Resolution of Unexpected Reference Standard Components of (5R,S)-isoprostane $F_{2\alpha}$ Type VI (iP$F_{2\alpha}$-VI) Using a Shallow UHPLC-MS/MS Gradient

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OVERVIEW

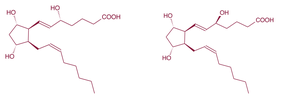
- Isomers of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are used as a measure of oxidative stress
- Ultra-High Performance Liquid Chromatography (UHPLC) has unprecedented resolving power
- Accurate quantification depends on well characterized reference standards

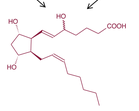
INTRODUCTION

A method improvement exercise for unconjugated iP$F_{2\alpha}$-VI in human urine, which incorporated an upgrade from conventional HPLC to UHPLC equipment, revealed a weakness in the certified characterization of the procured reference standard. The 5R/5S mixed diastereomer reference material used in the conventional method eluted as a single peak. However, the UHPLC method separated these known isomers as well as two additional unidentified and unexpected components in the reference material.

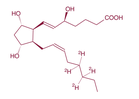
METHODS

Figure 1. Structures





(5R,S)-iP$F_{2\alpha}$-VI Reference Standard



d_4-5S-iP$F_{2\alpha}$-VI Internal Standard (IS)

The reference standard was described as 5-iP$F_{2\alpha}$-VI and provided with a formal name of: 5,9α,11α-trihydroxy-(8β)-prosta-6E,14Z-dien-1-oic acid. The material is not otherwise described as racemic and the certificate of analysis lists a purity of 100.00% by TLC.

The internal standard (IS) was described as iP$F_{2\alpha}$-VI-d4 and provided with a formal name of: 5S,9α,11α-trihydroxy-(8β)-prosta-6E,14Z-dien-1-oic-17,17,18,18-d4 acid. The HPLC purity was listed as 100.0%

The desired methodology was for unconjugated 5-F_{2t}-IsoP (5S-iP$F_{2\alpha}$-VI) in human urine. Method development was initiated by injecting the standard on a 15-F_{2t}-isoprostane (Type III) system that uses a 1.8 µm C18 column. The two main peaks, for (5R,S)-iP$F_{2\alpha}$-VI, were immediately observed, but the unexpected peaks only resolved as the column was changed, mobile phase modified, and the gradient made shallower.

Sample Processing

Concentrations were assayed from 1.0 mL samples of human urine. UriSub® served as the surrogate matrix for the preparation of calibration standards, which were freshly spiked for each batch using 20x working standards prepared in ethanol. The d_4-stable labeled internal standard in ethanol (25 µL) was added to samples prior to acidification by addition of dilute HCl.

- Samples were applied to Waters Oasis MAX, 60 mg, in 96-well format
- The plate was washed with dilute HCl, an aqueous solution of methanol, a neutral buffer, methanol, and weak formic acid prior to elution
- The eluate was evaporated and sample reconstituted in dilute acetonitrile

Instrumentation

- Mobile Phase A: 0.5% HCOOH
- Mobile Phase B: 56:38:6 MeOH:ACN:500 mM ammonium acetate
- Column: Waters BEH Shield RP18 column, 2.1 x 100 mm, 1.7 µm, 50°C
- Gradient: 11%B over 5.8 minutes
- Detection: SCIEX Q-TRAP® 5500 Negative APCI, 353.3→115.0 (analyte), 357.3→115.0 (IS)

Some of the presented work was performed by negative mode ESI using slightly different gradient conditions with less acidic mobile phase A.

RESULTS

The IS was described as a pure 5S enantiomer, and only one peak was observed. Therefore the 1st analyte isobaric peak eluting closest to the retention time of the IS peak was assumed to have the corresponding 5S configuration, while the second large analyte peak was the 5R isomer (Figure 2a). This elution order was consistent with a weakly acidic reversed phase gradient outlined in Larose, et al. (1). All four peaks observed in the reference material were also present in urine lots that were assayed, as shown in Figure 2b.

Figures 2a and 2b.

