



Quantification of LTE4 in Human Urine with a 2 pg/mL LLOQ Using a UPLC and LC/MS/MS

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Introduction

Leukotriene E4 (LTE4) is a biomarker that is monitored to help determine efficacy for various pharmaceutical applications. An assay was developed with a 2 pg/mL LLOQ to quantify the endogenous levels of LTE4 present in human urine. In method development, we had to solve problems that are often present in biomarker assay development, such as achieving a low level of quantitation, circumventing the endogenous levels of the analyte of interest in the matrix, and eliminating any matrix effects observed with LC/MS/MS studies.

Methodology

SAMPLE PREPARATION

- SPE: Strata™-X
- Aliquot Size: 1000 µL plasma
- Linear range: 2.00-500 pg/mL
- Load: Acidic
- Wash: Water:MeOH
- Elution: MeOH



Methodology (continued)

INSTRUMENTATION

- Mass Spec: Sciex API 5000™ (Negative ion mode)
- Source Type: ESI
- Temperature: 500 °C
- LC Column: Kinetex® PFP 1.7μ 50x2mm
- LC program: Gradient
- Mobile Phase: A: 1% Acetic Acid in water
B: MeOH
- LTE4 438.2 → 333.1
- LTE4-d3 441.2 → 336.1
- LTE4-d5 443.2 → 338.1

METHOD VALIDATION

- Fully validated for regulated analysis
- Passed all required tests
- Used isotopic labeled compounds as a surrogate analyte with a mass balance check against the unlabeled compounds

Results and Discussion

METHOD DEVELOPMENT

- **Chromatography development:** We initially started with a hexyl phenyl column which had good peak shape using 0.1% acetic acid in water and MeOH; however, we could not achieve the LLOQ of 2 pg/mL with the neat solutions. We discovered that the analyte had better ionization with more acetic acid present in the mobile phase and switched to 1% acetic acid in water and MeOH. We also discovered we had better retention and a sharper peak when we switched from the hexyl phenyl to the Kinetex® PFP, which gave us enough sensitivity for the desired LLOQ.
- **Surrogate Matrix vs. Surrogate Analyte (i.e. stable-labeled analogs):** In the initial period of method development, we screened several lots of human urine to see if there was an internal source of matrix with low levels of LTE4 and thus avoid using a surrogate matrix. The lowest source found in house was at an estimated concentration of 4 pg/mL: when the same source was tested a few days later, it had increased to 20 pg/mL. In order to have constant access to a matrix without LTE4 present, we switched our focus from human urine to synthetic urine. With QCs made and qualified against a fresh standard curve, we believed that the assay could go into validation. We then took a lot of human urine with a known amount of LTE4, used this as the dilution QC, diluting it at a factor of 10 with synthetic urine. After extraction and analysis, we observed a large fluctuation in the IS response between samples that contained human urine and samples that did not.



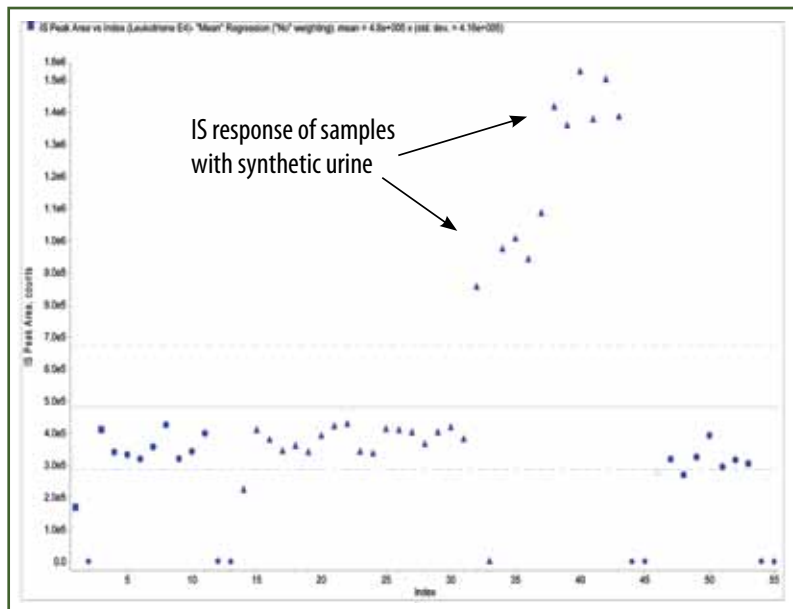
Results and Discussion (continued)

