Highly Sensitive and Accurate Quantification of Isoprostane Oxidative Biomarkers using a High-Resolution Workflow

Increasing selectivity with high-resolution multiple reaction monitoring (MRM^{HR}) improves the signal-to-noise ratio for better accuracy, dynamic range, and LLOQ when quantitating isobaric prostaglandin-$D_{2\alpha}$ isoforms

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Key challenges of isoprostane biomarker assays

- **Lack of sensitivity** – Quantification is poorly reproducible at low picogram levels in complex biological matrices.
- **Overlapping interferences** – Assay selectivity is hampered by interfering, co-eluting peaks.
- **Multicomponent analysis in single assay** –Single isomer measurement is a poor indicator of oxidative stress due to rapid degradation and variable isomer formation. **Substandard data quality** – Precision and accuracy are compromised at very low biomarker levels, giving results below accepted bioanalytical standards.

Key benefits of MRM^{HR} for quantifying isoprostanes

- **Maximized sensitivity** – LLOQ of 5 pg/mL was an ~10-fold improvement over the triple quad MRM method.
- **Increased selectivity and specificity** – Reduced background noise enhances S/N ratios and reproducibility.
- **Wider dynamic range** – Measurements (5–10,000 pg/mL) are linear over 4-orders of magnitude (r = 0.9994).
- **All-inclusive assay in one injection** – Both known and unknown oxidative stress markers can be monitored simultaneously with a high-resolutionTOF-MS scan.

Key features of the MRM^{HR} workflow on the TripleTOF® System

- **MRM-like quantitation** – High-specificity is obtained using a narrow extraction width to mine high resolution TOF data.
- **Simultaneous, multicomponent analysis** – Fast acquisition rates can collect full-scan, MS/MS spectra for multiple precursors without additional cycle time.
- **Fast cycle times maintained** – Processing speed allows for sufficient peak coverage, even with fast LC separations.

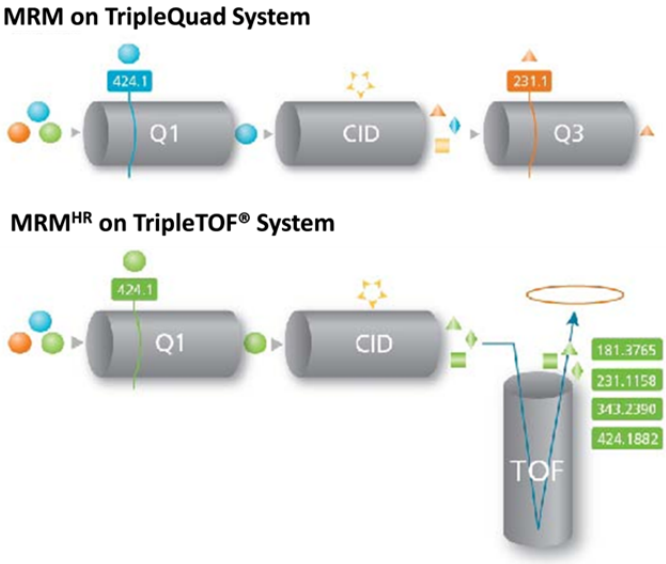

Figure 1. Schematic overview of MRM^{HR} acquisition technique on a TripleTOF® System compared to standard triple quadrupole mass spectrometer.

Introduction

Evaluating pharmaceutical candidates for adverse downstream effects, such as the induction of oxidative stress, is an essential part of the early drug discovery and development processes. Isoprostanes, end-products of non-specific lipid peroxidation, are mediators of oxidative cellular damage and serve as effective biomarkers of oxidative stress. These stable, prostaglandin-like compounds are a class of oxidative products that are abundantly formed both *in vitro* and *in vivo* during free-radical reactions with arachidonic acid in a cyclooxygenase-independent pathway (**Figure 2**). During oxidative conditions, isoprostane formation surpasses that of the physiologically formed metabolites of arachidonic acid (prostaglandins, thromboxanes, and leukotrienes), promoting potent biological activity and stimulating inflammatory responses *in vivo*, including platelet and granulocyte activation. This isoprostane-dependent activity has been linked to a number of human diseases, and elevated levels of isoprostanes have been detected in damaged tissues and can serve as an index of *in vivo* oxidative stress.[1]

