Sensitive and Accurate Quantitation of Retinoic Acid Isomers in Biological Samples

Using the QTRAP® 6500 System with SelexION® Technology

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Retinoic acid (RA) is a vitamin-A metabolite that serves as a mediator of growth and development by regulating gene transcription under control of the RA receptors and retinoid X receptors.[1-4] There are multiple isomers of retinoic acid, each defined by a specific double-bond stereochemistry and a unique biological role. Resolution of retinoic acid isomers is critical since each isoform has different affinities for nuclear receptors thus may afford different biological actions.[4,7] RA quantitation is commonly achieved with liquid chromatographic separation of isomers and sensitive mass spectrometric detection[7-8], which may suffer from co-extracted and co-eluted matrix interference.

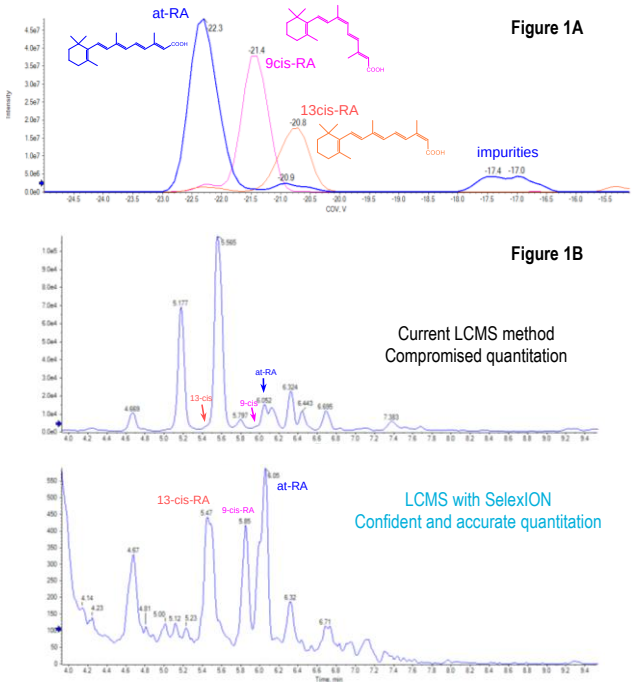

Figure 1. Advantage of SelexION Technology for the Quantitation of Retionic Acid Isomers in Complex Matrices. (Top) SelexION Technology can be used to separate the three RA Isomers without chromatography. (Middle) Isomers can be separated using LC however method is prone to interferences when quantifying in complex matrices. (Bottom) Chromatography in combination with DMS separation can improve the confidence and quality of retinoic acid quantitative.

Here, a novel approach using SelexION technology is presented to effectively resolve the challenge of matrix and isomeric interferences and for qualitative and quantitative Retinoic acid analysis.

Key Features of SelexION Technology for Retinoic Acids Analysis

- SelexION Technology is a planar differential mobility device (DMS) that separates analytes based on differences in their chemical properties, prior to entering the instrument orifice, thus providing an orthogonal level of selectivity
- SelexION technology can separate three retinoic acid isomers without chromatographic separation; however, when coupled to chromatographic separation, DMS drastically reduces matrix interferences and improves quantitative results
- SelexION technology improves quantitation performance for biological samples with heavy matrix interferences

Materials and Methods

Sample Preparation: Mouse plasma, liver, and intestine samples were collected and prepared as described[11] then stored at -80 ºC. Stock standard solutions were prepared in ethanol at a final standard concentration of 100 µg/mL. Calibration standards were prepared by serial dilution from the stock solution to 8 levels from 12 to 300,000 pg/mL for each retinoic acid isomer.

LC-MS/MS Analysis: The LC-MS/MS analysis for retinoic acids was performed on a HPLC system consisting of a binary high pressure mixing gradient pump with degasser, a thermostated autosampler, and a column oven. Chromatographic separation was achieved on an Ascentis Express RP-Amide column (2.1×100mm, 2.7 µm, Supelco, Cat#53913-U) held at 25 ºC. A flow rate of 1.0 mL/min was used with an injection volume of 10 µL using the gradient below.

Time (min)	%A	%B
0	*40*	*60*
0.3	*40*	*60*
2.8	*5*	*95*
3.4	*5*	*95*
3.5	*40*	*60*
4.0	*40*	*60*

Mobile Phase: *A) Water, 0.1% formic acid*
B) Acetonitrile, 0.1% formic acid

Mass Spectrometry: The QTRAP 6500 system was equipped with an IonDrive™ Turbo V Source and the APCI (atmospheric pressure chemical ionization) probe. It was operated in multiple reaction monitoring (MRM) mode with the transition 301.2 → 205.1 used as the quantitative transition with collision energy set at 17 V and collision gas of 8. The source conditions were as follows: nebulizer current was 3 mA, source temperature was 425 ºC, curtain gas was 20 and Gas 1 was 70.

