QUANTIFICATION OF 11-DEHYDRO THROMBOXANE B_2 IN HUMAN URINE BY LC-MS/MS - SELECTIVE AND SENSITIVE

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INTRODUCTION

Thromboxane A_2 (TXA_2) is an important biomarker in multiple biological processes in the human body. Persistent biosynthesis of TXA_2 has been associated with several ageing-related diseases, including diabetes mellitus, obesity, cardio- and cerebrovascular or chronic inflammatory diseases. TXA_2 is difficult to measure since it is rapidly metabolized to Thromboxane B_2 (TXB_2) and further to 11-dehydro TXB_2, which is excreted in urine. Therefore, quantification of 11-dehydro TXB_2 in urine is a suitable readout of TXA_2 synthesis in the human body. Here we present a fully validated SPE-LC-MS/MS assay for the quantification of 11-dehydro TXB2 in human urine in the range of 25.0 – 2500 pg/mL using a sample volume of 1 mL.

pKa 4.27

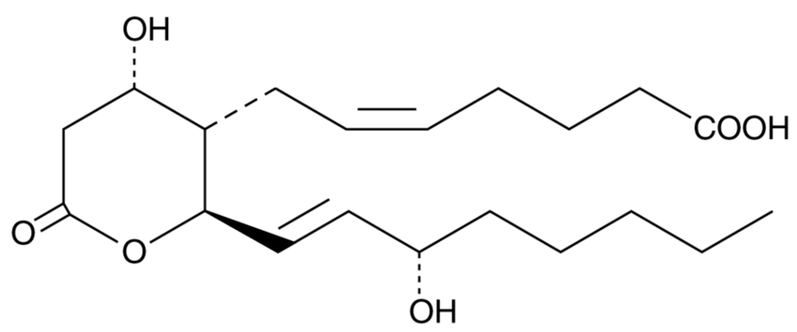


Figure 1: Structure of 11-dehydro Thromboxane B_2

PREPARATION OF STANDARD (STD) AND QUALITY CONTROL (QC) SAMPLES

Because 11-dehydro TXB_2 is an endogenous compound, calibration standard samples were prepared in an analyte-free surrogate matrix (Urisub®). For QC sample preparation, in-house collected individual urine samples were screened for 11-dehydro TXB_2 concentration levels. Selected urine samples were then pooled to reach QC Low level. For preparation of QC Med, QC High and dilution quality control (DQC) samples, aliquots of the QC Low pool were spiked with 11-dehydro TXB_2. QC LLOQ samples were prepared in Urisub®.

SAMPLE EXTRACTION PROCEDURE

Due to the chemical properties of the analyte, samples were extracted using a mixed mode anion exchange solid phase extraction (MAX SPE) plate. This optimized procedure showed high recovery of the analyte (91.0 – 96.0% in human urine) resulting in high sensitivity of the assay.

Loading
- 1 mL of STD / QC / Urine sample
- Add 50 µL of isotope labeled internal standard
- Add 1.0 N Hydrochloric acid (HCl) and incubate for 30 min
- Add methanol (MeOH) to each sample
- Condition plate with a mixture of methanol and Hydrochloric acid
- Load samples on SPE plate

Step 1:

Removal of hydrophilic interferences
- Wash sorbent using a mixture of HCl / water / MeOH (Binding of 11-dehydro TXB_2 to the sorbent is based on hydrophobic interaction)
- Wash sorbent with water

Step 2:

Removal of hydrophobic interferences
- Add Acetate buffer adjusted to pH 6.0 using Ammonia solution (Shift in pH creates the carboxylate anion of 11-dehydro TXB_2 that interacts with the quaternary ammonium function of the sorbent)
- Wash sequentially with water, methanol, acetonitrile and dichloromethane (DCM)

Elution
- Elute analyte using DCM / Formic acid (Shift in pH protonates 11-dehydro TXB_2, which consequently elutes from the sorbent)
- Evaporate to dryness
- Reconstitute in water / methanol