We then attempted to use rat urine as a surrogate matrix because LTE4 was not present at the assay's detectable levels. Fresh QCs were made and qualified against a fresh standard curve and we believed that the assay could go into validation. We calculated we would need at least 2 liters of rat urine in order to make enough pooled QCs and standards using a 1 mL aliquot. Because the lead time for validation of the assay was short, we also projected that this was not going to meet our timelines.

In order to keep the matrix the same as the study samples, we used LTE4-D3 as a surrogate analyte and tried to find a suitable labeled IS. We obtained two analogues, N-Acetyl-LTE4 and LTD4, which had reported low levels in human urine and extracted a PSAE; however, we observed divergent curves for N-Acetyl-LTE4 and a high level of LTD4 in the matrix blanks. We identified a commercial source for LTE4-D5 and used this isotope for the internal standard. Upon analysis, a mass balance check was performed to ensure the instrument response of the unlabeled LTE4 matched the instrument response of the D3 labeled LTE4. The peak area of the D3 labeled LTE4 was used to assess the regression and quality controls for the incurred samples.

Once the problems of sensitivity, endogenous presence of the analyte being studied, and matrix effects had been resolved, the assay was validated under GLP criteria and is currently being used to analyze clinical samples.

FIGURE 1: Human Urine Versus Synthetic Urine





Results and Discussion (continued)

ASSAY VALIDATION

- **Accuracy/precision:** Demonstrated at n=6 at LLOQ, Low, Medium, High concentrations over 3 validation runs. (Tables 1 and 4)
- **Ability to dilute:** Demonstrated above ULOQ at DF=10. (Table 2)
- **Ionization Effects:** Demonstrated with blank, QC0 and low QC concentrations in six sources of human urine. (Tables 3)
- **Extraction recovery:** Evaluated for analyte at Low, Medium, High concentrations and IS at working concentration (n=6). (Table 5)
- **Carryover:** Evaluated in each run.
- **Stock solution stability:** Room temperature for analytes and the I.S. for 24 hrs 101 days at -20°C in EtOH
- **Stability in matrix:** Freezing/thawing, 4 cycles
Room temperature, 37hrs
196 days at -70°C and -20°C
- **Reinjection Reproducibility:** Batch reinjection stability for 136 hours at RT

