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LC-MS-MS quantitative analysis of 12 Retinoids, derivatives and metabolites in serum for clinical research use

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ABSTRACT

Introduction: Liquid chromatography triple quadrupole mass spectrometry is suited for rapid analysis of multiple analytes of similar and different structures and physicochemical properties. Retinoids are a diverse group of three different generations of biologically active compounds that physiologically impact the bodies' functions and include- retinol, retinal, tretinoin, isotretinoin, etretinate, acitretin, adapalene, bexarotene, tazarotene and metabolites. A sensitive and specific LC-MS/MS analytical research method was developed and optimized for the quantitation of retinoids and metabolites in serum. Simple sample preparation techniques were used that included a protein crash and liquid-liquid extraction. A one dimensional chromatographic configuration achieved the required sensitivity, accuracy and robustness to measure the compounds over their relevant dynamic range.

Method: A Thermo Scientific™ TSQ Endura™ tandem mass spectrometer in positive and negative Electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 200 ul of serum was used for the analysis of the retinoids. A Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 2.6 um column with a water:methanol mixture containing 0.1% formic acid was used. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive and negative mode due to the compounds different chemistries and structures. The accuracy of these methods were verified using standardized reference materials and from donor individuals.

Results: Good linearity and reproducibility were obtained with the concentration range from 1 ng/ml to 1000 ng/ml for the retinoids and metabolites with a coefficient of determination R2>0.98 or better for all compounds in the various matrices. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to the sub ng/ml levels of the retinoids and metabolites in biological samples. Excellent reproducibility was observed for all compounds (CV < 10%) for the techniques and configurations used.

Conclusion: A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry research method was developed and verified for the simultaneous measurement of retinoids and metabolites in serum. The sample preparation techniques are quick and easily applied for high throughput analysis.

INTRODUCTION

The retinoids are vitamers of vitamin A of which there are three generations that regulate epithelial cell growth. Retinoids are involved in vision, regulation of cell proliferation and differentiation, growth of bone tissue, immune function, and activation of tumor suppressor genes.

In this research study, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques 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. The sample preparation choices were kept simple and included protein crash and a one step liquid-liquid extraction. The methodologies were developed on the TSQ Endura tandem mass spectrometer in positive Electrospray ionization modes with a 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:	50 mg	Etretinate:	10 mg
All-trans-Retinal:	50 mg	Adapalene :	100 mg
All-trans-Retinoic Acid-Tretinoin:	50 mg	Bexarotene:	5 mg
Retinyl Acetate:	5 g	Tazarotene :	10 mg
Acitretin:	10 mg	Tazarotenic Acid:	5 mg

Sigma-Aldrich Corp. St Louis, MO-

Retinyl Palmitate:	1 g
13-Cis-Retinoic Acid-Isotretinoin:	100 mg

Cambridge Isotopes Laboratories, Inc., Tewkesbury, MA-

Retinla-D6:	0.01 g
Retinoic Acid-D6:	0.01 g
Retinol-D6:	0.01 g
Vitamin A Acetate-D6:	0.01 g

Reagents

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

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

The standards and internal standards were made up in Methanol.

Sample Preparation- Protein Crash

- 200 µL of fat soluble Vitamin depleted serum/HSA mixture calibrators, controls and serum sample were added to 1.5 ml eppendorf tubes and 20 µL of Retinoid ISTD mixture at 1000 ng/mL were added to each tube and vortexed briefly
- 400 µL of Acetonitrile was added to each tube 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 added to a test tube and 20 µL of Retinoid ISTD mixture at 1000 ng/mL were added to each and vortexed briefly
- 200 µL of Acetonitrile was added to each tube and vortexed for 1 min
- 1.2 mL of Methyl-Tert-Butyl Ether was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper 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.

Data Analysis

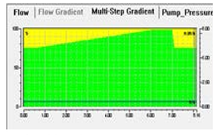
The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Endura Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

METHOD

HPLC Conditions-

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

Column:	Accucore C18, 100 x 2.1 mm, 2.6 µm
Column Temperature:	30 ºC
Injection Volume:	10 µL
Sampler Temperature:	4 ºC
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
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

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
Tazarotenic Acid	0.86	Positive	324.2	294/308	34.3/36.4	191
Tazarotene	1.67	Positive	352.2	324.01294	26.4/40.1	206
Acitretin	2.51	Positive	327.2	176.9/159	10.3/17.4	101
13-Cis-Retinoic Acid	2.90	Positive	301.2	283.1/122.9	10.3/15.9	95
Retinal	3.07	Positive	285.2	161/193	10.3/10.3	94
Retinal-D6	3.09	Positive	291.3	167.1/196.1	10.3/10.3	92
Retinol	3.10	Positive	269.3	93/119	10.3/20.2	116
Retinol-D6	3.09	Positive	275.2	95.9/121.9	19.7/18.9	109
Bexarotene	3.16	Positive	349.2	279.1/146.9	25.5/36.5	176
Retinoic Acid	3.27	Positive	301.3	283.1/122.9	10.3/16	96
Retinoic Acid-D6	3.24	Positive	307.3	289.2/123.1	10.3/16.8	102
Etretinate	4.28	Positive	355.2	309.1/131	10.3/21.5	102
Retinyl Acetate	4.35	Positive	269.2	93/119	21.2/20.2	108
Retinyl Acetate-D6	4.32	Positive	275.3	95.9/121.9	16.9/12.5	112
Adapalene	4.53	Positive	413.2	134.9/93.1	24.7/39.1	167
Retinyl Palmitate	4.80	Positive	269.2	93/213.1	21.3/13.3	111

