



Single Analyte Mediated Quantitative Determination of Endogenous 5-HETE in Plasma Samples

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Introduction

Endogenous compound, 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), is a major metabolite of Arachidonic acid (AA) via the Lipoxygenase (LOX) pathway. 5-oxo-6,8,11,14-eicosatetraenoic acid (5-OXO-ETE), a secondary metabolite, is formed by the oxidation of 5-HETE by 5-hydroxyeicosanoid dehydrogenase. Research indicates that the biological activity of 5-HETE can be applied as a regulated messenger for certain diseases. This work aims to develop a quantitative method to determine the endogenous level of 5-HETE in biological samples using LC-MS/MS. The commonly accepted method for quantifying endogenous compounds is to use two stable-labeled isotopes as reference standard and internal standard (IS), respectively. This concept is based on the commonly accepted assumption that the test compound and its stable-labeled isotope have equivalent mass spectrometry ionization efficiency. Since only one stable-labeled isotope is commercially available for 5-HETE, the current methodology uses a labeled analog compound, such as 8-HETE-d8 or 15-HETE-d8 as the internal standard. Due to the difference of endogenous levels of analog unlabeled compound between matrix used to prepare a calibration curve and real samples, any matrix interference, such as ion suppression, will be unpredictable, leading to unreliable analytical results. In drug discovery, or early stage drug development, very limited effort is put into developing two stable-labeled isotopes during quantitation if they are not commercially available. Therefore, one labeled compound and a generic IS, or analog compound as IS is the common approach in the quantitation of endogenous compounds. Using a generic IS or analog IS raises the question of how well the IS can mirror the variation of the reference standard during sample preparation procedures, such as extraction efficiency, as well as with LC-MS/MS data collection, such as ion suppression or enhancement at the MS ionization source. But, why can we not utilize the endogenous analyte existing in the blank matrix when the



Introduction continued

level of endogenous analyte in the matrix used to prepare the standard curve is consistent. Here we propose a new regression approach that uses one stable-labeled isotope as both surrogate reference standard and internal standard in one sample batch. The calibrators are prepared using labeled compound as reference standard and unlabeled compound as IS. Samples are prepared using labeled compound as IS. The predicated sample concentrations are then calculated using the regression obtained from labeled compound. The average endogenous level of 5-HETE in blank matrix is subtracted from the calibrator IS for consistency between calibrators and samples. In this poster, we discuss a couple of cases that have had issues with using generic internal standard and labeled analog as internal standard. We will show some results that use authentic endogenous compound as the internal standard in the calculations, and discuss the benefits and issues.

Method

Stable-labeled isotope, 5-HETE-d8, was used as the surrogate analyte to quantify the endogenous levels of 5-HETE in mouse plasma. 5-HETE-d8 was spiked into pooled mouse plasma to prepare the calibration curve in a range of 0.5 to 250 ng/mL. 25 μ L of calibration standard was precipitated by 150 μ L of 25 ng/mL 5-HETE in Acetonitrile. Samples were spun down, then 75 μ L of supernatant was combined with 25 μ L of Water for LC-MS/MS analysis.

The QC samples were prepared using unlabeled 5-HETE as reference standard. The final level of 5-HETE in QC samples would be the amount of added plus the endogenous level. 5-HETE was spiked into the same lot of pooled mouse plasma, and precipitated by 25 ng/mL 5-HETE-d8 in Acetonitrile. In the above samples, a generic non-analog IS, Glyburide was added for comparison purposes.

Data acquisition was performed on a Sciex API 4000 platform using negative ionization with a Phenomenex PFP column. Mobile phase A: 0.1% formic acid (FA) in 95:5 Water:Acetonitrile; Mobile phase B: 0.1% FA in 50:50 Methanol:Acetonitrile. LC run was 3 minutes with a gradient profile from 60% B to 100 % B in 1.8 minutes.

Initial data processing was done using Analyst software for peak areas. Final data calculation was performed using Excel.