FIGURE 2: LTE4

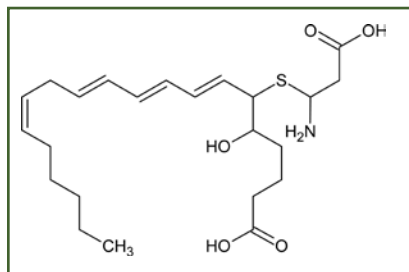


FIGURE 3: LTE4-D3

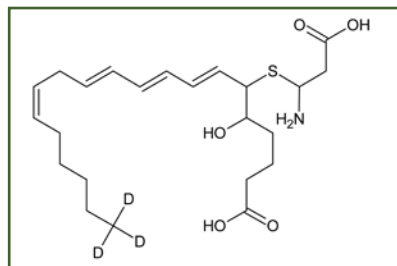


Figure 4: LTE4-D5

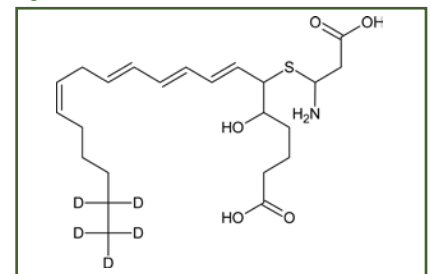


FIGURE 5: N-Acetyl-LTE4

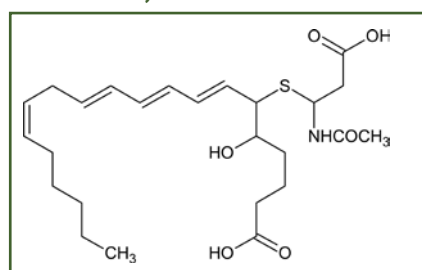
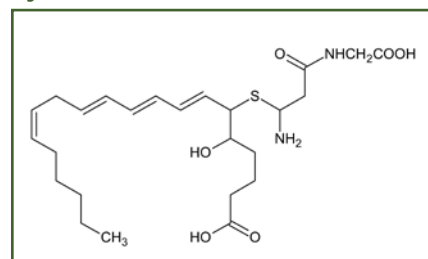


Figure 6: LTD4





Results and Discussion (continued)

FIGURE 7: LLOQ (LTE4-d3)

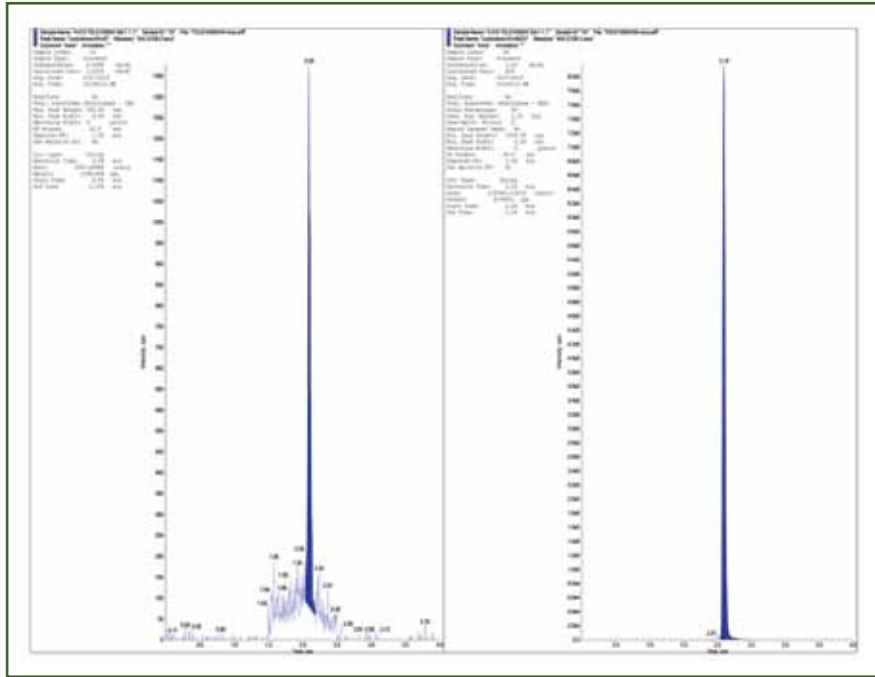
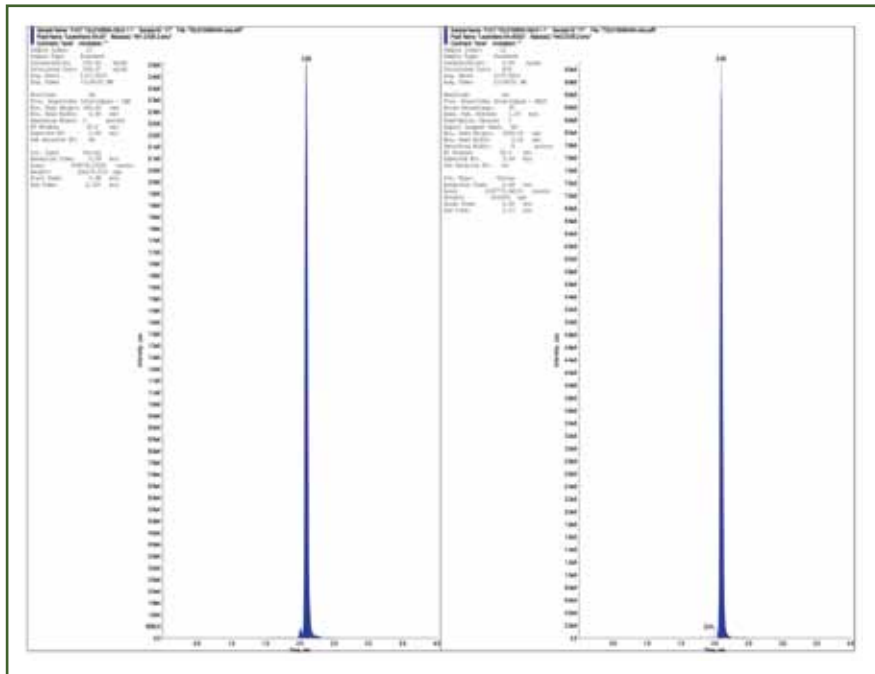


