Dienes Derivatization MaxSpec® Kit

Item No. 601510

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Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
21593	Dienes Derivatizing MaxSpec® Reagent	1 vial / 1.5 mg	-20°C
601511	Vitamin D_3 (solution)	1 vial / 10 µM in 1 ml ethanol	-20°C
710006	Autosampler Glass Vial Inserts (300 µl)	1 pack of 100 inserts	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Methanol, LCMS grade or equivalent
2. Acetonitrile, HPLC grade or equivalent
3. Deionized water
4. HPLC autosampler vials (such as Phenomenex p/n AR0-9921-13-C)
5. 1.7 mL polypropylene Eppendorf Tubes (such as VWR p/n 87003-294)
6. Waters Oasis HLB SPE cartridges, 30 g (p/n WAT094225)

Equipment

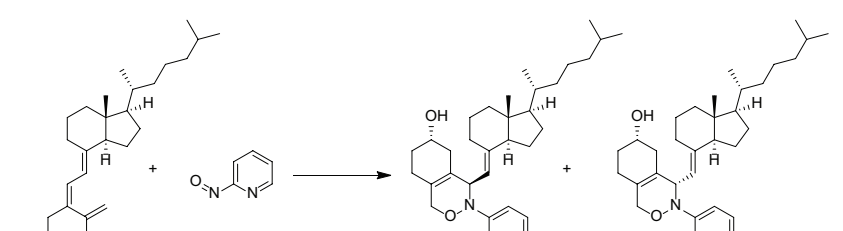
1. Oven or incubator controlled at 70°C
2. Microcentrifuge (optional)
3. Vacuum SPE manifold (optional)
4. SpeedVac concentrator (optional)
5. Pipettors (10 µl, 200 µl, and 1,000 µl)
6. LC-MS (a Waters Acquity UPLC and Xevo TQ-S micro triple quadrupole mass spectrometer were used to develop the procedures outlined in this kit)

INTRODUCTION

About This Assay

The Dienes Derivatization MaxSpec® Kit includes a chemically stable diene derivatization reagent, which derivatizes the diene compound of interest using a one-step chemical derivatization in methanol based on click chemistry. Vitamin D_3 is derivatized by the Derivatization Reagent and derivatization efficiency is 100%. The derivatized-Vitamin D3 provides 100-fold increase in sensitivity over underivatized Vitamin D_3 for liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a lower limit of detection of 10 pM. Excellent linearity was obtained in a concentration range of 10pM-25nM for the derivatized-Vitamin D3. This suggests the derivatization agent can be potentially used for accurate quantitation of trace diene-containing compounds like Vitamin D metabolites if the compounds can be efficiently extracted and purified from sample matrix.

A simple, rapid, and efficient protocol is provided in the kit to extract diene-containing compounds from sample matrices like plasma prior to sample derivatization for LC-MS/MS analysis. The extraction protocol is performed using a combination of protein precipitation and Waters HLB solid phase extraction (SPE) cartridges. After drying completely under vacuum, the extracted and concentrated diene compounds were directly dissolved in Derivatization Solution. After derivatization, the mixture can be directly injected for sensitive detection of the derivatized diene compounds of interest using LC-MS/MS. In order to demonstrate the applicability of the derivatization kit for sensitive detection and accurate quantification of the diene-containing compounds in biological matrix, Vitamin D metabolites were spiked into 4% bovine serum albumin (BSA). Using the protocol, the lower limit quantitation of 1, 25-dihydroxy Vitamin D_2 and 25-hydroxy Vitamin D_3 were determined to be 25 pM for each.



Scheme 1. The Derivatization Reaction and General Principles. Example derivatization reaction of pyr-NO (Derivatization Reagent) and Vitamin D_3. The reaction is regioselective and produces a primary pair of diastereomers in a ratio of about 1:4.A minor pair of regioisomers may also form as minor products of the reaction. For quantitation, only the single major diastereomer is integrated and is sufficient for accurate and sensitive quantitation of Vitamin D_3. The pyridine ring in the derivatized products significantly improves ionization and results in significant improvements in sensitivity over other methods.

