalization by ratio to osmolality,[26] to creatinine concentration,[27] and, for mass spectrometry (MS)-based applications, to the total signal.[28] Creatinine excretion is race, gender, and age dependent in healthy donors but stable for a particular donor.[27,29,30] The level of excreted creatinine is commonly used as normalizing factor for urinary volume in provocations to asthma patients (when the donor is free of renal diseases).[15,31]

One significant obstacle in analyzing urine is the level of complexity, including high salt levels, that can reduce the quality of the quantification of eicosanoid metabolites. Consequently urine shows high background variability that can affect the analysis by either matrix effects or dilution.[32,33] To address this issue, we have developed a method for the analysis of eicosanoid metabolites in urine by ultraperformance liquid chromatography (UPLC) coupled to electrospray ionization (ESI) tandem triple quadrupole MS (ESI-MS/MS). This method compensates for the inter- and intra-individual sample matrix effects via the use of a reference material.[32] The volume of urine to extract is calculated using the ratio of absorbance between the sample and the reference material, enabling the minimization of matrix effects.

EXPERIMENTAL SECTION

Chemicals Standards. Unless otherwise noted, eicosanoid standards were purchased from Cayman Chemical (Ann Arbor, MI): PGD_2, [$^2H_9$]-PGD_2, tetranor prostaglandin D metabolite (tetranorPGDM), [$^2H_6$]-tetranorPGDM, 11-β-$PGF_{2\alpha}$, [$^2H_4$]-11-β-$PGF_{2\alpha}$, 2,3-dinor-11-β-$PGF_{2\alpha}$, PGE_1, 13,14-dihydro-15-keto-PGE_1, [$^2H_4$]-13,14-dihydro-15-keto-PGE_1, PGE_2, [$^2H_9$]PGE_2, 13,14-dihydro-15-keto-PGE_2, [$^2H_4$]-13,14-dihydro-15-keto-PGE_2, tetranor prostaglandin E metabolite (tetranorPGEM), [$^2H_6$]-tetranorPGEM, $PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, [$^2H_4$]-13,14-dihydro-15-keto-$PGF_{2\alpha}$, 6-keto$PGF_{1\alpha}$, [$^2H_4$]-6-keto$PGF_{1\alpha}$, thromboxane B_2 (TXB_2), [$^2H_4$]-TXB_2, 11-dehydroTXB_2, 2,3-dinorTXB_2, 8-iso-$PGF_{2\alpha}$, [$^2H_4$]-8-iso-$PGF_{2\alpha}$, 2,3-dinor-8-iso-$PGF_{2\alpha}$, leukotriene (LT) B_4, 6-*trans*-LTB_4, LTC_4, LTD_4, LTE_4, EXC_4, and EXE_4. 2,3-dinor-6-keto$PGF_{1\alpha}$ and [$^2H_3$]-2,3-dinor-6-keto$PGF_{1\alpha}$ were from Enzo Life Sciences (Lausen, Switzerland). The isoprostanes 8,12-iso-iP$F_{2\alpha}$-VI and [$^2H_4$]-8,12-iso-iP$F_{2\alpha}$-VI were synthesized as described before.[34,35] EXD_4 was kindly provided by H. E. Claesson (Karolinska Institute). Creatinine, glycerol, ammonium acetate, methoxyamine hydrochloride, and isopropanol were purchased from Sigma-Aldrich (Saint Louis, MO). Milli-Q ultrapure deionized water was used (Millipore Corporation, Billerica, MA). Methanol and acetonitrile were from Rathburn (Walkerburn, Scotland). [$^2H_3$]-creatinine was from CDN Isotopes (Pointe-Claire, Quebec). Acetone, acetic acid, and formic acid were from Fisher. Aqueous ammonia 25% (w/v) was from Merck (Darmstadt, Germany).

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Table 1. Ionization Mode, On-Column Limits of Detection, and Linear Range of the Eicosanoid Metabolites

