LC-MS/MS Quantitative Analysis of 12 Retinoids, Derivatives and Metabolites in Serum for Clinical Research

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ABSTRACT

Purpose: An analytically sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) research method for the quantification of retinoids and metabolites in serum is presented. The method includes retinol, retinal, tretinoin, isotretinoin, etretinate, acitretin, adapalene, bexarotene, tazarotene and metabolites. Protein precipitation (PPT) and liquid-liquid-extraction (LLE) were compared as simple sample preparation approaches. A one-dimensional liquid chromatography approach allowed to achieve the required sensitivity, accuracy and robustness to quantify the compounds on a relevant dynamic range.

Methods: 200 µL of serum were extracted by protein precipitation or liquid-liquid extraction. A Thermo Scientific™ Vanquish™ LC system coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with heated electrospray source operated in polarity switching mode were used. Chromatographic separation was achieved by gradient elution on a 100 x 2.1 mm (2.6µ) Thermo Scientific™ Accucore™ C18 column using a water:methanol mixture containing 0.1% formic acid as a mobile phase. Acquisition was performed by selective reaction monitoring (SRM) for each analyte and internal standard in positive and negative acquisition mode. Accuracy for the PPT and LLE approaches was evaluated using standard reference material and donor samples.

Results: Good linearity and reproducibility were obtained, with a linear response between 1 and 1000 ng/mL for both the retinoids and the metabolites and a correlation factor (R2) always above 0.98. The lower limits of detection (LLOD) and lower limits of quantification (LLOQ) in serum were determined to the sub ng/mL levels for the retinoids and their metabolites. Excellent reproducibility was observed for all the compounds using both sample preparation approaches, with a maximum coefficient of variation (%CV) always below 10%.

INTRODUCTION

The retinoids are vitamers of vitamin A of which there are three generations that regulate epithelial cell growth. Various HPLC columns and solvent combinations as well as simple and easy sample preparation techniques were evaluated in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of 12 retinoids, derivatives and their metabolites that include tazarotenic acid, tazarotene, acitretin, isotretinoin (13-Cis-Retinoic acid), retinol, retinal, bexarotene, tretinoin (All-Trans-Retinoic acid), etretinate, retinyl acetate, adapalene and retinyl palmitate. Very simple sample preparation included protein precipitation and a one step liquid-liquid extraction. The methodologies were developed on a Thermo Scientific™ TSQ Endura™ tandem mass spectrometer in positive Electrospray ionization modes with a Thermo Scientific™ Vanquish™ HPLC system for a 8 minute analytical gradient.

MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from

Cayman Chemicals, Inc., Ann Arbor, MI-

Retinol- Vitamin A, All-trans-Retinal, All-trans-Retinoic Acid-Tretinoin, Retinyl Acetate, Retinyl Acetate, Acitretin, Etretinate, Adapalene, Bexarotene, Tazarotene, Tazarotenic Acid

Sigma-Aldrich Corp. St Louis, MO-

Retinyl Palmitate, 13-Cis-Retinoic Acid-Isotretinoin

Cambridge Isotopes Laboratories, Inc., Tewkesbury, MA-

Retinla-D6, Retinoic Acid-D6, Retinol-D6, Vitamin A Acetate-D6

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used

HPLC grade Water, Formic Acid, Methanol, Acetonitile, Methyl-Tert-Butyl-Ether (MTBE)

Sample Preparation

Sample Preparation-Protein Crash: 200 µL of fat soluble Vitamin depleted serum/HSA mixture calibrators, controls and serum sample were spiked with 20 µL of Retinoid ISTD mixture at 1000 ng/mL and vortexed briefly. 400 µL of Acetonitrile was added to each sample and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm. The supernatant was transferred to an MS vial and capped. All in-house calibrators were prepared in fat soluble vitamin depleted serum and water (Golden West Biological, Inc, Temecula, CA)

Sample Preparation- Liquid-Liquid Extraction : 200 µL of fat soluble Vitamin depleted serum/water mixture calibrators, controls and serum samples were spiked with 20 µL of Retinoid ISTD mixture at 1000 ng/mL and vortexed briefly. 200 µL of Acetonitrile was added to each sample and vortexed for 1 min. 1.2 mL of Methyl-Tert-Butyl Ether was added to each sample and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm. The organic layer was transferred to a new test tube and dried down under nitrogen at room temperature. The extract was reconstituted in 200 µL of 1:3 water and methanol. The supernatant was transferred to an MS vial and capped. The calibration curves ranged from 1 ng/mL to 1000 ng/mL and various pooled donor samples were used as control material.

