# ANALYTICAL

## Preparation and Storage of Samples

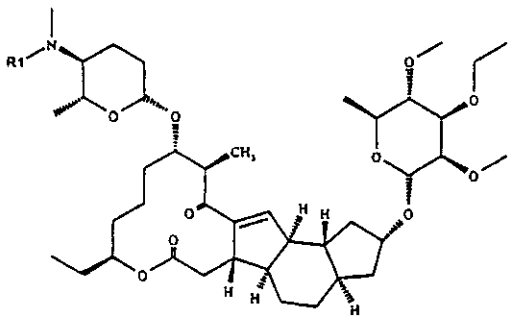
Water was collected on 26-Jul-05 in the morning from a pond in Ulm, located in Southern Germany. The appearance of the water was yellowish without any smell. The water was characterized for physical and chemical properties as follow: pH 7.9, total water hardness: 17° d (Deutsche Härtegrade, 3.05 mmol/L), dissolved organic carbon (DOC): 29 mg/L, turbidity: 2.27 NTU and filterable compounds: 5.2 mg/L.

## Preparation of Solutions and Standards

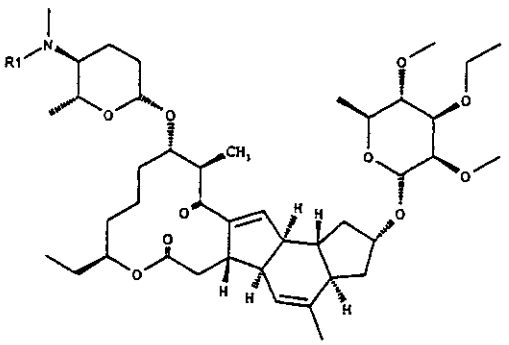
Reagents (obtained from Merck and Promochem) used were of equivalent specifications as described in Section 6.1 of method GRM 05.12. Solutions were prepared as described in Section 6.3 of method GRM 05.12.

The following analytical reference standard/test substances (obtained from the Sponsor) were utilized during the independent laboratory method validation:

**XDE-175 and Its Metabolites**



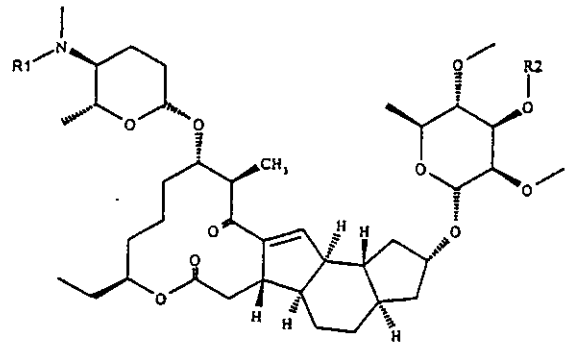
XDE-175-J, R1 = $CH_3$
XDE-175-*N*-Demethyl-J, R1 = H



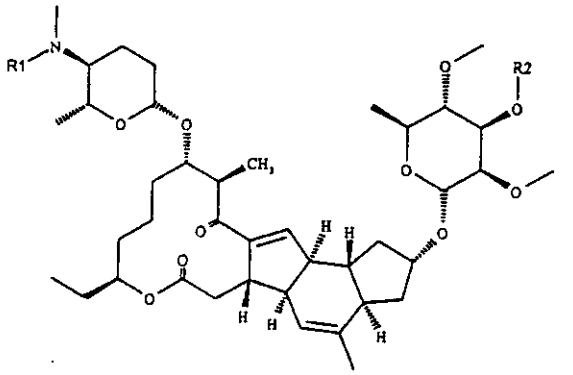
XDE-175-L, R1 = $CH_3$
XDE-175-*N*-Demethyl-L, R1 = H

| XDE-175-J | | XDE-175-L | |
|---|---|---|---|
| Molecular Formula: | $C_{42}H_{69}NO_{10}$ | Molecular Formula: | $C_{43}H_{69}NO_{10}$ |
| Nominal Mass: | 747.5 | Nominal Mass: | 759.5 |
| CAS Registry Number: | 187166-40-1 | CAS Registry Number: | 187166-15-0 |
| XDE-175-*N*-demethyl-J | | XDE-175-*N*-demethyl-L | |
| Molecular Formula: | $C_{41}H_{67}NO_{10}$ | Molecular Formula: | $C_{42}H_{67}NO_{10}$ |
| Nominal Mass: | 733.5 | Nominal Mass: | 745.5 |
| CAS Registry Number: | N/A | CAS Registry Number: | N/A |