biological origin of the analyte	eicosanoid	analytical method	ionization mode[a]	limit of detection (fmol)	linear range (fmol)
prostaglandin D pathway	PGD_2	A-2	neg	0.77	0.77−1149
	11-β-$PGF_{2\alpha}$	A-2	neg	3.17	3.18−2380
	2,3-dinor-11-β-$PGF_{2\alpha}$	A-2	neg	1.79	1.79−48.5
	tetranorPGDM	A-2	neg	5.34	5.34−2672
prostaglandin E pathway	PGE_1	A-2	neg	0.76	2.28−1142
	13,14-dihydro-15-keto-PGE_1	A-2	neg	2.53	2.53−212
	PGE_2	A-2	neg	0.77	0.77−574
	13,14-dihydro-15-keto-PGE_2	A-2	neg	7.70	7.7−3830
	tetranorPGEM	A-2	neg	15.4	15.4−7708
prostaglandin F pathway	$PGF_{2\alpha}$	A-2	neg	2.53	7.61−1904
	13,14-dihydro-15-keto-$PGF_{2\alpha}$	A-2	neg	3.81	11.4−952
prostacyclin pathway	2,3-dinor-6-keto$PGF_{1\alpha}$	A-1	neg	15.2	45.7−22900
thromboxane pathway	TXB_2	A-1	neg	0.85	0.85−1275
	11-dehydroTXB_2	A-2	neg	1.83	1.83−2748
	2,3-dinorTXB_2	A-1	neg	5.26	5.26−1314
oxidative stress	8-iso-$PGF_{2\alpha}$	A-2	neg	1.90	1.90−952
	2,3-dinor-8-iso$PGF_{2\alpha}$	A-2	neg	0.55	0.55−46
	8,12-iso-i$PF_{2\alpha}$-VI	A-2	neg	0.95	2.85−1428
5-lipoxygenase pathway	LTB_4	B	neg	4.45	13.4−3343
	6-*trans*LTB_4	B	neg	4.45	13.4−3343
	LTC_4	B	pos	4.51	13.5−3380
	LTD_4	B	pos	4.59	4.59−3445
	LTE_4	B	pos	4.50	4.50−1125
15-lipoxygenase pathway	EXC_4	B	pos	4.51	13.5−1125
	EXD_4	B	pos	3.02	9.06−755
	EXE_4	B	pos	4.50	4.50−1125
creatinine	N.A.[b]	C	pos	50 pg	50−2000 pg

[a]neg, ESI in negative mode; pos, ESI in positive mode. [b]Not applicable.

Analytical Methods. In all cases liquid chromatography coupled to mass spectrometry (LC-MS/MS) separation and quantification was carried out on a UPLC Acquity-Xevo TQ mass spectrometer system (Waters, Milford, MA). Dwell time was automatically adjusted in order to acquire 13 points per chromatographic peak; capillary voltage was 2.25 kV in negative mode and 2.50 kV in positive mode. The ionization polarity for each compound is provided in Table 1. Desolvation temperature and gas flow were set according to the instrument recommendations for the chromatographic flow. In all methods, calibration level aliquots were stored under argon at −80 °C in Wheaton prescored gold-band amber ampules (Sigma-Aldrich).[36] Sample chromatograms for each method are shown in Figure S-2.

Method A. *PG Metabolites and Isoprostanes.* Standards were dissolved in methanol, and eight calibration levels were prepared according to Table S-1 in methanol/water 50:50 (v/v). Solid phase extraction (SPE) of analytes was carried out in 3 cm^3/60 mg HLB Oasis SPE cartridges (Waters). SPE cartridges were conditioned with 3 mL of methanol and 3 mL of water with 0.1% of acetic acid. Urine was diluted with water to a total volume of 2 mL and mixed with 0.5 mL of water containing 0.5% of acetic acid, spiked with the internal standard mix solution, and loaded onto the SPE cartridge. Samples were washed with 3 mL of water with 0.1% of acetic acid, and the cartridges were dried in the SPE manifold under vacuum-induced air stream at −30 kPa for 30 min. Analytes were eluted with 3 mL of acetonitrile in tubes containing 6 μL of glycerol 30% (v/v) in methanol. After evaporation of the eluates under vacuum, samples were resuspended in 100 μL of methanol/water 50:50 (v/v). For the analysis of eicosanoid metabolites with tautomers (TXB_2, 2,3-dinorTXB_2, and 2,3-dinor-6-keto$PGF_{1\alpha}$), 40 μL of the resuspended material was reserved for derivatization (method A-1).[15] The remainder was used to analyze the metabolites that do not show tautomerism (method A-2). In both cases, the column used was an Acquity UPLC BEH C18 (2.1 × 150 mm, 1.7 μm, Waters), and 7.5 μL of sample were injected.