Figure 2: Sample extraction scheme for 11-dehydro TXB_2

LC-MS/MS CONDITIONS

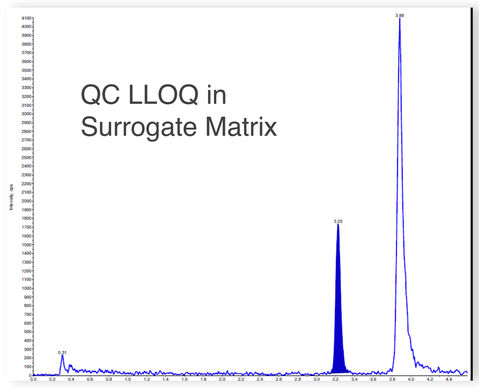
Chromatographic conditions

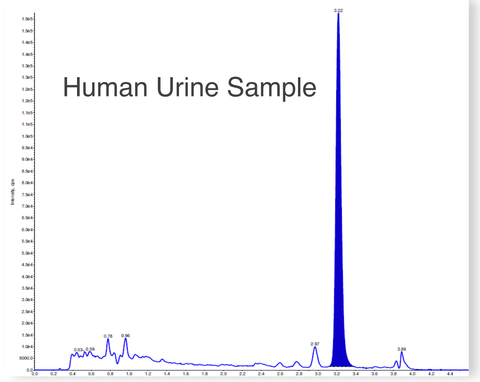
UHPLC	Waters ACQUITY UPLC™ I-Class
Analytical column	Waters ACQUITY UPLC™ BEH C18, 50 x 2.1 mm, 1.7 µm
Mobile phase A	Water / Acetic Acid (75:25 v/v)
Mobile phase B	Methanol / Acetonitrile (60:40 v/v)
Flow rate	0.45 mL/min
Column temperature	45 °C
Injection volume	20 µL
Total run time	5.0 min

MS/MS conditions

Mass spectrometers	SCIEX Triple QuadTM 5500 / SCIEX Triple QuadTM 6500
Source/Polarity	APCI / Negative
MRM transitions	m/z 367.0 -> 161.0 (11-dehydro TXB_2)
	m/z 371.0 -> 309.0 (11-dehydro TXB_2-IS)

CHROMATOGRAMS





Within-Batch and Between-Batch Precision and Accuracy Results

		QC LLOQ 25.0 pg/mL	QC Low 62.4 pg/mL	QC Med 256 pg/mL	QC High 1910 pg/mL	DQC* 6010pg/mL
Run 1	Accuracy (%) CV (%) n	106.4 4.1 6	101.3 1.6 6	98.8 0.8 6	99.0 1.2 6	102.2 1.4 6
Run 2	Accuracy (%) CV (%) n	105.6 3.3 6	101.0 1.1 6	98.8 0.8 6	99.5 0.6 6	
Run 3	Accuracy (%) CV (%) n	102.8 5.4 6	101.1 3.2	99.2 1.4 6	100.5 1.5 6	
Inter-batch Precision and Accuracy	Accuracy (%) CV (%) n	**104.8** **4.4** **18**	**101.1** **2.0** **18**	**98.8** **1.0** **18**	**100.0** **1.3** **18**	

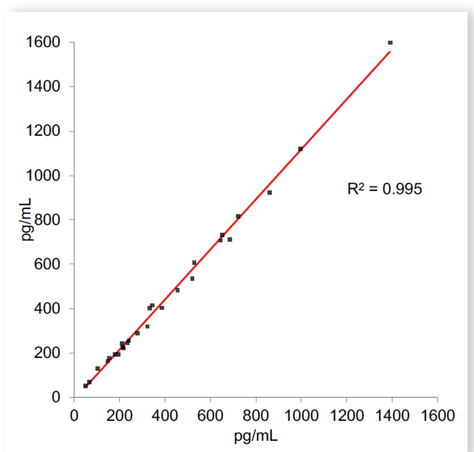


Figure 3: Incurred sample reanalysis (ISR) of 27 individual human urine samples analyzed at Celerion Switzerland AG and Celerion Inc.

Conclusions

Our SPE-LC-MS/MS assay for quantification of 11-dehydro TXB_2 in human urine was successfully validated according to international guidelines. This highly selective and sensitive method allows for quantification of endogenous 11-dehydro TXB_2 levels in a clinically relevant range of 25.0 – 2500 pg/mL.