Results and Discussion

IS ISSUE #1: *Bias generated by using a generic IS in the calculation of surrogate endogenous compounds*

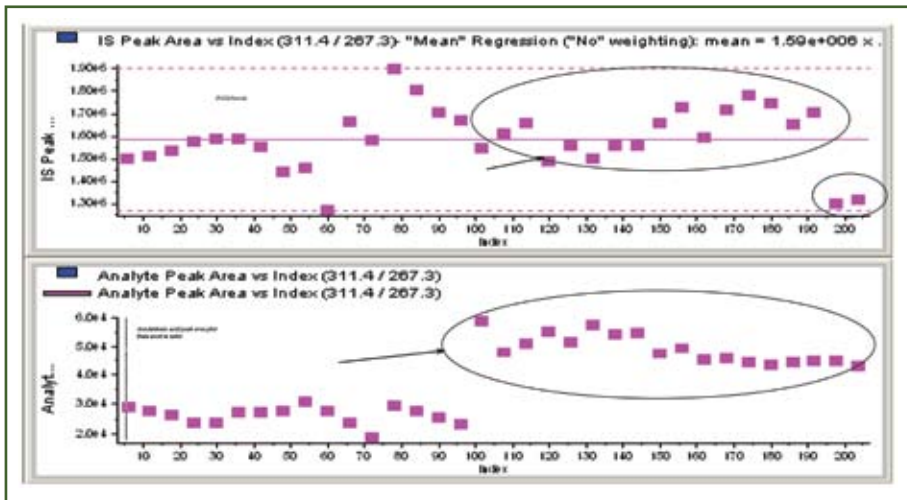
In an evaluation of retarding enzymatic oxidation of endogenous Arachidonic acid (AA), surrogate compound AA-d8 was used as a reference standard. Samples were prepared by adding different antioxidants at several levels to pooled mouse liver homogenate. The initial data was processed using the generic internal standard, Glyburide, due to the lack of a second commercially available stable-labeled isotope. After examining the IS (Glyburide) response carefully, a question was raised about whether the percentage of remaining AA-d8 after BHA addition was real or caused by matrix effects that were not caught by the IS. Since the endogenous AA level is the same throughout the experiment, any interference should affect AA and AA-d8 equally. The data was then reprocessed using the endogenous AA level as IS. Figure 1 shows the Glyburide and AA-d8 MS responses, and Table 1 shows the compared results.

TABLE 1: Calculated AA-d8 / IS Peak-Area Ratios

Sample Name	Initial Calculation		Re-calculation	
	AA-d8/Glyburide	% remaining	AA-d8/AA	% remaining
AAd8_msT_PPT_RT_36hr	0.019	57	0.007	59
AAd8_msT_PPT_RT_01BHT_36hr	0.016	48	0.006	50
AAd8_msT_PPT_RT_05BHT_36hr	0.016	48	0.007	53
AAd8_msT_PPT_RT_1BHT_36hr	0.018	55	0.007	57
AAd8_msT_PPT_RT_01BHA_36hr	0.021	64	0.007	59
AAd8_msT_PPT_RT_05BHA_36hr	0.013	39	0.006	46
AAd8_msT_PPT_RT_1BHA_36hr	0.015	46	0.008	65
AAd8_msT_PPT_RT_05M_36hr	0.014	43	0.007	57
AAd8_msT_PPT_FZ_36hr	0.034	101	0.013	102
AAd8_msT_PPT_FZ_01BHT_36hr	0.034	101	0.013	105
AAd8_msT_PPT_FZ_05BHT_36hr	0.036	107	0.013	109
AAd8_msT_PPT_FZ_1BHT_36hr	0.035	104	0.014	118
AAd8_msT_PPT_FZ_01BHA_36hr	0.029	85	0.012	101
AAd8_msT_PPT_FZ_05BHA_36hr	0.027	82	0.011	92
AAd8_msT_PPT_FZ_1BHA_36hr	0.025	74	0.011	89
AAd8_msT_PPT_FZ_05M_36hr	0.026	79	0.012	94
AAd8_msT_PPT_Fresh_20µLhr	0.033	100	0.012	100