Method A-1, analysis of the compounds with tautomerism. Twenty-five grams of methoxyamine hydrochloride[15] were dissolved in 50 mL of water. Five microliters of this solution were added to 40 μL of sample and calibration curve levels. After 15 min the excess of methoxyamine was quenched with 5 μL of acetone, and after another 15 min the reaction was basified with 5 μL of ammonia 12.5% (w/v). Column temperature was set at 60 °C, and the mobile phase was a gradient of solvents A (water with 0.1% of acetic acid) and B (acetonitrile/isopropanol 90:10, v/v) with a flow of 0.325 mL min^{-1}. The gradient initiated with 75% of A, which changed linearly to 66% at 6.5 min and to 58% at 9.5 min. The column was then washed with solvent B and equilibrated to initial conditions.

Method A-2, analysis of the compounds without tautomerism. Column temperature was 40 °C, and the mobile phases were the same as in Method A-1 with a flow of 0.375 mL min^{-1}. The gradient initiated with 95% of A, which was decreased linearly to 90% at 1.5 min, to 84% at 6.5 min, to 69% at 7 min, to 67% at 20 min, and to 61% at 24 min. The column was then washed with solvent B and equilibrated to initial conditions.

Method B. *Leukotrienes.* Seven calibration levels were prepared according to Table S-2 in methanol/water 85:15 (v/v). The same SPE cartridges as in Method A were used and

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Table 2. Urine Concentration-Dependent Matrix Effects for Internal Standards[a]

		% ME[b]			% RE[c]			% OPE[d]		
internal standard	method	sample A	sample B	sample C	sample A	sample B	sample C	sample A	sample B	sample C
$[^2H_9]$-PGD_2	A-2	64.3	94.9	110	81.3	81.0	71.7	52.3	76.8	79.1
$[^2H_6]$-tetranorPGDM	A-2	72.5	117	126	76.4	62.5	59.1	55.4	72.8	74.7
$[^2H_4]$-11-β-$PGF_{2\alpha}$	A-2	85.9	104	111	70.8	77.7	69.5	60.8	80.5	77.1
$[^2H_4]$-13,14-dihydro-15-keto-PGE_1	A-2	97.7	101	103	82.8	78.9	86.9	81.0	80.0	89.9
$[^2H_9]$-PGE_2	A-2	92.9	109	120	76.5	82.4	69.8	71.0	89.6	83.4
$[^2H_4]$-13,14-dihydro-15-keto-PGE_2	A-2	110	106	102	80.1	86.1	83.5	88.3	91.6	85.0
$[^2H_6]$-tetranorPGEM	A-2	46.1	73.6	63.2	89.2	26.1	68.6	41.1	19.2	43.4
$[^2H_4]$-13,14-dihydro-15-keto-$PGF_{2\alpha}$	A-2	102	94.1	95.5	70.7	86.8	76.7	71.9	81.7	73.2
$[^2H_4]$-8-iso-$PGF_{2\alpha}$	A-2	57.7	87.6	105	83.2	84.6	72.4	48.0	74.2	76.3
$[^2H_4]$-8,12-iso-$iPF_{2\alpha}$-VI	A-2	91.8	104	111	74.7	75.0	61.6	68.6	77.7	68.6
$[^2H_3]$-2,3-dinor-6-keto$PGF_{1\alpha}$	A-1	53.0	83.7	102	84.7	88.9	94.6	44.9	74.4	96.9
$[^2H_4]$-TXB_2	A-1	59.3	90.2	97.8	64.1	68.7	77.5	38.0	61.9	75.8
$[^2H_4]$-LTB_4	B	84.1	107	103	81.2	83.3	60.6	68.3	89.4	62.2
$[^2H_5]$-LTC_4	B	106	120	109	68.8	70.0	47.5	72.8	83.8	51.9
$[^2H_5]$-LTD_4	B	82.8	111	106	62.6	62.1	34.3	51.8	68.7	36.5
$[^2H_5]$-LTE_4	B	109	175	112	66.4	61.7	33.0	72.1	108	37.0

[a]For methods A-1 and A-2, 1 mL of each sample was analyzed. For method B, 2.7 mL of sample were analyzed. Both analyses were performed in triplicate. [b]ME, matrix effect. [c]RE, recovery of extraction. [d]OPE, overall process efficiency.

conditioned as described above. Urine was mixed with water to 2.7 mL of total volume and 0.3 mL of water containing 1% acetic acid, spiked with the internal standards, and loaded onto the SPE cartridge. Samples were washed with 1 mL of methanol/water 50:50 (v/v) and 3 mL of water with 0.1% of acetic acid. The cartridges were dried in the SPE manifold under vacuum-induced air stream at −30 kPa for 30 min, and the analytes were eluted with 1 mL of methanol in tubes containing 3 μL of glycerol 30% (v/v) in methanol. After evaporation of the eluates under vacuum, samples were resuspended in 50 μL of methanol/water 85:15 (v/v).