Accurate detection of isoprostanes is currently the accepted bioanalytical standard for evaluating oxidative damage. Conventional analyses of isoprostanes have relied upon triple quadrupole mass spectrometers and sample-specific purification and enrichment techniques, but these processes have provided only limited sensitivity and selectivity.[1] To improve detection limits and eliminate interfering species more effectively, selected reaction monitoring acquisition techniques coupled with the fast acquisition speeds and high resolution of hybrid triple quadrupole/TOF technology have been shown to provide sufficient selectivity for separations that are unattainable with a standard triple quadrupole instrument.[2] The high resolution multiple reaction monitoring (MRM^{HR}) method carried out on a fast-scanning high resolution mass spectrometer allows for accurate quantification due to the acquisition of a large number of data points per peak and wide linear dynamic range. This technical application note describes how traditional MRM acquisition can be used with the high resolution TripleTOF® 5600+ System to achieve increases in sensitivity and lowered limits of quantitation for the detection and separation of isoprostane species in oxidatively-damaged hepatocytes.

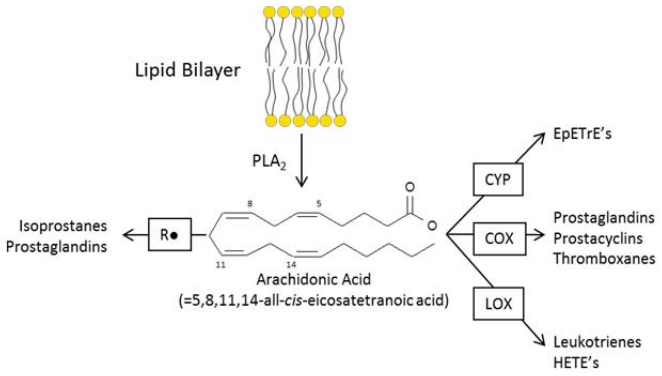

Figure 2. Schematic overview of eicosanoid generation from membrane phospholipids. Arachidonic acid (released from cellular membranes by the enzyme PLA_2) is subsequently converted to epoxyeicosatrienic acids (EpETrE's) via cytochrome P450 enzymes (CYP); to prostaglandins, prostacyclins and thromboxanes via cyclooxygenases (COX); and to leukotrienes and hydroxyeicosatetraenic acids (HETE's) via lipoxygenases (LOX). The formation of isoprostanes from arachidonic acid is catalyzed by free radicals (R˙);increases have been implicated in a variety of human diseases.

Methods

Sample preparation

After incubation with the hepatotoxin ferric nitrile triacetate (FeNTA), hepatocyte samples were subject to protein precipitation with one volume of acetonitrile that also contained internal standard. The supernatant from centrifuged samples was decanted and directly injected into the LC/MS/MS system.

Chromatography

LC System:	Agilent 1100 series LC pump
Trap Column:	YMC AQ, 5µm (20 x 2.1 mm) YMC Europe
Flow rate:	0.25 µl/min
Analytical Column:	Atlantis T3, 3 µm (100 x 2.1 mm)
Mobile Phase A:	Water (0.2% formic acid)
Mobile Phase B:	Acetonitrile (0.2% formic acid)

An aliquot of the sample was loaded onto a trapping column (TC) (YMC AQ, 20 x 2.1 mm, 5um, YMC Europe) of an HPLC column-switching system, using water containing 0.2 % formic acid (eluent A2) at a flow rate of 0.25 mL/min for 5.1 minutes using an Agilent 1100 series LC pump. The sample was further diluted by online addition of eluent A3 at a flow rate of 1.75 mL increasing to 2.25 mL within 5.1 minutes to TC, via a T-piece. Following this, TC was switched in line with an analytical column (AC) (Atlantis T3, 100 x 2.1 mm, 3 um, Waters) and the retained analytes were transferred to AC using gradient elution.