FIGURE 8: ULOQ (LTE4-d3)





Results and Discussion (continued)

FIGURE 9: IS only (LTE4-d5)

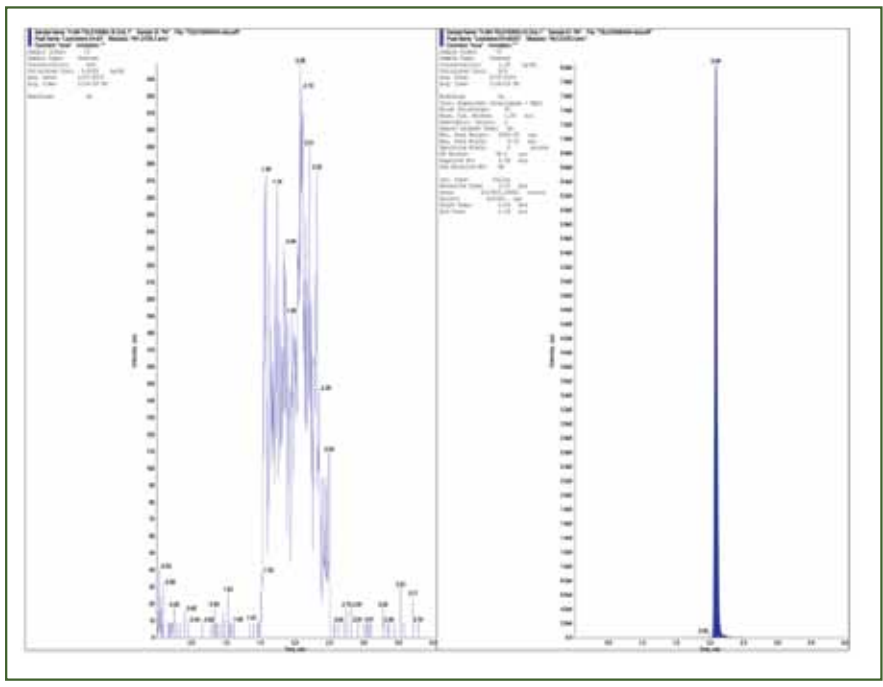
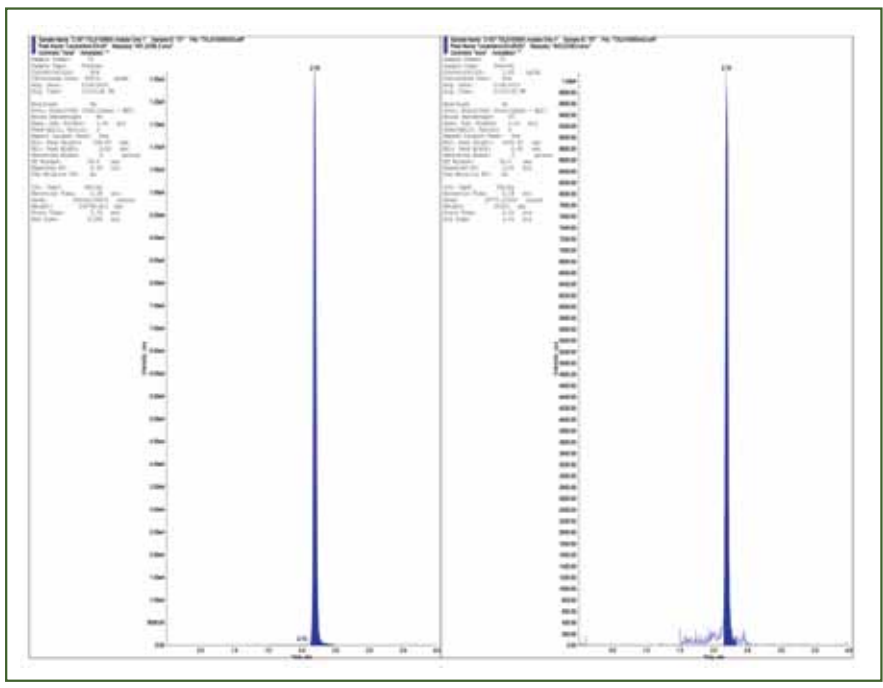