Separation was performed in an Acquity UPLC BEH C18 (2.1 × 150 mm, 1.7 μm) column at 60 °C with a gradient of solvents C (water containing 0.2% of formic acid) and D (acetonitrile/isopropanol 90:10, v/v, plus 0.2% of formic acid) with a flow of 0.45 mL min^{-1}. The gradient was initiated with 60% of C and changed linearly to 50% at 4.25 min. The column was washed with solvent D and equilibrated to initial conditions. A 7.5 μL amount of resuspend samples was injected.

Method C. *Creatinine.* Seven different levels were prepared in water in concentrations of 20, 40, 100, 200, 400, 600, and 800 ng mL^{-1} with 300 ng mL^{-1} of internal standard, $[^2H_3]$-creatinine. Aliquots were stored at −20 °C. Urine was diluted 1:8000 (v/v), and 2.5 μL was injected and separated in a XBridge HILIC (2.1 × 50 mm, 2.5 μm, Waters) at 25 °C in a gradient of solvents E (acetonitrile) and F (100 mM ammonium acetate aqueous solution) with a flow of 0.4 mL min^{-1}. The gradient was initiated with 85% of E and decreased linearly to 20% at 1 min.

Absorbance. The absorbance of urine was used as a surrogate indicator of relative concentration. Reference material was generated by preparing a pool of urine to have a representative absorbance (0.273 in a dilution 1:40 [v/v]) in an Epoch microplate spectrophotometer (Biotek, Vermont, NE). Reference material had a concentration of 735 μg mL^{-1} of creatinine. The absorbance ratio of urine compared to a Milli-Q water blank was maximum at 300 nm (Figure S-1), which was subsequently employed for all urinary measurements. Prior to extraction, the absorbance of 150 μL of urine diluted 1:40 (v/v) was measured in 96-well plates, with results used to determine appropriate extraction volumes. The volume of initial sample required was calculated according to Equation S-1. When the aliquot to extract was <2 mL, it was diluted with Milli-Q water to give a final volume of 2 mL.

Clinical Samples. For model samples A, B, C, and reference material, midstream urine from 3 healthy female and 3 healthy male donors (age 25−35 years) was collected. Samples A, B, and C had absorbances of 0.363, 0.281, and 0.029, respectively, which corresponded to urinary colors of deep, normal, and light yellow. After optimization, 0.400 mL of the reference material were extracted for method A and 2.70 mL for method B.

For the assessment of metabolite excretion of eicosanoid metabolites during atopic asthma exacerbation, 16 subjects with mild atopic asthma and airway hyperresponsiveness to methacholine (MCh) and demonstrating allergen-induced early phase bronchoconstriction were challenged with rising doses of allergen to produce a 20% drop in lung function measured as forced expiratory volume in 1 s (FEV_1). The methodology was performed as described in Roquet et al.[37] Urine was collected before provocation and 1 h after challenge. In all cases donors were free of renal diseases. This study was approved by The South Ethical Review Board of Karolinska Institutet decision 2009/959-31-4 with ClinicalTrials.Gov Identifier: NCT01002690.

RESULTS AND DISCUSSION

Method Development. In order to achieve the necessary selectivity and sensitivity, the chromatographic separation, the derivatization, and the extraction were individually optimized. TetranorPGDM and tetranorPGEM are the key compounds for separation due to their similar chromatographic and spectrometric characteristics. They have previously been screened with a neutral loss of 18 units because it is the most abundant product ion;[38] however, the transition m/z 327/143 was chosen (Table S-3) as more selective for both compounds. A good chromatographic separation was needed in order to achieve selectivity for these two isomers. Both compounds evidenced low affinity for the C18 stationary phase, but baseline

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Table 3. Range of Maximum Internal Standard Recovery and Analyte Correction in Model Urine Samples[a]