## XDE-175 and Its Metabolites (Internal Standards)




XDE-175-J, R1 = $^{13}CD_3$, R2 = $C_2D_5$
XDE-175-*N*-Demethyl-J, R1 = H, R2 = $C_2D_5$




XDE-175-L, R1 = $^{13}CD_3$, R2 = $C_2D_5$
XDE-175-*N*-Demethyl-L, R1 = H, R2 = $C_2D_5$

| Common Name of Internal Standard | | | |
|---|---|---|---|
| XDE-175-J IS | | XDE-175-L IS | |
| Molecular Formula: | $C_{41}{}^{13}CH_{61}D_8NO_{10}$ | Molecular Formula: | $C_{42}{}^{13}CH_{61}D_8NO_{10}$ |
| Nominal Mass: | 756.5 | Nominal Mass: | 768.5 |
| CAS Registry Number: | N/A | CAS Registry Number: | N/A |
| XDE-175-*N*-Demethyl-J IS | | XDE-175-*N*-Demethyl-L IS | |
| Molecular Formula: | $C_{41}H_{62}D_5NO_{10}$ | Molecular Formula: | $C_{42}H_{62}D_5NO_{10}$ |
| Nominal Mass: | 738.5 | Nominal Mass: | 750.5 |
| CAS Registry Number: | N/A | CAS Registry Number: | N/A |

| Test Substance/ Analytical Standard(s) | AGR/TSN No. | Percent Purity | Certification Date | Reference |
|---|---|---|---|---|
| XDE-175-J | TSN104472 | 97.6 | 22-MAR-2004 | FA&PC 043028 |
| XDE-175-L | TSN104480 | 96.1 | 24-MAR-2004 | FA&PC 043036 |
| XDE-175-*N*-demethyl-J | TSN105114 | 98.0 | 11-APR-2005 | FA&PC 053239 |
| XDE-175-*N*-demethyl-L | TSN105124 | 99.0 | 14-APR-2005 | FA&PC 053249 |
| XDE-175-J-IS[1] | TSN104657 | 96.0 | 17-JUN-2004 | FA&PC 043195 |
| XDE-175-L-IS[1] | TSN104658 | 96.0 | 17-JUN-2004 | FA&PC 043196 |
| XDE-175-*N*-demethyl-J-IS[1] | TSN104663 | 96.0 | 17-JUN-2004 | FA&PC 043203 |
| XDE-175-*N*-demethyl-L-IS[1] | TSN104664 | 93.0 | 17-JUN-2004 | FA&PC 043204 |

[1] Internal standard

Standard solutions and calibration standard solutions were prepared as described in Section 7 of method GRM 05.12.

Fortification of Recovery Samples

One ILV trial of the method was run and consisted of the following:

1 reagent blank (containing no matrix or analyte)
2 unfortified control samples
5 control samples fortified at 0.05 µg/L with XDE-175 and its metabolites (the LOQ of the method)
5 control samples fortified at 0.5 µg/L with XDE-175 and its metabolites (10 x LOQ).

Fortification solutions were prepared as described in Section 7.1 of the residue analytical method GRM 05.12.

Sample Extraction, Purification and Analysis

The ILV trial was conducted as described in Section 9.3 of method GRM 05.12, with negligible

variations due to slightly different laboratory equipment and practices.

Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent laboratory conducted preliminary studies necessary for establishing acceptable performance of the chromatographic instrumentation to be used. These preliminary studies included establishing that adequate HPLC retention times of the analytes and MS/MS detector sensitivity could be achieved. Verification of a lack of XDE-175 and its metabolites contamination in the control sample matrices was not conducted prior to the method trial.