General Information

- This provided method was optimized for the extraction and analysis of Vitamin D and metabolites. Other analytes will likely require modifications to the provided method and should be verified to ensure extraction and analysis conditions are appropriate.
- The Suitability Standard is provided for use as a positive control to ensure proper kit function and may be used to troubleshoot issues with extraction recovery, the derivatization reaction, chromatography, or mass spectrometry settings. Most method related issues may be resolved using this standard.
- Use of plastic inserts will reduce sensitivity due to the introduction of leachates during the derivatization step. Sensitivity issues are otherwise not expected to be related to this kit and are most likely related to issues with the mass spectrometer.

PRE-ASSAY PREPARATION

Derivatization Reagent Preparation

NOTE: Vitamin D_3 is known to be sensitive to light therefore all derivatization reactions were performed in the dark and derivatized standards and samples were stored at -20°C in the dark for no more than 1 week.

1. Derivatization Stock Solution

Prepare a 25 mM solution of the Derivatization Stock Solution by adding 556 µl of methanol to the 1.5 mg Dienes Derivatizing MaxSpec® Reagent (Item No. 21593) supplied in the kit and vortex or mix until homogenous. Once prepared, this stock solution may be stored at -20 °C for up to 3 months.

2. Derivatization Solution

Prepare a 2.5 mM Derivatization Solution by dissolving 1 volume of Derivatization Reagent Stock Solution with 9 volumes of methanol. This solution will be used to derivatize samples and should be prepared as needed from the Stock Solution.

ASSAY PROTOCOL

Derivatization

If the sample is dissolved in solvent, dry the sample completely in a speed vacuum. Re-dissolve the dried sample in 50 µl of the 2.5 mM Derivatization Solution by vortexing for 1 minutes. The reconstituted sample solution was transferred into a glass HPLC vial with a glass insert. Incubate the mixture at 70°C for 1 hour for derivatization. Allow to cool to room temperature. The sample can be directly injected for LC-MS/MS analysis.

For samples in biological matrix such as plasma or serum, sample extraction and purification is required before derivatization. At Cayman, we developed a simple and fast extraction method to extract Vitamin D metabolites from plasma using protein precipitation and solid phase extraction. After sample preparation, the lower limit of quantitation of 25-hydroxy Vitamin D_3 and 1,25-dihydroxy Vitamin D_2 were approximately 25 pM. A detailed procedure is provided on page 9 for your reference.

NOTE: It has been determined that the derivatized vitamin D products are light sensitive and should be stored dark at -20°C for no longer than 1 week prior to analysis.

System Suitability Experiment for Sample Derivatization

NOTE: This procedure may be used as a control experiment to verify proper experimental set up prior to and/or during sample analysis. The conditions provided are suggested only and all method parameters should be optimized and verified prior to sample analysis.

Prior to derivatization, the provided 10 µM vitamin D_3 solution is further diluted for 100-fold to make a 100 nM Vitamin D_3 test solution. 50 µl of the 100 nM solution is dried under speed vaccum or under nitrogen stream. 50 µl of the derivatization solution is added into each dried sample to reconstitute the dried samples. After vortexing for 30 seconds, the reconstituted solutions are transferred into glass inserts of HPLC autosampler vials. Incubate the mixture at 70°C for 1 hour for derivatization. The derivatized sample can be directly injected for LC-MS/MS analysis.

1. LC-MS/MS Analysis

A Waters Acquity UPLC and Xevo TQ-S micro mass spectrometer were used for LC-MS/MS analysis of Vitamin D_3 and derivatized-Vitamin D_3. This approach can be adapted to a conventional LC-MS/MS system however, sensitivity may be affected by any changes to the supplied method.

2. HPLC setup

An Acquity UPLC 1.7 µm BEH C8 column (2.1 x 100 mm) operated at ambient temperature is used for the chromatography. Set up a gradient elution method with Mobile Phase A (water with 0.1% formic acid) and Mobile Phase B (acetonitrile with 0.1% formic acid) as outlined in Table 1. The HPLC flow rate was 400 µL/minute. Sample injection volume is 5 µl.

Time (minutes)	%A	%B
0-0.4	98	2
3	30	70
4	1	99
7	1	99
7.1-9	98	2

Table 1. HPLC Gradient for Vitamin D_3 and derivatized-Vitamin D_3.