			μg of creatinine extracted		
		method	sample A	sample B	sample C
optimum range of % OPE for internal standards[b]	$[^2H_9]$-PGD_2	A-2	50.0−100	50.0−100	50.0−450
	$[^2H_6]$-tetranorPGDM	A-2	300−750	300−750	50.0−200
	$[^2H_4]$-11-β-$PGF_{2\alpha}$	A-2	50.0−750	50.0−750	50.0−750
	$[^2H_4]$-13,14-dihydro-15-keto-PGE_1	A-2	50.0−750	50.0−750	50.0−750
	$[^2H_9]$-PGE_2	A-2	50.0−750	50.0−750	200−750
	$[^2H_4]$-13,14-dihydro-15-keto-PGE_2	A-2	300−750	300−750	200−750
	$[^2H_6]$-tetranorPGEM	A-2	50.0−200	50.0−100	50.0−100
	$[^2H_4]$-13,14-dihydro-15-keto-$PGF_{2\alpha}$	A-2	50.0−750	50.0−750	200−750
	$[^2H_4]$-8-iso-$PGF_{2\alpha}$	A-2	50.0−450	50.0−450	50.0−450
	$[^2H_4]$-8,12-iso-$iPF_{2\alpha}$-VI	A-2	50.0−750	50.0−750	200−750
	$[^2H_3]$-2,3-dinor-6-ketoPGF$_{1\alpha}$	A-1	50.0−200	50.0−200	50.0−200
	$[^2H_4]$-TXB_2	A-1	50.0−300	50.0−300	50.0−300
	$[^2H_4]$-LTB_4	B	500−4000	500−4000	–
	$[^2H_5]$-LTC_4	B	500−4000	500−4000	–
	$[^2H_5]$-LTD_4	B	500−4000	500−4000	–
	$[^2H_5]$-LTE_4	B	500−4000	500−4000	–
range in which the internal standards correct the response[c]	tetranorPGDM	A-2	75.0−750	50.0−450	50.0−300
	2,3-dinor-11-β-$PGF_{2\alpha}$	A-2	50.0−200	50.0−300	50.0−200
	tetranorPGEM	A-2	50.0−750	50.0−750	50.0−100
	$PGF_{2\alpha}$	A-2	100−600	75.0−600	200−600
	8-iso$PGF_{2\alpha}$	A-2	100−750	50.0−750	100−600
	2,3-dinor-8-iso$PGF_{2\alpha}$	A-2	50.0−300	50.0−300	50.0−300
	8,12-iso-$iPF_{2\alpha}$-VI	A-2	50.0−300	50.0−600	50.0−750
	TXB_2	A-1	300−750	200−750	200−750
	2,3-dinorTXB_2	A-1	50.0−750	50.0−750	50.0−600
	2,3-dinor-6-ketoPGF$_{1\alpha}$	A-1	100−750	100−600	300−750
	LTE_4	B	500−3000	500−2000	–

[a]For method A, the volume of urine with 50, 75, 100, 200, 300, 450, 600, and 750 μg of creatinine of every sample was extracted from samples A, B, and C. For method B, the volume of urine with 500, 1000, 1500, 2000, 3000, and 4000 μg of creatinine was extracted from samples A and B. [b]The optimum range of overall process efficiency (OPE) for the signals of the internal standard. [c]The range of linear relation between the concentration calculated and the amount of creatinine. The experiments were carried out in duplicate.

separation was achieved using a shallow gradient until 6.5 min in method A-2 (Figure S-2B).

The derivatization of those compounds with tautomers that lead to nongaussian chromatographic peaks was also optimized. Methoxyamine derivatization has been used prior to SPE;[15] however, it increases the probability of observing artifacts for all analytes, not only for those that show tautomers, as well as the cost of the analysis. If the excess methoxyamine hydrochloride was quenched with acetone and solution basified with ammonia, the analytes were stable at 4 °C for 24 h (data not shown). No differences in the internal standard signals of method A-1 were observed between a 15 and 30 min reaction time, so subsequently 15 min was chosen. Therefore, the sample resuspension in method A was split, and method A-1 was applied to this aliquot in order to analyze TXB_2, 2,3-dinorTXB_2, and 2,3-dinor-6-ketoPGF$_{1\alpha}$.

Due to the complexity of the physicochemical properties of the analytes, two different SPE protocols were required (method A and B). Method A isolated all the eicosanoid metabolites from urine, with the exception of the cysLTs. Analytes were eluted from the cartridge with acetonitrile, while the main polar components (e.g., cysLTs) were retained. In method B, the cartridge was washed with methanol/water 50:50 (v/v). This solution washed away the majority of the compounds that color urine (putatively urobilin), but cysLTs were retained. These analytes were eluted in the last step with methanol. Attempts were made to perform a single extraction, and this wash and elution in method B were performed after elution with acetonitrile in method A; however, the cysLT recoveries decreased, halving the performance of the analysis.