The instrumental conditions used during the ILV trial were conducted as described in Section 8 of method GRM 05.12, with minor adaptations as given below:

Liquid Chromatography Operating Conditions

| | |
|---|---|
| Instrumentation: | CTC Analytics HTC PAL Autosampler<br>Agilent Model 1100 binary pump<br>Agilent Model 1100 degasser |
| Column (used for quantitation): | YMC ODS- AM<br>50 x 4.6 mm, i.d., 5 µm particle size<br>Securityguard:<br>Waters Hypersil Gold, 10 x 3 mm, 5 µm particle size |
| Column (used for confirmation): | Phenomenex Synergi Polar-RP<br>75 x 4.6 mm i.d., 4 µm particle size<br>Securityguard:<br>Phenomenex Polar-RP, 4 x 3 mm, 4 µm particle size |
| Column Temperature: | 25 °C |
| Injection Volume: | 95 µL |
| Run Time: | 16 minutes |
| Mobile Phase: | A – acetonitrile/methanol (1/1, v/v) + 2 mM $NH_4$acetate<br>B – water +2 mM $NH_4$acetate |
| Flow Rate: | 400 µL/min |

| Gradient: | Time, min | A, % | B, % |
|---|---|---|---|
| | 0.00 | 30 | 70 |
| | 1.00 | 30 | 70 |
| | 3.00 | 100 | 0 |
| | 13.00 | 100 | 0 |
| | 13.10 | 30 | 70 |
| | 16.00 | 30 | 70 |

Mass Spectrometry Operating Conditions

| | |
|---|---|
| Instrumentation: | Applied Biosystems API 3000 LC/MS/MS System<br>Applied Biosystems Analyst 1.3.1 data system |
| Interface: | TurboIonSpray |
| Scan Type: | MRM |
| Resolution: | Q1 – Unit, Q3 – Unit |
| Curtain Gas (CUR): | 12 |
| Collision Gas (CAD): | 4 |
| Temperature (TEM): | 425 °C |
| Nebulizer Gas (NEB): | 14 |
| Run time: | 16 minutes |
| Polarity: | Positive |
| IonSpray Voltage (IS:) | 5500 |

| Compound: | Ion, m/z | | Dwell Time, ms | Collision Energy, V |
|---|---|---|---|---|
| | Q1 | Q3 | | |
| XDE-175-J | 748.7 | 142.1 | 120 | 45 |
| XDE-175-L | 760.7 | 142.1 | 120 | 45 |
| XDE-175-*N*-demethyl-J | 734.7 | 127.9 | 120 | 42 |
| XDE-175-*N*-demethyl-L | 746.8 | 127.9 | 120 | 45 |
| XDE-175-J IS | 757.9 | 146.2 | 120 | 45 |
| XDE-175-L IS | 769.9 | 146.2 | 120 | 45 |
| XDE-175-*N*-Demethyl-J IS | 739.9 | 128.2 | 120 | 42 |
| XDE-175-*N*-Demethyl-L IS | 751.7 | 128.2 | 120 | 45 |

Calculations

Linear regression equations using internal standards were generated for XDE-175 and its metabolites by injecting calibration standards. Regression calculation was performed by the Analyst software, with 1/x weighting, using the concentration ratio (analyte standard)/(internal standard), in (ng/mL)/(ng/mL), for the X-axis, versus the peak area ratio (analyte peak

area)/(internal standard peak area) for the Y-axis (see Figure 1 to Figure 2).

Calibration standards (see Figure 3 to Figure 5 for examples) with 0.0075, 0.025, 0.075, 0.25, 0.5, 2.5, and 5.0 ng/mL of the analytes, all containing 0.5 ng/mL of the internal standards were prepared in water/acetonitrile (2/8, v/v, see GRM 05.12, Section 7.3.1.). The concentrations of the above calibration standards corresponded to the following residue concentrations: 0.015, 0.05, 0.15, 0.5, 1.0, 5.0, and 10 µg/L (see Section 7.3.1. of method GRM 05.12).