3. Mass spectrometer setup

The eluted Vitamin D_3 and derivatized-Vitamin D_3 analytes were ionized by electrospray ionization in positive ion mode with nitrogen as the desolvation gas and argon as the collision gas. The following mass spectrometric parameters are set: source spray voltage, 2.5 kV; cone voltage, 40 V; ion source temperature, 150°C; desolvation temperature 400°C. The eluted Vitamin D_3 and derivatized-Vitamin D_3 ions are selectively monitored using multiple reaction monitoring in the same LC-MS/MS analysis. The MS/MS transitions and MS/MS conditions are listed in Table 2. For best sensitivity, MS/MS parameters should be optimized using manufacturer recommended tuning procedures and a freshly prepared derivatized standard.

Name	Mass (Da)	Transitions (m/z)	Retention (minutes)	Cone (V)	Collision Energy (V)
Vitamin D_3	384.3	385.6>259.4	5.50	26	14
Derivatized-Vitamin D_3	492.4	493.4>231.1	4.26, 4.59	24	20
Derivatized-Vitamin D_3	492.4	493.4>227.1	4.73, 4.91	24	20

Table 2. LC-MS/MS Conditions Used to Detect Vitamin D_3 and Derivatized Vitamin D_3

Results

Detection of the Derivatized-Vitamin D_3

There are four isomers of the derivatized-Vitamin D_3 produced in system suitability test. The four isomers were separated and detected under current LC-MS/MS conditions, shown in Figure 1.

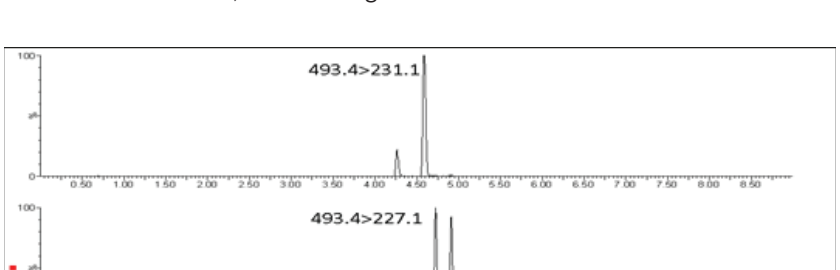


Figure 1. Reconstructed Mass Chromatograms of four isomers of the derivatized-Vitamin D_3.

Enhanced Sensitivity of the Derivatized-Vitamin D_3 for LC-MS/MS Analysis

In Figure 1, the peak at 4.59 minutes arising from the product ion at m/z 231.1 Da is the most intense of the four isomers produced in the derivatization reaction. This transition was used to evaluate the improvement in sensitivity of derivatized-Vitamin D_3 compared to the original Vitamin D_3 and is the recommended transition for quantitation. Using the developed LC-MS/MS method, a lower limit of detection (LOD S/N=3) and lower limit of quantification (LOQ S/N=10) of Vitamin D_3 and the derivatized Vitamin D_3 were determined and compared. The data is shown in Figure 2. By this protocol, the LOD and LOQ of the derivatized-Vitamin D_3 were determined to be 10 pM (3.8 pg/ml) and 25 pM (9.6 pg/ml) respectively which was found to be approximately 100-fold more sensitive than underivatized Vitamin D_3.

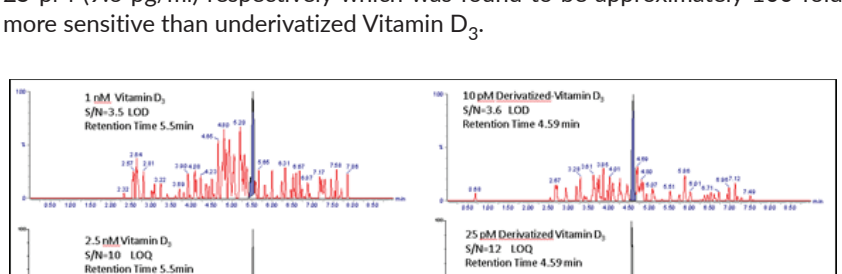


Figure 2. Comparison of LOD and LOQ of Vitamin D_3 and the derivatized-Vitamin D_3

Derivatization Efficiency

To verify 100% derivatization efficiency, Vitamin D_3 was monitored at two concentrations (25 nM and 50 nM) before and after derivatization. No un-derivatized Vitamin D_3 was detected in the derivatized samples.