FIGURE 10: Analyte Only (LTE4-d3)





Results and Discussion (continued)

FIGURE 11: Matrix blank

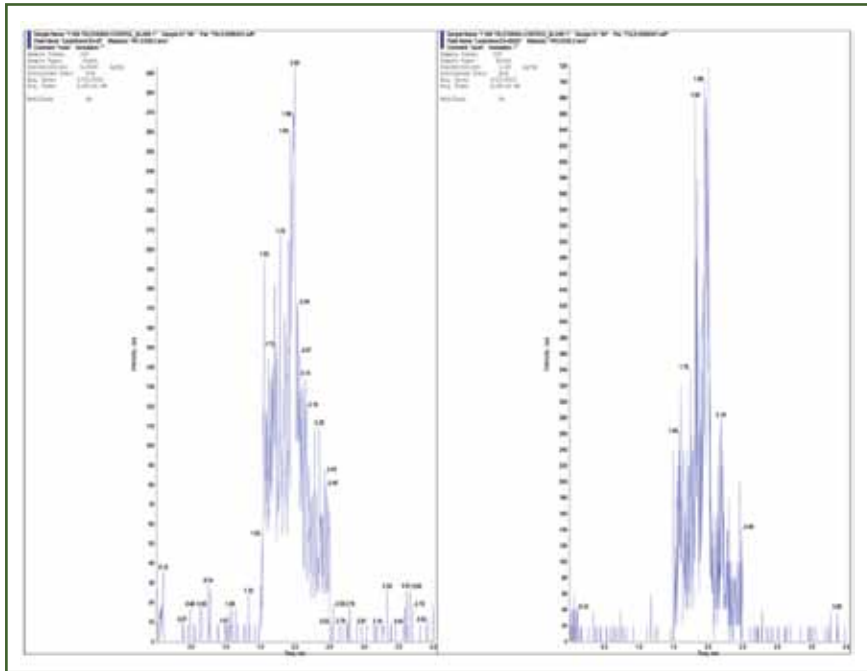
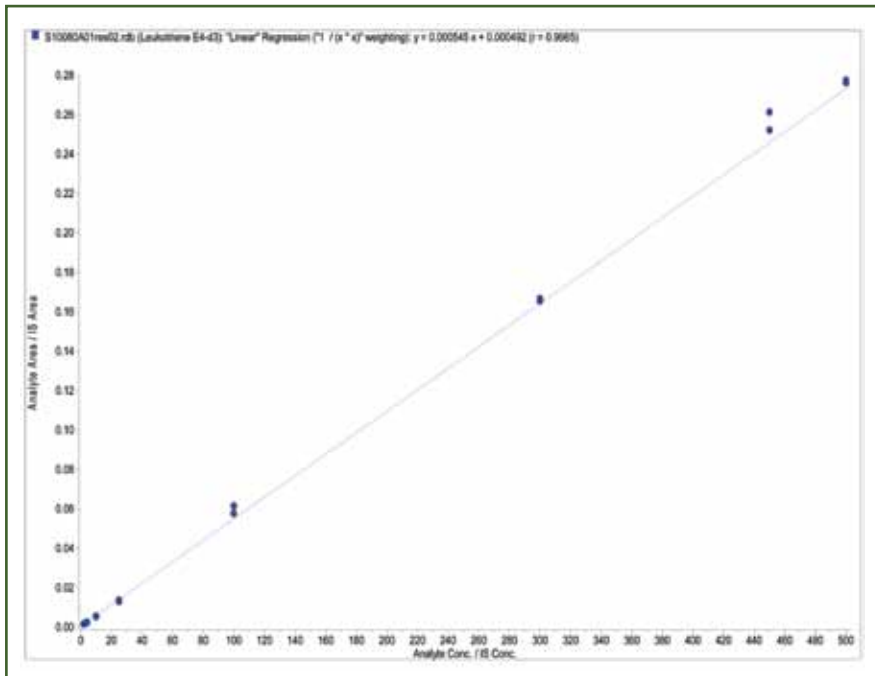