Results and Discussion continued

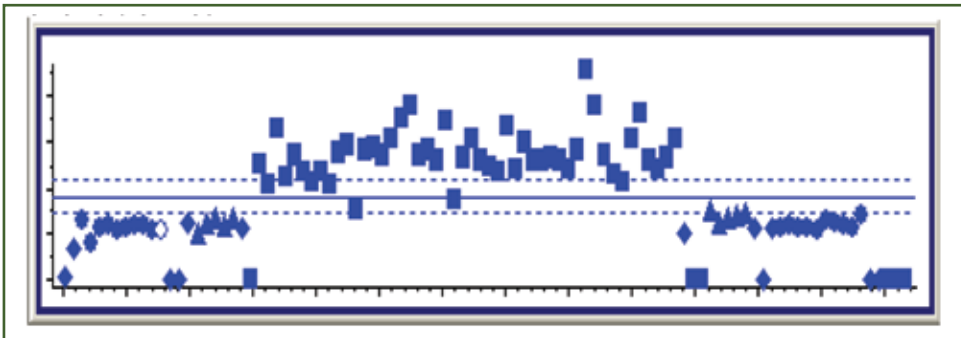
FIGURE 1. Generic IS MS Response vs. Endogenous IS MS response



IS ISSUE #2: Internal standard response enhanced by authentic endogenous compound

In this study, a surrogate stable-labeled isotope was used as the reference standard and a stable-labeled analog was used as the internal standard. Both compounds were endogenous compounds in the mouse plasma. The standard curve was prepared by spiking stable-labeled isotope into commercially available matrix. After samples were analyzed, different IS responses were observed between calibration standards and biological samples. The cause was found to be the difference in endogenous level of unlabeled IS in blank plasma and sample plasma. The endogenous level of authentic analog enhanced the surrogate analog IS, causing the data calculation to be incorrect. No good solutions have yet to be found.

FIGURE 2. IS response in biological samples using stable-labeled analog compound



Results and Discussion continued

Utilizing a targeted endogenous compound as the IS in a calibration curve

We thus propose a new approach to apply in the situation that there is only one stable-labeled isotope available to be used in sample analysis. To quantitatively determine the endogenous 5-HETE levels in mouse plasma, a calibration curve was prepared using 5-HETE-d8 as the reference standard and 5-HETE in Acetonitrile as the IS. The representative samples were prepared by spiking 5-HETE into mouse plasma and using 5-HETE-d8 in Acetonitrile (same concentration as 5-HETE in Acetonitrile) as the IS. All samples also included the generic IS, Glyburide as a marker. In this case, 5-HETE and 5-HETE-d8 were interchangeable in playing the role of internal standard. Any variation caused by sample processing and data collection should be better caught and accounted for in the calculation.

When using surrogate compounds in analysis, the sample concentrations were calculated using the calibration equation obtained from the surrogate compound curve using peak-area ratio. In our approach, all calculations were done in Excel. Peak areas for 5-HETE and 5-HETE-d8 were exported from Analyst. Average peak area of 5-HETE for matrix blanks were calculated and then subtracted from the calibration standards. The peak-area ratios of 5-HETE-d8 and 5-HETE were then calculated for the calibration standards. The following equations were used to obtain a calibration equation with a 1/x² weighing factor in Excel. The peak-area ratios of 5-HETE and 5-HETE-d8 from the samples were used to calculate the endogenous level of 5-HETE in the mouse plasma using the calibration equation obtained. We cross checked results with the ones obtained using the generic IS, Glyburide. The results were comparable. The generic IS works here because we are using the same lots of matrix. Experimental results are summarized in Table 2 & 3.