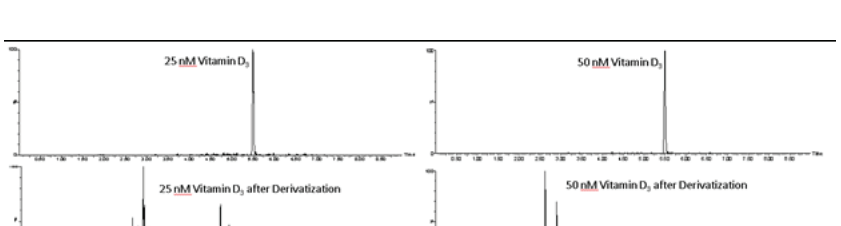


Figure 3. Mass Chromatograms of 25 nM Vitamin D_3 and 50 nM Vitamin D_3 before and after derivatization.

Linearity of the Derivatized-Vitamin D_3 Curve

In order to demonstrate the applicability of the diene-derivatization agent for accurate quantification, a series of Vitamin D_3 solutions over the range from 25 pM to 25 nM were derivatized and their responses were plotted against the initial concentration of Vitamin D_3. Excellent linearity was obtained for the derivatized-Vitamin D_3 over this range with a correlation efficient r=0.999.

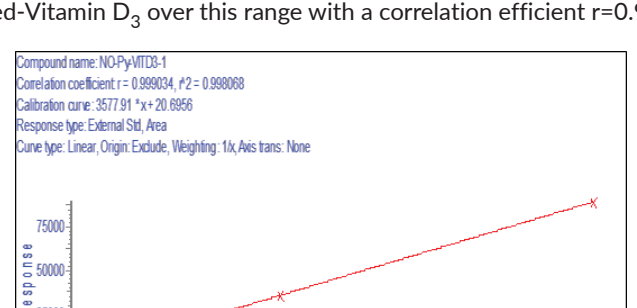


Figure 4. Calibration curve of the derivatized Vitamin D_3.

ANALYSIS

Sample Preparation and LC-MS/MS Method for Diene Compounds in Plasma

NOTE: The protocol described below is provided as a suggested protocol for the analysis of Vitamin D metabolites in plasma. This Dienes Derivatization MaxSpec® Kit is intended for Research Use Only and not intended to be used in the diagnosis or treatment of any disease. In order to ensure optimal performance of this Kit, all method parameters should be optimized and verified on your instrumentation. Vitamin D metabolites are present at extremely low levels in some samples and may not be detectable using this Kit if instrumentation and/or experimental methods are not optimal for the analysis. Many of the issues with this analysis may be mitigated by employing appropriately sensitive equipment and by optimization of the suggested methods included in this booklet.

This kit provides a simple and efficient protocol to extract and derivatize diene-containing compounds from biological matrices. There is growing interest in sensitive detection and quantitation of Vitamin D metabolites in plasma because of their important role as the biologically active forms of Vitamin D. In the study, 25-hydroxy Vitamin D_3 and 1,25-dihydroxy Vitamin D_2 were spiked in 4% BSA in PBS to mimic Vitamin metabolites in plasma samples. Different concentrations of Vitamin D metabolites were prepared and only 100 µl of sample was used for sample preparation. The detailed protocol for extraction and purification of Vitamin D metabolites is described below.

1. LC-MS/MS Analysis

- Add 300 µL of acetonitrile (MeCN) into 100 µl of sample in a 1.5 ml Eppendorf Tube to precipitate unwanted matrix proteins.
- Vortex the mixture vigorously for 1 minute.
- Centrifuge the mixture at 13,200 rpm for 10 minutes.
- Transfer all of the supernatant into a clean 1.5 ml Eppendorf tube.
- To prepare the sample for SPE, add 400 µl of 5% methanol in water to the supernatant.
- Repeat for all samples and standards.