The sensitivity of the analytical methods was assayed as the limit of detection (LOD) and linear range of the calibration curve (Table 1). When calculated as on-column injected amount of analyte, the calibration curve showed similar LODs to recently published work.[39]

Matrix Effect in Authentic Samples. The matrix effect of internal standards was calculated according to Matuszewski et al.[33] for three different model samples (samples A, B, and C, respectively, deep, normal, and light yellow) that mimic the sample variability of samples routinely obtained from patients, from very concentrated to very diluted. The samples were extracted with the internal standards added before and after extraction in order to calculate the matrix effect, the recovery of extraction, and the overall process efficiency[33] (Table 2).

Matrix effects were more prominent in sample A, possessing a deep color (i.e., greater absorbance) and higher creatinine concentration. In this sample, the matrix effect for compounds analyzed in negative mode resulted in ion suppression of on average 23.5%, while compounds analyzed in positive mode showed ion enhancement of on average 27.8%. In addition, when the matrix effect was compared to the retention time for the compounds analyzed by method A-2, compounds that eluted at shorter retention times evidenced higher ion suppression relative to those with greater column retention

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Table 4. Accuracy and Precision of the Endogenous Urinary Compounds Following Spiking of Model Samples A, B, and C[a]

		sample A		sample B		sample C	
analyte	method of analysis	% acc.[b]	% RSD[c]	% acc.[b]	% RSD[c]	% acc.[b]	% RSD[c]
tetranorPGDM	A-2	18.2	4.00	11.8	6.40	8.21	6.35
2,3-dinor-11-β-$PGF_{2\alpha}$	A-2	−29.3	4.33	−29.3	9.13	−26.7	4.69
tetranorPGEM	A-2	1.68	8.19	−4.78	1.47	−8.14	11.3
PGE_1	A-2	CC[d]	CC	9.02	9.09	CC	CC
PGE_2	A-2	CC	CC	−2.39	12.5	CC	CC
$PGF_{2\alpha}$	A-2	−8.79	5.13	−2.35	3.80	0.21	5.86
8-iso$PGF_{2\alpha}$	A-2	−7.65	0.913	15.6	10.4	5.28	7.18
2,3-dinor-8-iso$PGF_{2\alpha}$	A-2	−48.6	3.71	−51.3	8.96	−32.8	4.61
8,12-iso-i$PF_{2\alpha}$-VI	A-2	2.29	1.30	1.08	1.73	−6.26	0.32
TXB_2	A-1	−29.5	7.84	−36.0	3.40	−35.8	8.70
11-dehydroTXB_2	A-2	−8.79	5.13	−2.35	3.80	0.21	5.86
2,3-dinorTXB_2	A-1	3.05	10.4	8.62	3.53	−6.85	7.69
2,3-dinor-6-keto$PGF_{1\alpha}$	A-1	−17.4	29.2	−16.3	9.48	−15.6	27.0
LTE_4	B	−3.03	16.6	−4.94	8.00	−4.38	34.8

[a]The volume extracted from every sample was done according to Table S-4. [b]The accuracy (acc.) is expressed as percentage of difference between the external standard method and the standard addition method. [c]The precision was the relative standard deviation (RSD) of the concentration calculated by the external standard quantification. [d]CC (calibration curve) denotes that in the specified sample the analyte was not detected and the accuracy and precision are described in Table S-5. Experiments were conducted in triplicate.

(Figure S-3, Pearson's r = 0.60 between matrix effect and retention time). Sample B yielded lower ion suppression in negative mode and similar ion enhancement in positive mode (r = 0.12 between retention time and ion suppression in method A-2). Sample C exhibited the lowest matrix effects in both positive and negative mode (r = 0.07 between retention time and ion suppression in method A-2). The extraction recoveries were sample and compound dependent but on average were less dependent on the sample background (average percentage of recoveries of extraction for samples A, B, and C were 74.1, 71.9, and 67.2%, respectively) showing that the main consequence of the sample variability and background was the matrix effect. The overall process efficiency averages were 62.0, 73.9, and 69.2% for, respectively, samples A, B, and C.

The next step in the analysis of the matrix effect was the extraction of different volumes of the model samples. Response of the analytes detectable in urine against the equivalent amount of creatinine extracted was assessed. For this experiment the amount of internal standard was kept constant and the volume of urine extracted was variable. The range in which the ratio analyte signal/internal standard signal showed a linear relation with the amount of urine extracted was determined. In this range, the internal standard was capable of correction of proportional variation on matrix effects and extraction variability. The optimum range for the overall process efficiencies of the internal standards and the linear range of the concentration calculated against the equivalent of creatinine extracted are provided in Table 3. Whereas the ranges were analyte and sample dependent, extractions of the urine containing 300 μg of creatinine in method A and 2000 μg in method B were compromised.