The SelexION Technology was next installed to improve method selectivity, and operated in negative electrospray ionization (ESI) mode. Using the MRM transition, -299.2 → -255.1, a collision energy of -23 V and collision gas of 10 was used. The source conditions were as follows: ionspray voltage of -4500 V, source temperature was 600 ºC, curtain gas was 10, Gas 1 was 60 and Gas 2 was 90. The SelexION Technology conditions used were as follows: DMS temperature of 100 ºC, separation voltage of 4000 V, and a range of modifiers and compensation voltages were tested.

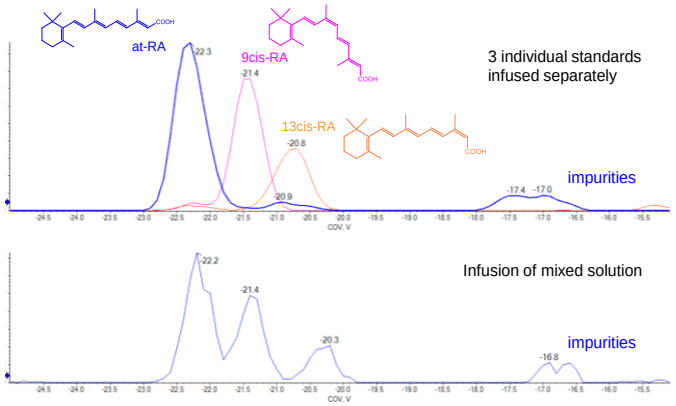

Figure 2. SelexION Technology Separation of Retinoic Acid Isomers. (Top) Three individual retinoic acid standards were infused separately to determine their distinctive CoV values. (Bottom) A mixed standard solution was then infused to show the isomers can be resolved by DMS.

Results

The purpose of this study was to establish a reliable method for the quantitation of retinoic acid isomers in biological samples with minimized adverse matrix effects. Previous reports have described the primary challenge in RA analysis is overcoming matrix interferences when analyzing biological samples[7,8]. Kane et al. reported atmospheric pressure chemical ionization (APCI) in positive mode is the preferred ionization technique for its better ionization efficiency, lower background, better linear dynamic range and better tolerance to adverse matrix effects such as ionization suppression.[7]

In this study, SelexION technology was used to add an orthogonal dimension of selectivity[9,10] to improve quantitative analysis of retinoic acid (RA) isomer in biological samples. Since infusion and chromatographic separation are the two commonly used workflows for lipid analysis, the applicability of SelexION technology was investigated for both workflows and optimized its performance to fit the assay's needs. Successful utilization of SelexION technology was achieved by balancing selectivity and sensitivity. The SelexION Technology device parameters can be optimized to achieve baseline resolution of the RA isomers; however, at this level of resolution, the observed peak intensity is noticeably decreased and is inappropriate for the requisite assay sensitivity. However, using both HPLC and the SelexION Technology set to operate with moderate selectivity considerably reduced matrix interference to produce a sensitive and selective quantitative method using ESI in the negative ion mode.

Direct Infusion Based Workflow

Direct infusion-based workflows are mainly used for qualitative and/or quantitative purposes, wherein the target analytes are present in relatively high concentrations relative to matrix. Using this workflow, the SelexION Technology was optimized to not only reduce matrix interference, but also to separate the three target RA isomers. DMS parameters such as separation voltage (SV), modifier type and flow rate, DMS cell temperature (DT), and resolution enhancement gas pressure (DR) were optimized while the compensation voltage (CoV) was ramped to monitor retinoic acid isomer separation. Using instrument parameters that maximized resolution, the three target RA isomers were separated with baseline resolution, as shown in the lower pane of Figure 2. The identity of each isomer was confirmed, appearing in the CoV ionogram with a specific CoV value: all trans-retinoic acid (at-RA) at CoV -22.3V, 9-cis-retinoic acid (9cis-RA) at CoV -21.4V, and 13-cis-retinoic acid (13cis-RA) at CoV -20.8V. It is worth noting that impurities, most likely mixed isomers and/or oxidation products, also resolved apart from the three targeted analytes.

The resolution of RA isomers using DMS can be greatly affected by the type of chemical modifier which is added to the ion source at atmospheric pressure. Use of chemical modifiers is unique to SelexION technology and adds an additional degree of freedom in resolving power by forming analyte-modifier clusters that behave differently in the asymmetric electric field than the analyte ions do by themselves. Thus, selecting the correct modifier can greatly affect the quality of analyte separation.