FIGURE 12: LTE4-d3 Curve





Results and Discussion (continued)

TABLE 1. Inter-Assay Precision and Accuracy of LTE4-d3 in Human Urine									
Run Date	Curve Number	LLOQ 2.00 pg/mL	%RE	QC-L 5.00 pg/mL	%RE	QC-M 90.0 pg/mL	%RE	QC-H 400 pg/mL	%RE
23-May-2010	1	2.10	5.0	5.52	10.4	95.0	5.6	456	14.0
		2.10	5.0	5.42	8.4	94.4	4.9	448	12.0
		2.21	10.5	5.57	11.4	93.2	3.6	447	11.8
		2.06	3.0	5.48	9.6	94.8	5.3	434	8.5
		**2.58	29.0	5.21	4.2	92.8	3.1	437	9.3
		1.93	-3.5	5.13	2.6	98.0	8.9	474	18.5
26-May-2010	4	2.07	3.5	5.06	1.2	88.4	-1.8	443	10.8
		2.23	11.5	5.10	2.0	88.8	-1.3	430	7.5
		2.18	9.0	4.88	-2.4	94.6	5.1	416	4.0
		2.18	9.0	5.67	13.4	87.0	-3.3	440	10.0
		2.08	4.0	5.40	8.0	88.1	-2.1	424	6.0
		2.22	11.0	5.36	7.2	89.3	-0.8	436	9.0
27-May-2010	5	1.87	-6.5	4.67	-6.6	89.5	-0.6	445	11.3
		2.35	17.5	5.37	7.4	94.5	5.0	444	11.0
		2.24	12.0	5.04	0.8	93.8	4.2	440	10.0
		2.32	16.0	4.72	-5.6	90.4	0.4	455	13.8
		2.17	8.5	5.69	13.8	93.8	4.2	439	9.8
		2.33	16.5	5.21	4.2	91.8	2.0	433	8.3
	Mean	2.18		5.25		92.1		441	
	S.D.	0.162		0.302		3.06		12.9	
	%CV	7.4		5.8		3.3		2.9	
	%Theoretical	109.0		105.0		102.3		110.3	
	%RE	9.0		5.0		2.3		10.3	
	n	18		18		18		18	

** > ±20% deviation from theoretical



Results and Discussion (continued)

TABLE 2. Dilution Integrity of LTE4-d3 in Human Urine

Run Date	Curve Number	QC-DIL 10x 1000 pg/mL	%RE
23-May-2010	1	***1180	18.0
		1090	9.0
		1130	13.0
		1140	14.0
		1120	12.0
		1100	10.0
	Mean	1130	
	S.D.	32.0	
	%CV	2.8	
	%Theoretical	113.0	
	%RE	13.0	
	n	6	