High pressure gradient elution was performed by a mixture of water containing 0.2 % formic acid (eluent A1) and 0.2 % formic acid in acetonitrile (eluent B1; 1/9 v/v). While eluent B1 was raised from 25 % to 60 % in 7.5 minutes in the trapping phase, eluent A1 was decreased complementarily. Following injection, B1 was increased to 95 % and maintained for 1.5 minutes. After

13.5 minutes, eluent B was decreased to 25 %. During this sequence after injection, TC was disconnected from AC after 5.1 minutes and flushed with 95 % of a solution of 0.2 % formic acid in acetonitrile (eluent B2; 1/9 v/v) and 5 % water containing 0.2 % formic acid (eluent A2) at a flow rate of 0.25 mL/min for 3.5 minutes. The total run time of the analysis cycle was 13.5minutes

Mass spectrometry

MS System:	TripleTOF® 5600+ System with a DuoSpray™ Ion Source
Ionization Mode:	ESI in negative ion mode
TOF MS range:	m/z 200-800 at 50 msec accumulation time
MRM^{HR}:	9 product ions, each 30 msec
Collision Energy Spread:	20 ±10 eV
Source Temperature:	20 ±10 eV
Calibration:	Automated, using external CDS

A 4000 QTRAP® was also operated in negative electrospray ionization, using a TurboV® Source set to 550°C and ionspry voltage of -4200 eV. Dwell time were 15 ms for individual transitions with an entrance potential kept at -10 eV Declustering potential and collision energy were optimized individually for each analyte ranging from -35 to -80 eV and -16 to -36 eV, respectively.

Table 1. Overview of the chosen precursor ions for acquisition of the isoprostane product ion spectra

Analyte	Precursor *m/z*	Mass error (ppm)
8-iso- Prostaglandin $F_{2\alpha}$	353.2334	3.1
5-iso-Prostaglandin $F_{2\alpha}$-VI	353.2334	1.4
dinor 8-iso Prostaglandin $F_{2\alpha}$	325.2042	1.0
Prostaglandin D_2	351.2177	0.2
Prostaglandin D_2-d_4	355.2428	1.2
13,14-dihydro-15-keto Prostaglandin D_2	351.2177	0.9

Quantitation was performed by preparation of a calibration curve using dilutions of standards against a dueterated internal standard of prostaglandin D_2-d_4 (**Figure 3**).

Data processing

Data was acquired with Analyst® 1.5.1 Software and data was reviewed in PeakView® 1.2 Software. Quantitation of measured analytes was performed with MultiQuant™ Software. The three most intense product ions were extracted (at 50 mDa intervals) and summed together. PD_2-d_4 was used as the internal standard.

Results and Discussion

Selectivity

Using the MRM^{HR} acquisition technique on the TripleTOF® 5600 System, selectivity comparable to an MRM scan was achieved. This is possible due to selecting high resolution product ions with a narrow extraction width (50 mDa) from the precursor molecular ion (Q1 unit selection). The result is spectrometric separation of isobaric prostaglandins $F_{2\alpha}$ (**Figure 4**).

Quantitation

A calibration curve of authentic standards (5–10,000 pg/mL) was created using MultiQuant™ Software (**Figure 5**). Results are described for three characteristic analytes: 5-iso Prostaglandin $F_{2\alpha}$-VI ($iPF_{2\alpha}$-VI), Prostaglandin D_2 (PD_2) and 13,14-dihydro-15-keto prostaglandin D_2 (dihydroketo PD_2). The detection limit (LOD) for all analytes was below the lowest measured concentration of 5 pg/mL. Linearity was achieved from 5-10,000 pg/mL with regression coefficients of 0.9994, 0.9996 and 0.9954 for $iPF_{2\alpha}$-VI, PD_2 and dihydro-keto PD_2, respectively. Five out of the six isoprostanes showed better LOQ and signal to noise ratio on TripleTOF® 5600$^+$ System compared to the 4000 QTRAP®.