Because tetranor metabolites are not stable at room temperature,[40,41] it was vital to minimize sample-handling time once defrosted. Toward this end, the relation between absorbance and creatinine content was assayed. At this point, a reference material was introduced with two purposes: control of batch-related variability and the estimation of the volume of every sample that was required. The later was done by the comparison of the absorbance of a sample to the reference material. Due to sample availability, even if the calculated volume of urine to extract surpassed these values, the maximum extracted volume was 2.00 mL with method A and 2.70 mL with method B (Table S-4).

Accuracy and Precision. In order to validate the method, the accuracy and precision of samples A, B, and C were spiked in triplicate with external standards at a concentration equivalent to the calibration curve in each method. The volume of sample to be extracted was calculated using the absorbance ratio between the samples A, B, and C and the reference material (Table S-4). The accuracy of the analytes that were present in nonspiked samples was determined as the relative deviation between the concentration calculated by the calibration curve and by sample addition (Table 4). The accuracy of the analytes that were not present in the nonspiked samples was assessed by relative deviation between the concentration found by calibration curve and the concentration added (Table S-5). In all cases, the precision was quantified by the relative standard deviation of the concentration determined with the calibration curve.

Overall, the method evidenced good precision and accuracy. However, compounds that were present in the nonspiked samples at low levels (2,3-dinor-6-keto$PGF_{1\alpha}$ and TXB_2) showed worse accuracy and precision. In addition, five analytes (PGE_2, PGD_2, 2,3-dinor-11-β-$PGF_{2\alpha}$, 2,3-dinor-8-iso$PGF_{2\alpha}$, and EXC_4) yielded worse accuracies than average with deviations higher than 30%. When analyzed, the lower accuracies (calculated either by sample addition or the concentration spiked) arose from different behavior between the analyte and the internal standard. In the case of PGE_2 and PGD_2, surrogates with nine deuteria were used as internal standards and exhibited different overall process efficiency than their native analogues, yielding a concentration inaccuracy. $[^2H]_4$-PGE_2 and $[^2H]_4$-PGD_2 were also evaluated, but they yielded a false positive signal on the native compound when injected on column. In order to reduce the potential for false positives, the internal standards with nine deuteria were used. In the case of the two dinor compounds with a $PGF_{2\alpha}$ ring, $[^2H_4]$-8-iso$PGF_{2\alpha}$ exhibited higher overall process efficiency than the analytes, yielding a calibration curve concentration

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lower than the authentic one. The same effect occurred for EXC_4; its internal standard ($[^2H_5]$-LTC_4) did not compensate well for the extraction and matrix effects. Nevertheless, the accuracy for these compounds was homogeneous in all three samples (A, B, and C), and the precision was generally <15%. Accordingly, the quantification can be applied to measure relative shifts in excretion levels, despite the inherent bias.

Analysis of Samples from Atopic Donors. The platform was applied to urine samples from atopic asthmatics. A total of 13 urinary eicosanoid metabolites were detected in at least 90% of the donors with atopic asthma. The parent eicosanoid PGE_2 was also detected; however, the excretion of this compound in urine is known to originate in seminal contamination and/or renal production.[42,43] The seminal contamination was detected in only one male patient. The eicosanoid metabolites have a systemic origin, including the lungs, which are highly perfused.[44] Therefore, in order to study the change of PGE_2 during the allergen provocation, tetranorPGEM was traced as the nonrenal metabolite of PGE_2. The excretion change of the eicosanoid metabolites in urine from the donors is shown in Table 5.

Table 5. Excreted Eicosanoid Metabolites in Urine before and after Allergen Provocation in Atopic Asthmatics[a]

	average ± standard deviation	
metabolite	before provocation	after provocation
tetranorPGDM	80.0 ± 52.6	257 ± 313*
2,3-dinor-11-β-$PGF_{2\alpha}$	52.0 ± 42.1	326 ± 302*
PGE_2	121 ± 399	26.0 ± 53.3
tetranorPGEM	335 ± 305	420 ± 365
$PGF_{2\alpha}$	67.6 ± 45.3	63.8 ± 31.1
2,3-dinor-6-keto$PGF_{1\alpha}$	176 ± 108	229 ± 116
TXB_2	2.80 ± 1.30	5.73 ± 3.23*
11-dehydroTXB_2	21.4 ± 17.6	15.9 ± 12.0
2,3-dinorTXB_2	34.1 ± 14.4	59.7 ± 29.9*
8-iso$PGF_{2\alpha}$	16.8 ± 4.59	20.1 ± 8.74
2,3-dinor-8-iso$PGF_{2\alpha}$	202 ± 149	313 ± 175*
8,12-iso-i$PF_{2\alpha}$-VI	395 ± 184	550 ± 259*
LTE_4	4.50 ± 3.37	14.0 ± 11.4*

[a]Data are expressed as nanogram of metabolite per mmol of creatinine excreted for 16 individuals. Analytes exhibiting significant change by paired-t test are indicated with an asterisk ($p < 0.05$).

