A New Methodology for Determination of Tretinoin in Rat Plasma Using Column Switching and LC/MS/MS Techniques

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INTRODUCTION

A method for determination of tretinoin (all-*trans*-retinoic acid) in rat plasma at concentrations approaching endogenous levels was needed for a preclinical study. It has been very challenging to determine low concentrations of tretinoin in biological matrices because of the interference of endogenous retinoic acids and their interconversion reactions. In our methodology, the pooled untreated rat plasma was used for preparation of standard and QC samples. The endogenous concentration of tretinoin in the pooled plasma was measured using standard addition method (Eq. 1) and then was added to the spiked concentration for the nominal value of each standard or QC sample. In this assay, we used column switching technique to separate tretinoin from isotretinoin (13-*cis*-retinoic acid) in the first column and to separate the tretinoin from other interfering species in the second column. The structures of tretinoin and isotretinoin are shown in Figure 1.

$$\frac{C_{end}}{R_1} = \frac{C_{end}+1}{R_2}, \quad C_{end} = \frac{R_1}{R_2 - R_1}$$

C_{end} = endogenous concentration of tretinoin
R_1 = mean area ratio (tretinoin/I.S.) measured for each individual lot of plasma blank
R_2 = mean area ratio (tretinoin/I.S.) measured for each individual lot of spiked plasma sample (1.0 ng/mL)

Equation 1. Standard Addition Method

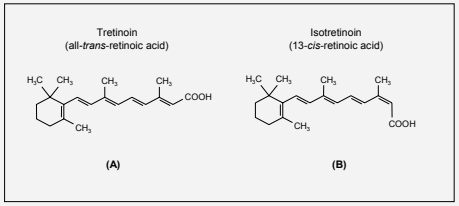

Figure 1. Structures of Tretinoin (A) and Its Major Interconversion Analog, Isotretinoin (B)

METHOD

Following a liquid-liquid extraction procedure for sample cleanup, the extract was chromatographed using a valve switching system consisting of two HPLC columns for enhancing the selectivity. Upon initial separation on an Aquasil C18 column, the fraction containing tretinoin was further separated with a Zorbax SB-C18 column while the first column was back flushed using high strength of mobile phase for robustness of chromatography. API 4000 LC-MS/MS system with a heated nebulizer was used under negative MRM mode for detection.

Liquid Chromatography

HPLC : Shimadzu 20AD
Autosampler : Perkin Elmer 200
Column 1 : Aquasil C18 (30 x 2.1 mm)
Column 2 : Zorbax SB-C18 (50 x 4.6 mm)
MP A : MeOH/10mM ammonium acetate pH 6.0 (10/90)
MP B : ACN/MeOH (30/70)
MP C : ACN/Acetone/DIH$_2$O (40/45/15)

Mass Spectrometry

AB Sciex API 4000
Polarity : Negative
Scan type : Multiple Reaction Monitoring
Tretinoin : 299 → 255
Tretinoin-d_5 : 304 → 260 (I.S.)

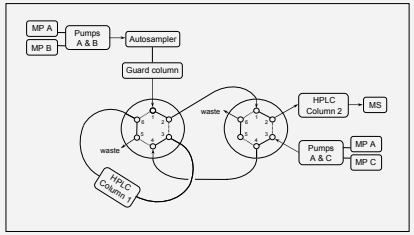

Figure 2. Diagram of LC/MS Instruments.

RESULTS

The method was validated over **a range of 1.0 to 112 ng/ml** above endogenous level with linear regression (1/x^2). The correlation coefficients for first three validation batches were 0.996 or better (Figure 3).

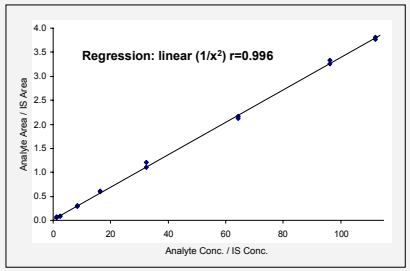

Figure 3. A Typical Calibration Curve for Tretinoin

Figures 4 through 6 show typical chromatograms for selected samples. The S/N ratio for tretinoin peak at LLOQ level was greater than 50. **Figure 7** shows endogenous peak in the extracted blank sample.