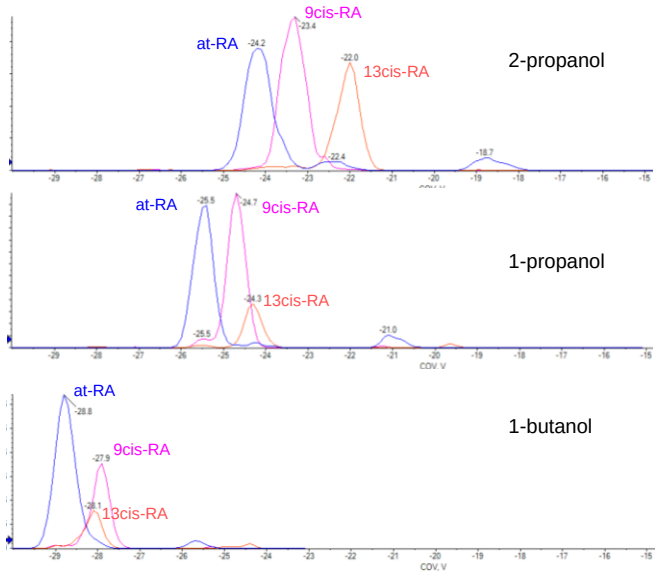

Figure 3. Chemical Modifiers Demonstrate Different Selectivity on DMS Separation. Three commonly used mobile phase solvents were used as chemical modifiers and investigated for their effects on retinoic acid separation. Different resolving characteristics were observed: the more linear modifier with longer carbon chain length (1-butanol) shifts all peaks to more negative side on CoV scale. And the shorter, branched 2-propanol provides better resolution among the isomers.

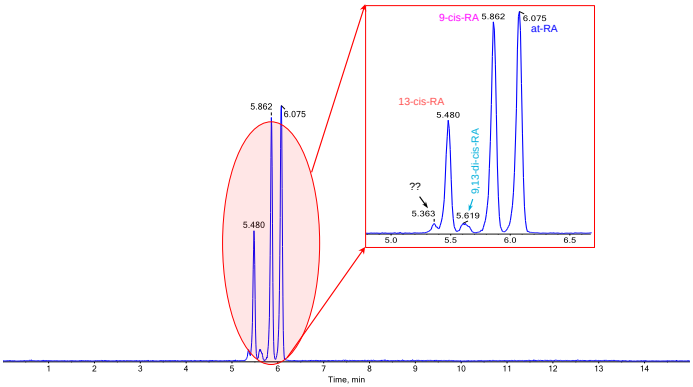

Figure 4. Example of Chromatographic Separation of Retinoic Acid Isomers. A well-established chromatographic method separates retinoic acid isomers within a reasonable time frame, but quantitation may be subject to adverse matrix interferences.

In this study, using ethanol as the modifier provided the best separation, as shown in Figure 2. Other commonly used solvents, such as 1-propanol, 2-propanol and 1-butanol, were investigated and the results are illustrated in Figure 3.

Chromatographic Separation Based Workflow

There are two potential scenarios when SelexION technology is coupled to chromatographic separation-based workflows. In the first scenario, the chromatographic separation for targeted analytes is already established, and DMS is used mainly to remove/eliminate matrix interferences to achieve better quantitation. In the second scenario, the separation of targeted analytes is not available or practical (e.g. hours-long chromatography run time, or with special mobile phase additives not compatible with MS detection), and SelexION technology is used to achieve separation of target analytes with better sensitivity than infusion-based workflows.

The use of SelexION technology in this study falls into the first scenario, wherein the chromatographic separation method was already established, but the quantitation suffered from matrix interferences, as shown in the top pane in Figure 1B. For low level quantitation of retinoic isomers in biological samples, chromatographic separation is required so the three retinoic acids can be better separated from each other as well as from matrix interferences. Figure 4 shows an example of LC separation of 5 possible retinoic acids.

The three main chromatographic peaks represent the three targeted retinoic acid isomers: 13cis-RA at 5.48 min, 9cis-RA at 5.86 min and at-RA at 6.08 min. Two other minor isomers were also separated: 9,13-di-cis-RA at 5.62 min and a possible unidentified isomer at 5.36 min.

Using a standard mix without matrix, all chromatographic peaks can be easily and reliably integrated for quantitative purposes in Figure 4. However, the matrix interference is overwhelming for complex samples, and accurate quantitation is compromised. As seen in Figure 5, where the pooled liver extract sample was analyzed, the RA isomer peaks co-elute with interfering peaks or are buried under interference peaks with greater intensity. In this low level quantitative scenario, the SelexION Technology was used to eliminate/reduce interferences. As seen in lower pane of Figure 5, the three prominent RA isomer peaks can be confidently quantified when the DMS is used to remove matrix interference. Comparing the data acquired with and without DMS, the observed overall improved sensitivity is attributed to noise reduction.