Linear regression equations with weighing factor (w) used in Excel calculation

$$y = bx + a$$

$$RSD_w^2 = \frac{\sum w(y - y(x))^2}{n-2} \quad MeanX_w = \frac{\sum xw}{n} \quad MeanY_w = \frac{\sum yw}{n}$$

$$b = \frac{\sum w(x - MeanX_w)(y - MeanY_w)}{\sum w(x - MeanX_w)^2} \quad a = MeanY_w - b * MeanX_w$$

Results and Discussion continued

TABLE 2 & 3: Results for 5-HETE estimation in mouse plasma

	Calibration Using Glyburide as IS			Calculation Using 5-HETE as IS	
	Std Conc. (ng/mL)	Calc Conc. (ng/mL)	%Accuracy	Calc Conc. (ng/mL)	%Accuracy
Std_A1	0.5	0.212	42.4*	0.19	37.2*
Std_A2	1	1.08	107.9	1.19	119
Std_A3	2	2.3	115	2.48	124
Std_A4	5	5.9	118.1	5.84	117
Std_A5	10	9.26	92.6	9.65	97
Std_A6	50	47.2	94.5	49.4	99
Std_A7	150	159	106.1	153	102
Std_A8	250	229	91.6	239	96
Std_B1	0.5	0.503	100.5	0.48	95
Std_B2	1	0.843	84.3	0.93	93
Std_B3	2	1.85	92.3	1.76	88
Std_B4	5	5.03	100.5	4.71	94
Std_B5	10	11.4	113.5	9.91	99
Std_B6	50	50.4	100.9	50.0	100
Std_B7	150	138	91.9	145	97
Std_B8	250	227	90.9	218	87

Calculation Using Glyburide as IS					Calculation Using 5-HETE-d8 as IS				
Spiked 5-HETE Conc. (ng/mL)	Corrected QC Conc.	Peak-area ratio 5-HETE & Glyburide	Calc. Conc. using EQ1	%Accuracy	Spiked 5-HETE Conc. (ng/mL)	Corrected QC Conc.	Peak-area ratio 5-HETE & 5-HETE-d8	Calc. Conc. using EQ2	%Accuracy
0.5	11.5	0.0042	9.8	85	0.5	11.5	0.038	10.0	87
0.5	11.5	0.0049	11.5	100	0.5	11.5	0.042	11.0	95
0.5	11.5	0.0047	11.1	96	0.5	11.5	0.040	10.4	90
1.5	12.5	0.0060	14.1	113	1.5	12.5	0.050	13.1	105
1.5	12.5	0.0059	13.9	111	1.5	12.5	0.049	12.9	103
1.5	12.5	0.0048	11.4	91	1.5	12.5	0.039	10.3	82
20	31	0.0140	33.0	107	20	31	0.112	29.3	95
20	31	0.0152	35.9	116	20	31	0.122	31.7	103
20	31	0.0170	40.1	129	20	31	0.117	30.4	99
200	211	0.0925	218.1	103	200	211	0.759	198.1	95
200	211	0.1017	240.0	114	200	211	0.815	212.7	102
200	211	0.1094	257.9	122	200	211	1.030	268.6	128
Average of 5-HETEs in blank matrix from 18 samples				11.0	Average of 5-HETEs in blank matrix from 18 samples				11.0



Conclusion

The experimental results indicate that surrogate compound and authentic endogenous compound can be used interchangeably under certain conditions. For this to work, the endogenous compound needs to be stable in biological matrix, stock checks need to be performed between unlabeled and labeled standards to make sure they are identical in order to obtain the same IS response, and Internal standard concentration considering endogenous level and labeled IS impurity must be considered in the experimental plan.

References

1. Poster "Determination of Lipoxygenase Products of Arachidonic Acid in Tissue and Plasma by LC-MS/MS", 2010 ASMS
2. "Weighted Least-Squares Regression in Practice: Selection of the Weighting Exponent". Hans-Joachim Kuss. *LC•GC Europe* December 2003