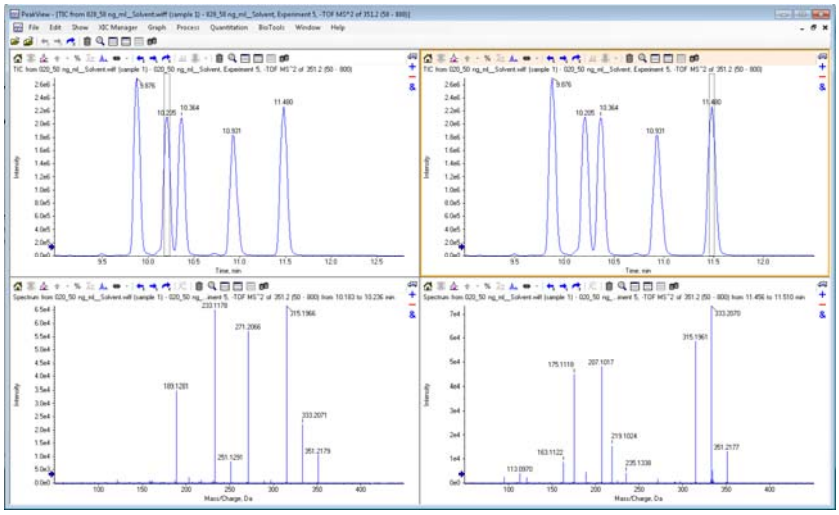

Figure 3. Recorded product ion spectra of prostaglandin D_2 (PD_2) and dihydroketo PD_2 shown in PeakView® Software. The three most intense fragment ions were chosen for the quantitation method.

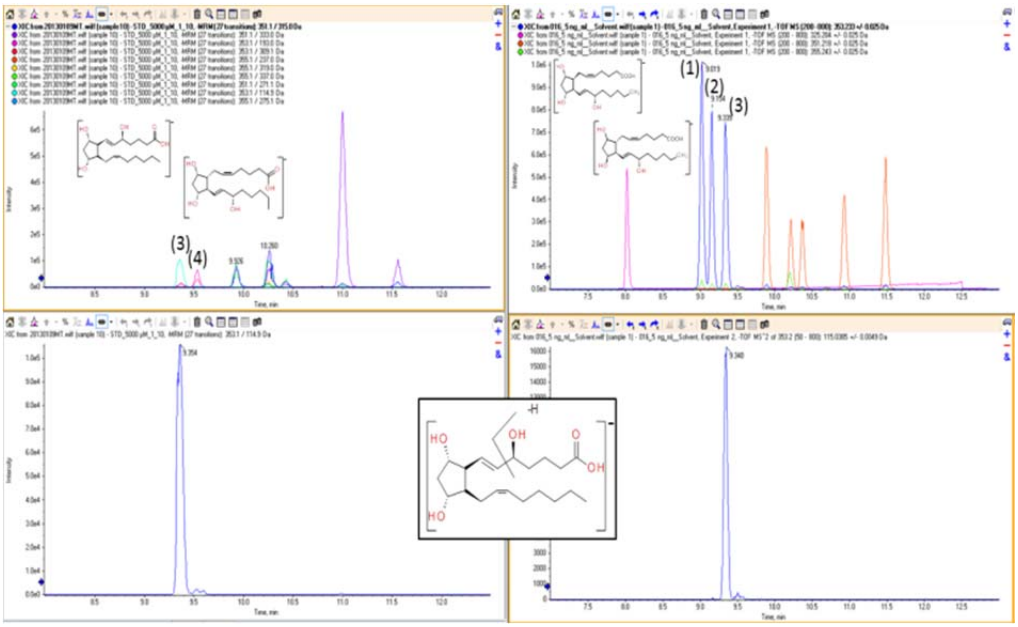

Figure 4. **Instrument-dependent specificity**. Specificity achieved with different scan techniques on a 4000 QTRAP® System (*left side*) and on a TripleTOF® 5600+ System (*right side*)**.**