2. SPE

- Condition the SPE cartridge using 1 mL of methanol, followed by 1ml of 5% methanol in water.
- Load the 800 µl supernatant mixture onto the SPE cartridge dropwise over 2-3 minutes.
- Wash the cartridge with 1 ml of 5% methanol in water. Repeat wash a second time.
- Wash the cartridge again using 1 ml 70% methanol in water solution. Repeat for a second time. 70% MeOH wash is critical to remove unwanted matrix interferences Using vacuum pressure, completely dry the cartridge for 3 minutes to remove water.
- Elute the analytes using 300 µl of methanol. Repeat and combine eluate.
- **Dry the eluate completely under nitrogen or on a Speedvac.

*The cartridge and SPE conditions provided were optimized for Vitamin D and its hydroxyl metabolites and may not be appropriate for other diene analytes.

**Unlike some competitor Kits, the Derivatization Reagent in this Kit is tolerant to the presence of water and so trace water remaining after SPE will not affect the analysis

3. Derivatization of Diene-Containing Compounds

- Reconstitute the dried sample in 50 µL of the prepared Derivatization Solution.
- Vortex the mixture vigorously for 1 minute.
- Transfer the mixture to an HPLC autosampler vial containing one of the provided 200 µL glass inserts.
- Place the vial in the preheated 70°C oven for 1 hour to derivatize the sample.
- After completely cooling the vial to room temperature the vial may be placed directly in the HPLC autosampler for analysis.

4. LC-MS/MS Analysis

HPLC setup:

Fit an Acquity UPLC CORTECS C18 column (2.1 x 100 mm, 1.6 µm) on an Acquity UPLC system at ambient temperature. Set up a gradient elution method with Mobile Phase A (water with 0.1% formic acid) and Mobile Phase B (Acetonitrile with 0.1% formic acid) as outlined in the Table 3. The HPLC flow rate was 400 µL/minutes. Inject 5 µl of supernatant obtained from sample preparation.

Time (minutes)	%A	%B
0-0.4	98	2
8	5	95
10	5	95
10.1	98	2
12	98	2

Table 3. HPLC Gradient for Separation of Derivatized-Vitamin D Metabolites

Mass spectrometer setup:

The derivatized-Vitamin D metabolites were ionized by electrospray ionization in positive ion mode under similar mass spectrometric conditions as described previously for Vitamin D_3 on the Waters TQ-S mass spectrometer. Nitrogen was used as the desolvation gas and argon was the collision gas. The MRM transitions are listed in the Table 4. MS/MS spectra of the derivatized metabolites were collected and the most abundant daughter ion for each analyte was selected as listed in the Table.

Name	Mass (Da)	Transitions (m/z)	Retention (minutes)	Cone (V)	Collision (V)
Derivatized-25(OH) Vitamin D3	508.4	509.4>231.2	5.3	24	18
Derivatized-1, 25(OH)2Vitamin D2	536.4	537.4>247.2	4.8	24	24

Table 4. Mass Spectrometric Conditions Used to Monitor the Derivatized-Vitamin D Metabolites

Using the protocol, 25 pM of 1,25-dihydroxy Vitamin D_2 and 25 pM of 25-hydroxy Vitamin D_3 were spiked in 4% BSA. 100 µl of the solution was extracted and purified using the developed method. Figure 5 shows the extracted mass chromatograms of derivatized Vitamin D metabolites. Based on their S/N (8 and 13), 25 pM is close to the LLOQ for both compounds in biological matrix.

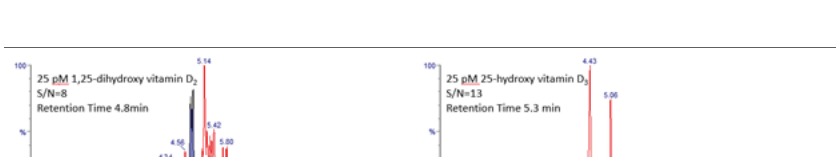


Figure 5. Extracted mass chromatograms of 1,25-dihydroxy Vitamin D_2 and 25-hydroxy Vitamin D_3.

Suggestions:

After method is developed and the retention time of derivatized diene compound is determined, the HPLC flow can be diverted from mass spectrometer to waste before and after the elution of the analyte to minimize source contamination.

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.