Concentrations of the analytes in the final extracts (resulting in ng/mL results) were determined by substituting the peak area ratios into the linear regression equation as shown below:

$Y = aX + b$

Y: Ratio: (Analyte peak area / IS peak area)

X: Ratio: (Analyte concentration $c_{End}$/ IS concentration)

The IS concentration was always 0.5 ng/mL.

Thus:

$c_{End} = ((Y - b) / a) \times$ IS concentration

$c_{End} =$ (((Analyte peak area / IS peak area) – b) / a) x IS concentration

The analyte concentration is thus obtained as residue R (in µg/L) by the following calculation:

$R = c_{End} \times (V_{Ex} \times V_{End} / V_{R1} \times W)$

$= c_{End} \times$ Multiplier M

where:

$c_{End}$: = Concentration of final extracts in ng/mL

$V_{Ex}$: = Extraction volume (20.0 mL)

$V_{End}$: = Volume of final extracts (20.1 mL)

$V_{R1}$: = Aliquot taken (20.0 mL)

W: = Specimen volume (10.0 mL)

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100\ \%$

*Example for Calculation of XDE-175-J:*

The calculation is exemplified with the water specimen PTRL-ID P865-30.

10 mL water was fortified at 0.05 µg/L (LOQ) by dosing 0.050 mL of the 10 ng/mL XDE-175 and its metabolites fortification solution.

After extraction ($V_{ex}$ = 20.0 mL), 100 µL of the internal standard solution were added to a 20.0 mL aliquot of the raw extract ($V_{End}$ = 20.1 mL).

The final extract was examined by LC/MS/MS in run file P865-021 (Figure 8), resulting in a XDE-175-J peak area of 4890 counts. The internal standard peak area was 47900 counts.

The Analyst software used the calibration function

Y = 2.76 x X - 0.022 (Figure 1, top)

which was established by injecting calibration solutions interspersed with final extracts, whereby:

Y: Ratio: (Analyte peak area / IS peak area)

X: Ratio: (Analyte concentration / IS concentration)
with IS concentration always 0.5 ng/mL.

Including the intercept b with – 0.022, the linear calibration function becomes:

$c_{End}$ = ((Y –b) / a) x IS concentration
= (((Analyte peak area / IS peak area) + 0.022) / 2.76) x 0.5 ng/mL
= (((4890 counts / 47900 counts) + 0.022) / 2.76) x 0.5 ng/mL
= 0.0225 ng/mL

The analyte concentration is thus obtained as residue R (in mg/kg) by the following calculation:

R = $c_{End}$ x ($V_{Ex}$ x $V_{End}$ / $V_{RI}$ x W)
= 0.0225 ng/mL x (20.0 mL x 20.1 mL / 20.0 mL x 10 mL)
= 0.0225 ng/mL x 2.01
= 0.045 ng/mL (µg/L)

Recoveries (Rec.) were calculated for the fortified specimens as follows:

Rec. = (R / $R_{fortified}$) x 100 %
= 0.045 µg/L / 0.050µg/L) x 100 %
= 90 %

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a fortification level of one matrix type was calculated using the "STDEV" function of the same spreadsheet

program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Confirmatory Evaluation

For confirmation of residues the final extracts were re-injected in the LC-MS/MS using the same HPLC and MS conditions but a HPLC column with a different stationary phase (Phenomenex Synergi Polar RP instead of YMC ODS-AM). The extracts were also evaluated by using internal calibration.