The cohort evidenced an increase in PGD_2 detected by the increase of its excreted metabolites, tetranorPGDM and 2,3-dinor-11-β-$PGF_{2\alpha}$. In addition, excreted LTE_4, which reflects total cysLT production (Figure 1), was significantly higher. However, EXE_4, which reflects LT production via 15-LOX (Figure 1), was not detected in urine either before or after exacerbation. Both PGD_2, and cysLTs are produced by mast cells that are activated during allergen provocation.[45] The TXB_2 and 2,3-dinorTXB_2 metabolites of TXA_2 increased significantly following the provocation. This increase in TXA_2 after allergen provocation in atopic asthmatics has been previously described[46] and reported in basal levels and following exacerbation.[47,48]

The AA auto-oxidative metabolites also increased with the allergen provocation (2,3-dinor-8-iso$PGF_{2\alpha}$ and 8,12-iso-i$PF_{2\alpha}$-VI). The production of these metabolites is related to the levels of oxidative stress by reaction with reactive oxygen species. The increase of excreted isoprostanes could be explained by (1) an increase of the level of oxidative stress during the provocation, (2) an increase of the free AA liberated by cytosolic phospholipase A_2 ($cPLA_2$) in inflammatory cells, or (3) the liberation of isoprostanes esterified in glycerophospholipid pools[49] (or a combination of these factors). Accordingly, despite the fact that oxidative stress is known to increase in patients with exacerbations,[50] there are other potential sources of the higher excretion of isoprostanes during an asthmatic event. An increase in F_2 isoprostanes excretion in urine from atopic asthmatics after peak exacerbation has been reported to be triggered by the allergen, but not by methacholine, in a COX-independent manner.[51] Interestingly, levels of the commonly measured isoprostane 8-iso$PGF_{2\alpha}$ did not change with provocation; however, the more abundant isoprostane 8,12-iso-i$PF_{2\alpha}$-VI significantly increased following allergen provocation. Accordingly, the platform of urinary mediators quantified in the current methods possesses the ability to detect oxidative stress-related shifts in isoprostanes that are not detectable by the current gold standard of lipid peroxidation. No significant changes in excreted $PGF_{2\alpha}$ were observed following allergen provocation, but the source of urinary $PGF_{2\alpha}$ is unclear because it has been reported to originate via auto-oxidation rather than the COX pathway.[52] It can also be speculated that the kidney is the source of $PGF_{2\alpha}$ in urine, explaining why its excretion does not change with lung exacerbation.

CONCLUSIONS

Herein we describe the development, validation, and application of a method to assess shifts in excreted eicosanoid metabolites in human urine. The use of a representative reference material was beneficial for assessing batch variability as well as determining the appropriate volume of urine to extract. This approach accounts for both matrix effects and sample dilution, while simultaneously providing good accuracy and precision. The incorporation of novel high abundance isoprostanes increased the ability of the method to detect differences associated with allergen provocation.

The use of deuterium labeled compounds as internal standards is a common approach and exhibits palpable advantages; however, the accuracy using these structural surrogates should not always be assumed. The lower accuracy observed for 2,3-dinor-11-β-$PGF_{2\alpha}$ and 2,3-dinor-8-iso$PGF_{2\alpha}$ warrants the need for specific 2,3-dinor internal standards because $[^2H]_4$-$PGF_{2\alpha}$ did not correct appropriately for these two compounds. However, even when the internal standards are exact structural analogues, a high number of deuteria (e.g., $[^2H]_9$-PGD_2 and $[^2H]_9$-PGE_2) can affect the accuracy. Consequently, the minimum number of deuteria with sufficient isotopic enrichment to avoid false positives is optimal.

This method compensates for the matrix effects of urine and possesses the advantage of reducing ion suppression in samples with high mass spectrometry background while simultaneously extracting sufficient analyte in diluted samples. In addition, this seminormalization by absorbance ratio to reference material could be applied to other methodologies, such as metabolomics, in order to adjust for the effects of the intrinsic sample variability of urine.

ASSOCIATED CONTENT

Supporting Information

Additinal information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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