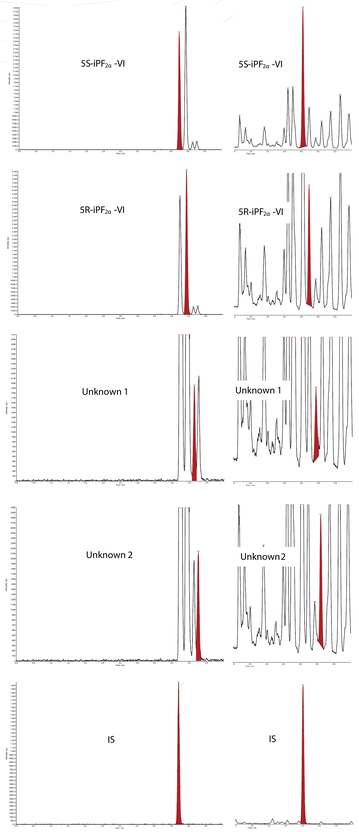


Figure 2a. Reference Solutions

Figure 2b. Urine Extract

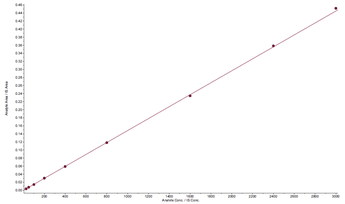
The first eluting peak was linear across the analytical range of 25 – 3000 pg/mL (Figure 3).

Relative peak responses between the total known species (i.e. 5R,S-iP$F_{2\alpha}$-VI) and total unknown species and the relative retention times (i.e. between the first eluting known species and first eluting unknown species) were consistent between extracted and unextracted (neat) solutions of the reference material (Table 1).

Table 1. Peak Area Comparisons

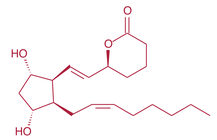
Reference Standard	Sample	Area	Summed Area	Area %	Function
Extracted	5S	68327	149833	6.4	Unk1/5S
Extracted	5R	81507			
Extracted	Unk1	4397	9624	6.4	Unk2/5R
Extracted	Unk2	5227			
				6.4	(Unk1+Unk2)/(5R+5S)
Reference Standard	**Sample**	**Area**	**Summed Area**	**Area %**	**Function**
Unextracted (Neat)	5S	117665	258036	6.3	Unk1/5S
Unextracted (Neat)	5R	140371			
Unextracted (Neat)	Unk1	7410	15843	6.0	Unk2/5R
Unextracted (Neat)	Unk2	8433			
				6.1	(Unk1+Unk2)/(5R+5S)

Figure 3. Typical Calibration Curve



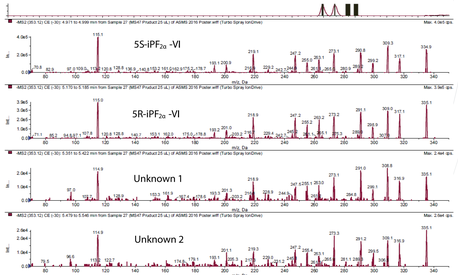
The 5-series isoprostanes may form intramolecular lactones or esters with alcohols under acidic conditions (Figure 4). Both conversion products would be less polar than the free acid form and later eluting peaks would be expected in reversed phase chromatography. The reference solution was supplied and stored in ethanol, and formation of an ethyl ester was conceivable but unlikely without adding strong acid. Samples were acidified during processing, but the relative abundance of the peaks in extracts of the reference standard was similar to that of diluted (neat) reference standard solutions. Negative mode declustering potential scans with both ESI and APCI ionization yielded no observed lactone or ester M-H ion or simple adduct m/z that would generate a 115 m/z product.

Figure 4. 5S-iP$F_{2\alpha}$-VI Condensed into a Lactone Form



The product spectra of each peak confirm structural homology of all peaks (Figure 5).

Figure 5. Product Ion Spectra.



CONCLUSIONS

No connection was verified between observed peaks in the reference material and potential late-eluting ethyl ester or lactone components. The consistency of product ion spectra would suggest that the contaminants were other diastereomers. The project is on hold while further characterization or alternate sourcing of reference material is debated internally.

Characterization of reference materials used for quantitation may prove inadequate or incomplete when the analytical method has greater resolving power than the method used for the purity determination of the material.

REFERENCES

1. Larose et al., "Analysis of F2-isoprostanes in plasma of pregnant women by HPLC-MS/MS using a column packed with core-shell particles"; Journal of Lipid Research Volume 54, 2013.

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