# Appendix A Excerpts from Dow AgroSciences LLC Method GRM 05.12

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

GRM: 05.12
EFFECTIVE: 22-June-2005
SUPERSEDES: New

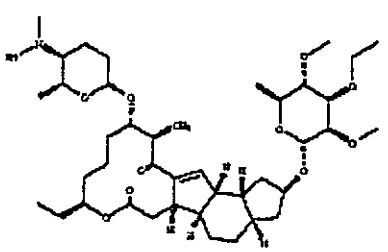
Dow AgroSciences

## Determination of Residues of XDE-175 and its Metabolites in Water by Liquid Chromatography with Tandem Mass Spectrometry

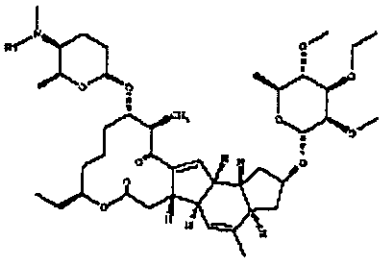
G. E. Schelle and M. J. Hastings

### 1. SCOPE

This method is applicable for the quantitative determination of XDE-175-J and XDE-175-L and their metabolites XDE-175-*N*-demethyl-J and XDE-175-*N*-demethyl-L in water (drinking water, ground water and surface water). The method was validated over the concentration range of 0.05-50.0 µg/L. The validated limit of quantitation was 0.05 µg/L.



XDE-175-J, R1 = $CH_3$
XDE-175-*N*-Demethyl-J, R1 = H



XDE-175-L, R1 = $CH_3$
XDE-175-*N*-Demethyl-L, R1 = H

Common and chemical names along with other identifying information are given in Table 1.

### 2. PRINCIPLE

Residues of XDE-175 and its metabolites in water samples are analyzed by liquid chromatography with positive ion electrospray ionization (ESI) tandem mass spectrometry (LC/MS/MS) after the addition of acetonitrile and a mixed stable isotope internal standard solution.

3. SAFETY PRECAUTIONS

3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

3.2. Acetonitrile and methanol are flammable and volatile and should be used in well-ventilated areas away from ignition sources.

3.3. Formic acid is corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be used when handling all chemicals.

4. EQUIPMENT (Note 12.1.)

4.1. Laboratory Equipment

4.1.1. Balance, analytical, Model AE100, Mettler-Toledo, Inc., Hightstown, NJ 08520.

4.1.2. Dispenser, Bottle-Top, adjustable, Brinkmann, 20-100 mL, catalog number 13-688-136, Fisher Scientific, Pittsburgh, PA 15219.

4.1.3. Pipettor, adjustable, Gilson Microman M100, 10-100 µL, catalog number F148504, Gilson Inc., Middleton, WI 53562.

4.1.4. Pipettor, adjustable, Gilson Microman M250, 50-250 µL, catalog number F148505, Gilson Inc.

4.1.5. Pipettor, adjustable, Gilson Microman M1000, 100-1000 µL, catalog number F148506, Gilson Inc.

4.1.6. Vortex mixer, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716.

4.2. Chromatographic System

4.2.1. Column, analytical, YMC ODS-AM, 50 x 4.6 mm, 5-µm, catalog number AM12S05-0546WT, Waters, Milford, MA 01757.

4.2.2. Column, confirmatory, Synergi Polar RP, 75 x 4.6 mm, 4-µm, catalog number 00C-4336-E0, Phenomenex, Torrance, CA 90501.