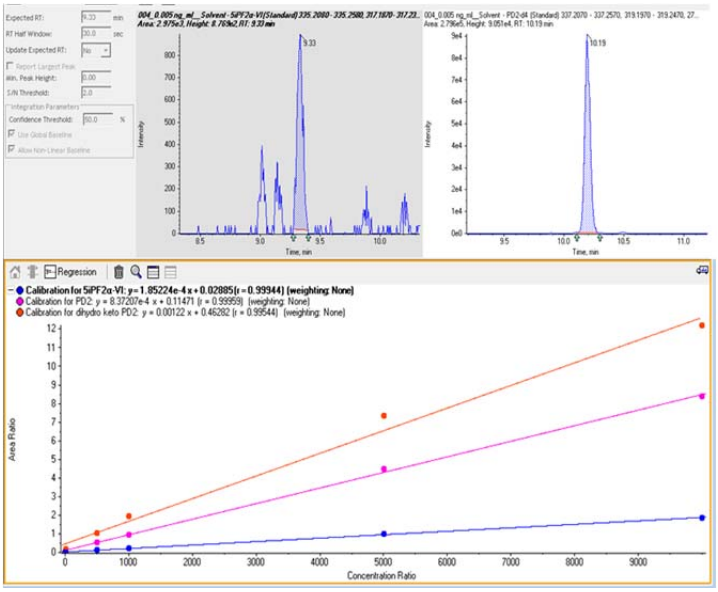

Figure 5. **Processing of calibration data for isoprostane species.** MultiQuant™ Software was used to measure peak areas corresponding to MRM^{HR} signals (*top*). Calibrations curves are shown for standards for isoprostane species, 5-iso-prostaglandin $F_{2\alpha}$, prostaglandin D_2, and dihydroketo prostaglandin D_2 (*bottom*).

Biological application

This method was evaluated in biological samples, and concentrations of isoprostanes were determined using the MRM^{HR} method based on the lower limit of detection. For *in vitro* experiments, hepatocytes were incubated with the model drug FeNTA, a known redox cycling agent and initiator of oxidative stress. *In vivo* experiments were also conducted with Fischer F344 rats that were i.p. dosed with FeNTA. Increases in isoprostane species, 15*R* PD_2 and $iPF_{2\alpha}$-VI, were observed for both *in vitro* and *in vivo* experimental setups when using MRM^{HR} methods for detection (**Figure 6**).

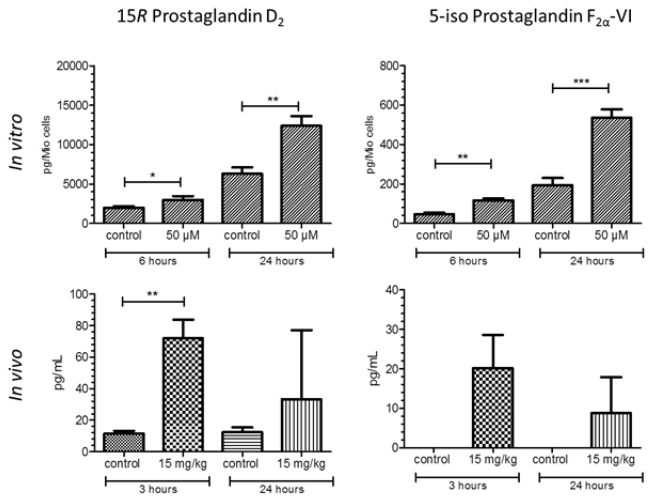

Figure 6. **Changes in isoprostane concentration after treatment with FeNTA.** (*Top panels*) The *in vitro* concentration of isoprostane species,15*R* prostaglandin D_2 (15*R*-PD_2, *left*) and 5-isoprostoglandin $F_{2\alpha}$-VI ($iPF_{2\alpha}$ –VI, *right panel*), were evaluated from hepatocytes over time using MRM^{HR} methods on a TripleTOF® 5600 System**.** (*Bottom panels*) The *in vivo* concentration of isoprostane species in plasma was determined in Fischer F344 rats.

Conclusion

This study demonstrates that hybrid triple quadrupole-TOF instruments can be used successfully for quantitative biomarker analysis. In addition, QTOF instrumentation offers the possibility for generic detection of unknown analytes using high resolution full scan data and allows for multiplexing biomarker discovery and quantitation in drug discovery workflows.

- Method transfer from a triple quadrupole to a TripleTOF 5600 System was straightforward.
- Sensitivity gains were observed with the improved selectivity of high resolution, accurate mass data peaks.
- Significant changes in isoprostane concentrations were observed *in vitro* after treatment with the oxidative stress initiating agent, FeNTA.
- This technique will allow multiplexing biomarker discovery and quantitation of future experiments in drug discovery

References

[1] Milne G, Yin H, Morrow JD. “Human Biochemistry of the Isoprostane Pathway.” *J. Biol. Chem*. June 6, 2008; 283(23): 15533-15537.

[2] Albanese J, Hunter C. “Increasing the Multiplexing of High-Resolution, Targeted Peptide Quantification Assays.” AB SCIEX technical note, 6260212-01.

Publication number: : 8540113-01

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