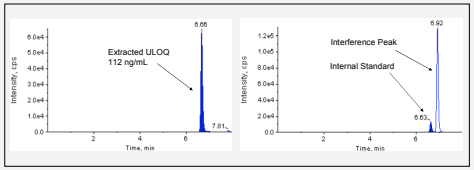

Figure 4. Chromatogram of an Extracted ULOQ Sample (112 ng/mL).

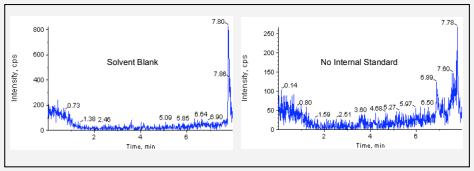

Figure 5. Chromatogram of a Solvent Blank Sample (No I.S.) Injected after ULOQ.

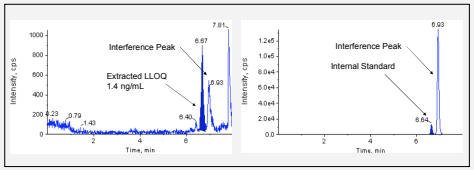

Figure 6. Chromatogram of an Extracted LLOQ sample (1.4 ng/mL).

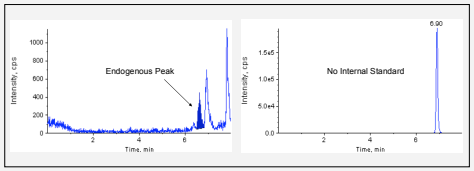

Figure 7. Chromatogram of an Extracted Plasma Blank Consisting of 0.4 ng/mL Tretinoin as Determined by Standard Addition Method.

The inter-batch statistics results for QC samples are shown in **Table 1**, which demonstrates excellent precision and accuracy. The endogenous concentrations of tretinoin were measured by standard addition method (Eq. 1) and the results are shown in **Table 2**.

Table 1. Precision and Accuracy Results (Inter-Batch) for Tretinoin Quality Control Samples.

	1.40 ng/mL (LLOQ)	3.40 ng/mL (Low)	24.4 ng/mL (Mid)	80.4 ng/mL (High)
n	18	18	18	18
Mean	1.45	3.59	25.9	86.9
Precision (cv %)	7.6	3.8	2.7	3.1
Accuracy (bias %)	3.7	5.6	6.2	8.0

Table 2. Endogenous Level Measured by Standard Addition Method.

Sample Name	Plasma blank		Spiked plasma blank#		Cal. conc. of endogenous level (C_{end} in ng/mL)
	R_1	% CV of 6 replicates	R_2	% CV of 6 replicates*	
Blank #1	0.0331	17.98	0.114	5.77	0.407
Blank #2	0.0246	19.49	0.117	10.55	0.266
Blank #3	0.0271	12.14	0.114	7.37	0.312
Blank #4	0.0265	7.53	0.115	6.68	0.300
Blank #5	0.0247	14.45	0.123	5.76	0.251
Blank #6*	0.0353	14.70	0.124	6.31	0.399

Spiked tretinoin concentration (1.0 ng/mL), * Back-calculated results based on the standard addition method. * Blank #6 is a pool of the combination of 11 different lots of rat plasma including Blank #1-5.

The selectivity and sensitivity of the method were confirmed by measuring spiked LLOQ levels of tretinoin (1.0 ng/mL) in 6 lots of human plasma (**Table 3**).

Table 3. Statistics for Spiked LLOQs (1.0 ng/mL) in 6 lots of Plasma Obtained Based on Calibration Curve Method.

	Nominal Conc. (ng/mL)	n	mean	CV (%)	Bias (%)
Lot 1	1.407	6	1.28	5.8	-8.8
Lot 2	1.266	6	1.31	10.5	3.8
Lot 3	1.312	6	1.28	7.3	-2.5
Lot 4	1.300	6	1.29	6.7	-0.9
Lot 5	1.251	6	1.38	5.7	10.0
Lot 6	1.399	6	1.39	6.3	-0.8

CONCLUSION

A reliable LC/MS/MS method for the determination of tretinoin at a range of 1.0 – 112 ng/mL above varied endogenous levels in rat plasma was developed and validated. This method has combined the standard addition method for determination of endogenous tretinoin and the calibration curve method for easy and robust quantitation. The methodology is novel in using untreated pool plasma to prepare standard and QC samples for analysis of tretinoin at concentrations approaching endogenous levels. The method showed acceptable accuracy, precision, selectivity, reproducibility and stability (data are not shown due to the limit of space), and is suitable for determination of tretinoin in preclinical rat samples.

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