4.2.3 Liquid chromatograph, Symbiosis Pharma, Spark Holland Inc., Plainsboro, NJ 08536.

4.2.4. Mass spectrometer, Model API 4000, MDS/Sciex, Foster City, CA 94404.

4.2.5. Mass spectrometer data system, Analyst 1.4, MDS/Sciex.

5. GLASSWARE AND MATERIALS (Note 12.1.)

5.1. Bottle, 1.0-L, media bottle, catalog number 06-423-3D, Fisher Scientific, Fisher Scientific, Pittsburgh, PA 15275.

5.2. Bottle, 2.0-L, media bottle, catalog number 06-423-3E, Fisher Scientific.

5.3. Collection plate, 96-well, 2-mL, catalog number 121-5203, Argonaut Technologies, Inc., Redwood City, CA 94063.

5.4. Collection plate sealing cap, catalog number 121-5205, Argonaut Technologies, Inc.

5.5. Cylinder, graduated, 100-mL, catalog number C7000-100, National Scientific Company, Lawrenceville, GA 30243.

5.6. Cylinder, graduated, 500-mL, catalog number C7000-500, National Scientific Company.

5.7. Cylinder, graduated, 1000-mL, catalog number C7000-1L, National Scientific Company.

5.8. Cylinder, graduated, 2000-mL, catalog number C7000-2L, National Scientific Company.

5.9. Flask, volumetric, 100-mL, catalog number 161-8987, National Scientific Company.

5.10. Pipet, polyethylene disposable transfer, 3-mL, catalog number, 13-711-7, Fisher Scientific.

5.11. Pipet, volumetric, 0.5-mL, catalog number 261-6010, National Scientific Company.

5.12. Pipet, volumetric, 1.0-mL, catalog number 261-6011, National Scientific Company.

5.13. Pipet, volumetric, 2.0-mL, catalog number 261-6012, National Scientific Company.

5.14. Pipet, volumetric, 3.0-mL, catalog number 261-6013, National Scientific Company.

5.15. Pipet, volumetric, 5.0-mL, catalog number 261-6015, National Scientific Company.

5.16. Pipet, volumetric, 10.0-mL, catalog number 261-6020, National Scientific Company.

5.17. Pipetter tips, Gilson Microman CP100, catalog number F148414, Gilson Inc.

5.18. Pipetter tips, Gilson Microman CP250, catalog number F148114, Gilson Inc.

5.19. Pipetter tips, Gilson Microman CP1000, catalog number F148560, Gilson Inc.

5.20. Vial, 40-mL, with PTFE-lined screw cap, catalog number B7800-6, National Scientific Company.

6. REAGENTS, STANDARDS, AND PREPARED SOLUTIONS (Note 12.1.)

6.1. Reagents

6.1.1. Acetonitrile, ChromAR HPLC grade, catalog number 2856, Mallinckrodt-Baker, Inc., Paris, KY 40361.

6.1.2. Ammonium acetate, HPLC grade, catalog number A639-500, Fisher Scientific.

6.1.3. Formic acid, 96%, ACS grade, catalog number 251364, Sigma-Aldrich, Milwaukee, WI 53201.

6.1.4. Methanol, ChromAR HPLC grade, catalog number 3041, Mallinckrodt-Baker Inc.

6.1.5. Nitrogen, refrigerated liquid, BOC Group Inc., Murray Hill, NJ 07974.

6.1.6. Water, HPLC grade, catalog number WX0004-1, EM Science, Gibbstown, NJ 08027.

6.2. Standards

6.2.1. Analytical standard information for XDE-175-J, XDE-175-L, XDE-175-*N*-demethyl-J, and XDE-175-*N*-demethyl-L are listed in Table 1.

Compounds can be obtained from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

6.2.2 Stable isotope labeled internal standards information for XDE-175-J, XDE-175-L, XDE-175-*N*-demethyl-J, XDE-175-*N*-demethyl-L are listed in Table 1.

Obtain from Specialty Synthesis Group, Dow AgroSciences LLC, 9330 Zionsville Road, Building 306, Indianapolis, IN 46268-1054. Dow AgroSciences will provide the stable isotope labeled internal standard free of charge.

6.3. Prepared Solutions

6.3.1. acetonitrile/methanol (1:1) containing 2 mM ammonium acetate

Weigh 0.15 g of ammonium acetate into a 40-mL vial and quantitatively transfer with 30 mL of methanol into a 1-L bottle. Rinse the vial with two additional 30 mL aliquots of methanol into the 1-L bottle. Add a further 410 mL of methanol to the bottle. Measure 500 mL of acetonitrile using a 500-mL graduated cylinder and then transfer to the 1.0-L bottle. Cap the bottle and mix. Allow the solution to equilibrate to room temperature before use.

6.3.2. acetonitrile/water (80:20)

Measure 800 mL of acetonitrile using a 1-L graduated cylinder and then transfer into a 1.0-L bottle. Measure 200 mL of water using a 500-mL graduated cylinder and then transfer into the 1.0-L bottle. Cap the bottle and mix. Allow the solution to equilibrate to room temperature before use.