In other situations where the chromatographic separation is not readily available, especially in high-throughput environments where a long chromatographic run time is not desirable, SelexION technology needs to not only reduce/eliminate matrix interferences, but also to resolve the target analytes. In this study, the additional DMS-related selectivity helps to resolve retinoic acid isomers based on their characteristic CoV values.

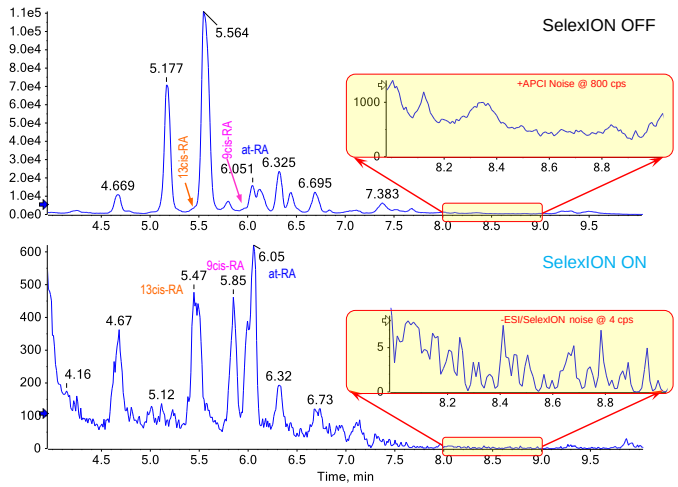

Figure 5. Improved Sensitivity for Retinoic Acid Quantitation in Biological Samples. (Top) Substantial matrix interference is observed and all three retinoic acid isomers co-elute with or are buried under interfering peaks with greater intensity, making quantitation compromised. (Bottom) With the DMS parameters optimized, interfering peaks were significantly reduced and the resolved retinoic acid isomers can be accurately quantified. (Insert) The DMS separation substantially reduces background noise to ensure sensitive quantitation.

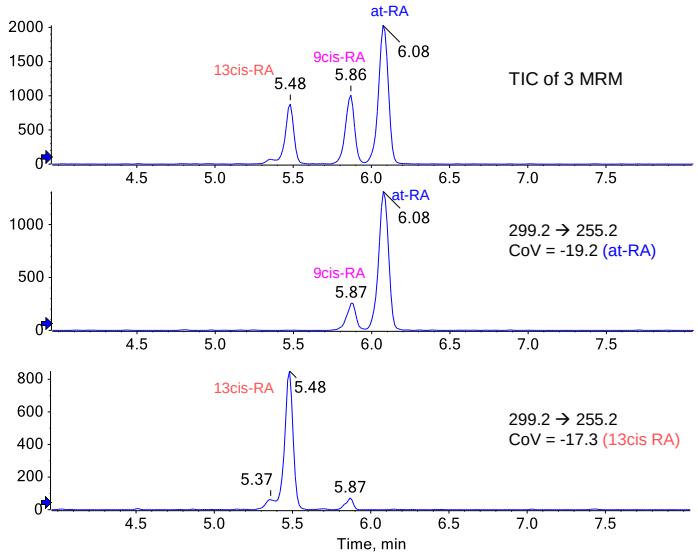

Figure 6. Combined Orthogonal Selectivity of Chromatographic Separation and DMS. The DMS device was operated in most selective mode in this example. (Middle) 13cis-RA was selectively removed and 9cis-RA signal considerably suppressed when the CoV was set at at-RA's specific Cov of -19.2V. (Bottom) Here, 9cis-RA and at-RA were selectively removed when CoV set at 13cis-RA's specific CoV of -17.3V.

As shown in Figure 6, three targeted RA isomers have the same MRM transition (299.2 → 255.2) and appear as three peaks in the monitored MRM TIC. When the compensation voltage is set to -19.2V, the characteristic value of at-RA, the 13cis-RA peak disappears. When the compensation voltage is set to 13cis-RA's characteristic value of -17.3V, the at-RA peak disappeared and only 13cis-RA remained as the major peak.

This experiment is an excellent example that demonstrates the additional selectivity that is possible using SelexION Technology. For previously mentioned scenarios where chromatographic separation is not readily available, incorporating the additional DMS separation to the existing LC/MS method can be a powerful solution to remove interferences from matrix to improve the selectivity and sensitivity of your assay to provide a biologically-relevant quantitative method.

Summary

In this study, SelexION Technology was used to introduce an additional dimension of selectivity with and without chromatography:

- For infusion-based workflows, SelexION technology successfully separates retinoic acid isomers based on their characteristic compensation voltages, enabling confident identification and accurate quantitation
- In chromatographic separation-based workflows, the incorporation of SelexION Technology to existing LC-MS/MS methods may add additional selectivity to improve quantitation of individually targeted retinoic acid isomers in biological samples

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