RESULTS

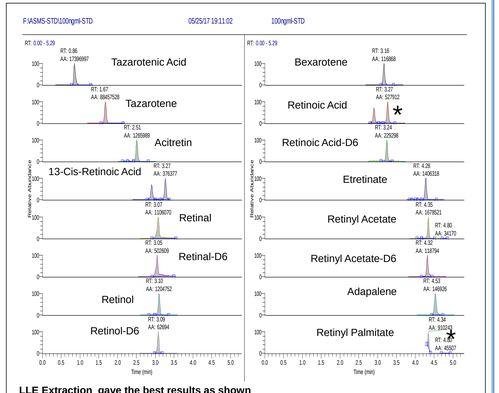
Linearity/Sensitivity

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 (R^2) 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 (R^2) > 98 for each sample extraction technique and the %CV for each calibration point were all <10% in order to be accepted.

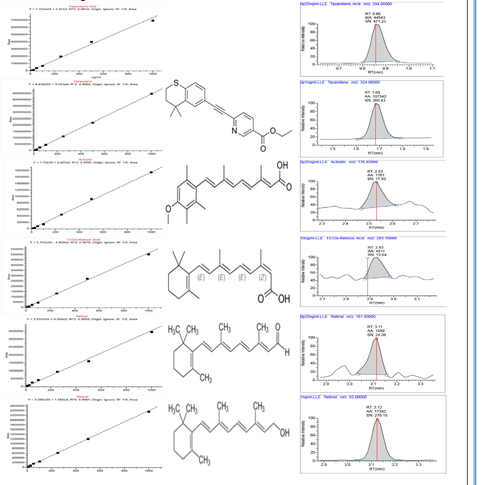
Table 2- Linearity and Sensitivity

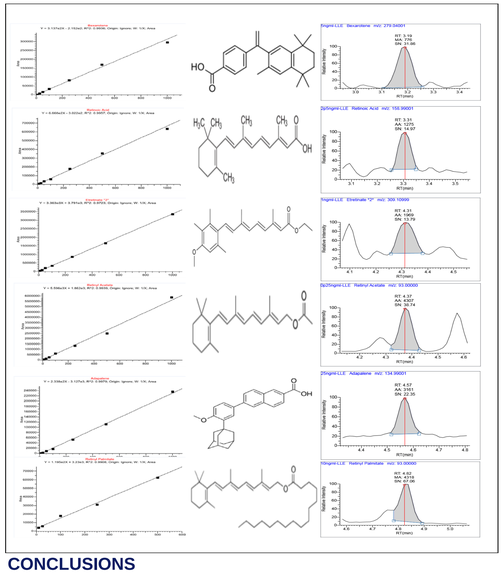
Compound	Linearity	LOQ (ng/ml)
Tazarotenic Acid	0.25 ng/ml – 1000 ng/ml PPT 0.025 ng/ml – 1000 ng/ml LLE	0.25 0.025
Tazarotene	0.5 ng/ml - 1000 ng/ml PPT 0.1 ng/ml -1000 ng/ml LLE	0.5 0.1
Acitretin	1 ng/ml – 1000 ng/ml PPT 0.25 ng/ml – 1000 ng/ml LLE	1 0.25
13-Cis-Retinoic Acid	25 ng/ml – 1000 ng/ml PPT 2.5 ng/ml – 1000 ng/ml LLE	25 10
Retinal	2.5 ng/ml – 1000 ng/ml PPT 0.25 ng/ml – 1000 ng/ml LLE	2.5 0.25
Retinol	1 ng/ml – 1000 ng/ml PPT 1 ng/ml – 1000 ng/ml LLE	1 1
Bexarotene	10 ng/ml -1000 ng/ml PPT 5 ng/ml -1000 ng/ml LLE	10 5
Retinoic Acid	2.5 ng/ml – 1000 ng/ml PPT 1 ng/ml – 1000 ng/ml LLE	2.5 2.5
Etretinate	1 ng/ml – 1000 ng/ml PPT 1 ng/ml – 1000 ng/ml LLE	1 1
Retinyl Acetate	1 ng/ml – 1000 ng/ml PPT 0.1 ng/ml – 1000 ng/ml LLE	1 0.1
Adapalene	25 ng/ml – 500 ng/ml PPT 25 ng/ml – 1000 ng/ml LLE	25 25
Retinyl Palmitate	25 ng/ml – 1000 ng/ml PPT 10 ng/ml – 1000 ng/ml LLE	25 10

Figure 1: Chromatograms and Retention time



LLE Extraction gave the best results as shown





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 of calibration curves with better accuracy, precision and reproducibility in positive mode but some of these compounds are structurally similar and thus can cause ion suppression, artifact co-elution, etc
- 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

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REFERENCES

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TRADEMARKS/LICENSING