6.3.3. acetonitrile/water (30:20) containing 0.1% formic acid

Measure 800 mL of acetonitrile using a 1-L graduated cylinder and then transfer into a 1.0-L bottle. Measure 200 mL of water using a 500-mL graduated cylinder and then transfer into the 1.0-L bottle. Pipet 1.0 mL of formic acid into the bottle. Cap the bottle and mix. Allow the solution to equilibrate to room temperature before use.

6.3.4. water containing 2 mM ammonium acetate

Weigh 0.15 g of ammonium acetate into a 40-mL vial and quantitatively transfer with 30 mL of HPLC water into a 1-L bottle. Rinse the vial with two additional 30 mL aliquots of HPLC water into the 1-L bottle. Add a further 910 mL of HPLC water to the bottle. Cap the bottle and mix. Allow the solution to equilibrate to room temperature before use.

7. PREPARATION OF STANDARD SOLUTIONS

7.1. Preparation of XDE-175 and Metabolite Spiking Solutions

7.1.1. Weigh 0.0100 g of each XDE-175 analytical standard (XDE-175-J, XDE-175-L, XDE-175-*N*-demethyl-J, XDE-175-*N*-demethyl-L) and quantitatively transfer each standard to separate 100-mL volumetric flasks with acetonitrile. Dilute to volume with acetonitrile to obtain a 100-µg/mL stock solution of each analyte.

7.1.2. Pipet 10.0 mL of each 100-µg/mL solution (Section 7.1.1.) into the same 100-mL volumetric flask. Dilute to volume with acetonitrile to obtain a 10.0-µg/mL mixed XDE-175 and metabolite spiking solution. Further dilute the 10.0-µg/mL mixed XDE-175 and metabolite spiking solution with acetonitrile according to the following

suggested scheme:

| Concentration of Initial Stock Solution | Aliquot of Stock Solution | Final Soln. Volume | Spiking Soln. Final Conc. | Equivalent Sample Conc.[a] | Volume of Spiking Soln. |
|---|---|---|---|---|---|
| µg/mL | mL | mL | µg/mL | µg/L | µL |
| 10.0 | 10.0 | 100 | 1.0 | 50.0 | 500 |
| 1.0 | 10.0 | 100 | 0.1 | 5.0 | 500 |
| 0.1 | 10.0 | 100 | 0.01 | 0.05 | 50 |
| -- | -- | -- | 0.01 | 0.015 | 15 |

[a] The equivalent sample concentration is based on fortifying a 10-mL water sample.

7.2. Preparation of XDE-175 and Metabolite Stable Isotope Internal Standard Solutions

7.2.1. Weigh 0.0100 g of each XDE-175 stable isotope standard (XDE-175-J IS, XDE-175-L-IS, XDE-175-*N*-demethyl-J IS and XDE-175-*N*-demethyl-L IS) and quantitatively transfer each standard to separate 100-mL volumetric flasks with acetonitrile. Dilute to volume with acetonitrile to obtain a 100-µg/mL stock solution of stable isotope standard.

7.2.2. Pipet 10.0 mL of each 100-µg/mL solution (Section 7.2.1.) into a 100-mL volumetric flask. Dilute to volume with acetonitrile to obtain a 10.0-µg/mL mixed XDE-175 and metabolite stable isotope internal standard solution.

7.2.3. Pipet 1.0 mL of the 10.0-µg/mL mixed XDE-175 stable isotope internal standard solution (Section 7.2.2.) into a 100-mL volumetric flask. Dilute to volume with acetonitrile/water (80:20) to obtain a 0.1-µg/mL mixed XDE-175 and metabolite stable isotope internal standard solution.

7.2.4. Pipet 0.5 mL of the 0.1-µg/mL mixed XDE-175 stable isotope internal standard solution (Section 7.2.3.) into a 100-mL volumetric flask. Dilute to volume with acetonitrile/water solution (80:20) to obtain a 0.5-ng/mL mixed XDE-175 and metabolite stable isotope internal standard solution.