Test Method(s)

HPLC Conditions - Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column: x 2.1 mm, 2.6 mm	Thermo Scientific™ Accucore™ C18, 100	
Column Temperature:	30 ºC	
Injection Volume:	10 µL	
Sampler Temperature:	4 ºC	
Needle Wash: seconds	Flush port (50%Methanol:50%Water) 10	
Mobile Phase A:	0.1% Formic Acid	
Mobile Phase B:	Methanol	
Flow Rate:	0.5 ml/min	
Gradient:	0.0 min-	25%A:75%B
	0.5 min-	25%A:75%B
	6.0 min-	2%A:98%B
	7.0 min-	2%A:98%B
	7.1 min-	25%A:75%B
Run time:	8 mins	

MS and Ion Source Conditions - TSQ Endura triple quadrupole mass spectrometer

Ion mode:	Positive Electrospray (H-ESI) Mode
Vaporizer Temperature:	325 ºC
Ion Transfer Tube Temperature:	250 ºC
Sheath Gas:	70
Aux Gas:	20
Sweep Gas:	1
Spray Voltage:	Positive Ion (V):4000 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle time (sec):	0.8
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Data Analysis

The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Endura Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

RESULTS

Linearity/Sensitivity

The assay was linear over the calibration curve for the Retinoids in fat soluble vitamin depleted serum/HAS mixture as shown in the table with their mean of coefficient of determinations (R2) for positive and for both sample preparation techniques. The linearity of each extraction was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R2) > 98 for each sample extraction technique and the %CV for each calibration point were all <10% in order to be accepted.

Table 1. Linearity and Sensitivity - PPT

Compound	Linearity - PPT	LOQ (ng/mL)
Tazarotenic Acid	0.25 ng/ml – 1000 ng/ml	0.25
Tazarotene	0.5 ng/ml - 1000 ng/ml	0.5
Acitretin	1 ng/ml – 1000 ng/ml	1
13-Cis-Retinoic Acid	25 ng/ml – 1000 ng/ml	25
Retinal	2.5 ng/ml – 1000 ng/ml	2.5
Retinol	1 ng/ml – 1000 ng/ml	1
Bexarotene	10 ng/ml -1000 ng/ml	10
Retinoic Acid	2.5 ng/ml – 1000 ng/ml	2.5
Etretinate	1 ng/ml – 1000 ng/ml	1
Retinyl Acetate	1 ng/ml – 1000 ng/ml	1
Adapalene	25 ng/ml – 500 ng/ml	25
Retinyl Palmitate	25 ng/ml – 1000 ng/ml	25

Table 2. Linearity and Sensitivity – LLE

Compound	Linearity - LLE	LOQ (ng/mL)
Tazarotenic Acid	0.025 ng/ml – 1000 ng/ml	0.025
Tazarotene	0.1 ng/ml -1000 ng/ml	0.1
Acitretin	0.25 ng/ml – 1000 ng/ml	0.25
13-Cis-Retinoic Acid	2.5 ng/ml – 1000 ng/ml	10
Retinal	0.25 ng/ml – 1000 ng/ml	0.25
Retinol	1 ng/ml – 1000 ng/ml	1
Bexarotene	5 ng/ml -1000 ng/ml	5
Retinoic Acid	1 ng/ml – 1000 ng/ml	2.5
Etretinate	1 ng/ml – 1000 ng/ml	1
Retinyl Acetate	0.1 ng/ml – 1000 ng/ml	0.1
Adapalene	25 ng/ml – 1000 ng/ml	25
Retinyl Palmitate	10 ng/ml – 1000 ng/ml	10