*** > ±15% deviation from theoretical

TABLE 3. Ionization Effect Evaluation of LTE4-d3 in Human Urine

Run Date	Curve Number	QC-L Lot 1		QC-L Lot 2		QC-L Lot 3		QC-L Lot 4		QC-L Lot 5		QC-L Lot 6	
		5.00 pg/mL	%RE	5.00 pg/mL	%RE	5.00 pg/mL	%RE	5.00 pg/mL	%RE	5.00 pg/mL	%RE	5.00 pg/mL	%RE
23-May-2010	1	4.45	-11.0	4.40	12.0	***6.23	24.6	4.50	-10.0	***6.27	25.4	4.86	-2.8
		4.95	-1.0	5.13	2.6	4.67	-6.6	4.95	-1.0	5.46	9.2	4.85	-3.0
		5.51	10.2	5.47	9.4	4.79	-4.2	4.43	-11.4	5.20	4.0	5.35	7.0
	Mean	4.97		5.00		5.23		4.63		5.64		5.02	
	S.D.	0.530		0.547		0.868		0.282		0.558		0.286	
	%CV	10.7		10.9		16.6		6.1		9.9		5.7	
	%Theoretical	99.4		100.0		104.6		92.6		112.8		100.4	
	%RE												
	n	-0.6		0.0		4.6		-7.4		12.8		0.4	

*** > ±15% deviation from theoretical



Results and Discussion (continued)

TABLE 4. Back-Calculated Calibration Standard Concentration Data for LTE4-d3 in Human Urine

Run Date	Curve Number	STD1 2.00 pg/mL	%RE	STD2 4.00 pg/mL	%RE	STD3 10.0 pg/mL	%RE	STD4 25.0 pg/mL	%RE	STD 5 100 pg/mL	%RE	STD6 300 pg/mL	%RE	STD7 450 pg/mL	%RE	STD8 500 pg/mL	%RE
23-May-2010	1	2.11	5.5	*3.27		9.08	-9.2	23.1	-7.6	111	11.0	303	1.0	476	5.8	503	0.6
		2.03	1.5	3.76	-6.0	9.30	-7.0	24.1	-3.6	104	4.0	301	0.3	459	2.0	505	1.0
26-May-2010	4	1.82	-9.0	4.03	0.8	9.69	-3.1	25.2	0.8	101	1.0	287	-4.3	441	-2.0	506	1.2
		2.10	5.0	4.34	8.5	9.89	-1.1	24.9	-0.4	101	1.0	293	-2.3	443	-1.6	529	5.8
27-May-2010	5	1.81	-9.5	4.10	2.5	9.47	-5.3	23.5	-6.0	100	0.0	294	-2.0	433	-3.8	511	2.2
		2.14	7.0	4.18	4.5	10.2	2.0	25.8	3.2	101	1.0	289	-3.7	461	2.4	530	6.0
Mean		2.00		4.08		9.61		24.4		103		295		452		514	
S.D.		0.149		0.214		0.407		1.04		4.15		6.38		15.9		12.3	
%CV		7.5		5.2		4.2		4.3		4.0		2.2		3.5		2.4	
%RE		0.0		2.0		-3.9		-2.4		3.0		-1.7		0.4		2.8	
n		6		5		6		6		6		6		6		6	

* Standard calibrator outside acceptance criteria and excluded from regression

TABLE 5. Extraction Recovery of LTE4-d3 in Human Urine

	Low (3.00 pg/mL)	Medium (200 pg/mL)	High (400 pg/mL)
Mean Recovery for Oc-treotide	63.7%	62.4%	64.6%
Recovery for Desmopressin	53.7%		

Conclusion

A sensitive and selective method was developed to quantify the biomarker Leukotriene E4 (LTE4) in human urine.