Figure 1. Calibration curves and chromatograms - LLE

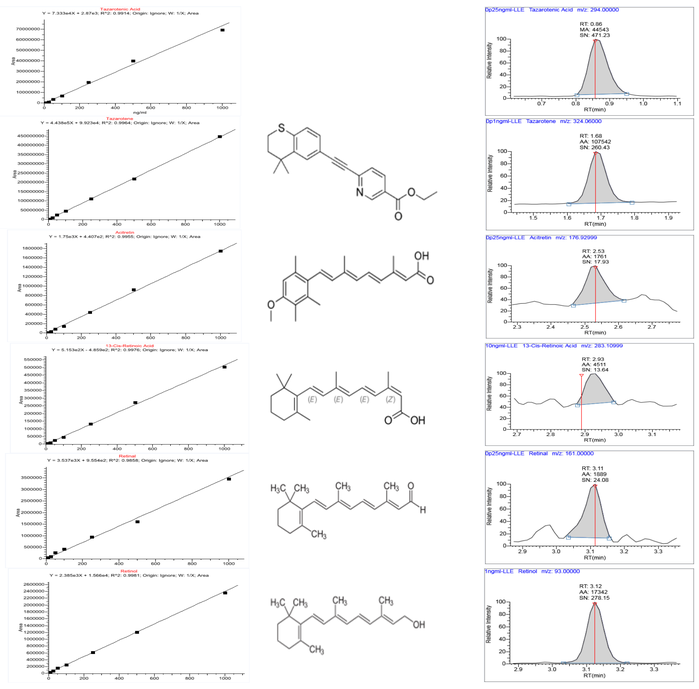
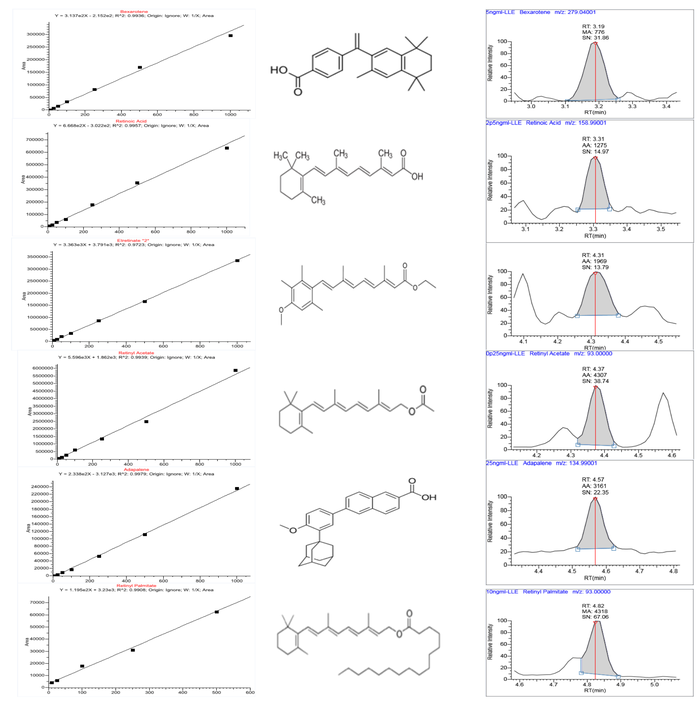


Figure 2. Calibration curves and chromatograms - PPT



CONCLUSIONS

- Baseline separation of 12 Retinoids, derivatives and their metabolites in 8 minutes with good LOQ in positive mode and a clean serum matrix is required to achieve the desired calibration curve and LOQ as the natural retinoids and their derivatives can be found in serum at significant levels.
- Very good linearity with better accuracy, precision and reproducibility in positive mode but some of these compounds are structurally similar and thus can cause ion suppression and artifact co-elution.
- Further evaluate other sample preparation techniques for improved Retinoid, derivatives and metabolite determinations and maximize the efficiency of the method as well as investigate whether these compounds can be extracted to the same degree with other fat soluble vitamins

REFERENCES

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TRADEMARKS/LICENSING

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