7.3. Preparation of Mixed XDE-175 and Metabolite Calibration Solutions

7.3.1. Prepare calibration standard solutions by pipeting 0.5 mL of the 0.1-µg/mL mixed XDE-175 and metabolites stable isotope solution, prepared in Section 7.2.3, into each volumetric flask and diluting the 1.0, 0.1 and 0.01-µg/mL mixed XDE-175 spiking solutions (Section 7.1.2.) with acetonitrile/water (80:20) to give calibration standards over the range 0.0075–10 ng/mL. Calibration standards may be prepared following the suggested scheme:

| Concentration of Stock Solution | Aliquot of Spiking Solution | Final Soln. Volume | Calibration Soln. Final Conc. | Equivalent Sample Conc.[a] |
|---|---|---|---|---|
| µg/mL | mL | mL | ng/mL | µg/L |
| 1.0 | 1.0 | 100 | 10.0 | 20.0 |
| 1.0 | 0.5 | 100 | 5.0 | 10.0 |
| 0.1 | 2.5 | 100 | 2.5 | 5.0 |
| 0.1 | 0.5 | 100 | 0.5 | 1.0 |
| 0.01 | 2.5 | 100 | 0.25 | 0.5 |
| 0.01 | 0.75 | 100 | 0.075 | 0.15 |
| 0.01 | 0.25 | 100 | 0.025 | 0.05 |
| 0.01 | 0.075 | 100 | 0.0075 | 0.015 |

[a] The equivalent sample concentration is based on extracting a 10-mL water sample.

7.4. Preparation of Mixed XDE-175 and Metabolites and Mixed XDE-175 and Metabolites Stable Isotope Crossover Standard Solutions

7.4.1. Prepare the mixed XDE-175 and metabolite crossover standard solution by pipeting 0.1 mL of the 0.1-µg/mL mixed XDE-175 and metabolites solution, prepared in Section 7.1.2, into a 20-mL volumetric flask and diluting with acetonitrile/water (80:20) to give a crossover standard of 0.5 ng/mL.

7.4.2. Prepare the mixed XDE-175 and metabolite stable isotope crossover standard solution by pipeting 0.1 mL of the 0.1-µg/mL mixed XDE-175 and metabolites stable isotope solution, prepared in Section 7.2.3, into a 20-mL volumetric flask and diluting with acetonitrile/water (80:20) to give a stable isotope crossover standard of 0.5 ng/mL.

8. LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

8.1. Typical Liquid Chromatography Operating Conditions (Note 12.2.)

| | |
|---|---|
| Instrumentation: | Spark Holland Symbiosis Pharma<br>MDS/Sciex API 4000 LC/MS/MS System<br>MDS/Sciex Analyst 1.4 data system |
| Column: | YMC ODS-AM, 50 x 4.6 mm, 5-µm (Quantitation)<br>Synergi Polar RP, 75 x 4.6 mm, 4-µm (Confirmation) |
| Column Temperature: | Ambient |
| Injection Volume: | 100 µL |
| Autosampler Wash Program: | Autosampler loop and needle washed with:<br>1) 500 µL of acetonitrile/water (80:20) containing 0.1% formic acid<br>2) 2 x 500 µL of acetonitrile/water (80:20) containing 0.1% formic acid with valve wash |

| | |
|---|---|
| | 3) 2 x 500 µL of methanol with valve wash<br>4) 500 µL of acetonitrile/water (80:20) containing 0.1% formic acid |
| Run Time: | Approximately 7 mins |
| Mobile Phase: | A –acetonitrile/methanol (1:1) containing 2 mM ammonium acetate<br>B –water containing 2 mM ammonium acetate |
| Flow: | 1.0 mL/min (approx 200 µL/min split to source) |

Gradient:

| Time, (min:secs) | A, % | B, % |
|---|---|---|
| 00:01 | 70 | 30 |
| 03:00 | 100 | 0 |
| 05:30 | 100 | 0 |
| 05:45 | 70 | 30 |
| 07:00 | 70 | 30 |

Flow Diverter Program: 1) 0.0→3.0 min: flow to waste
2) 3.0→6.0 min: flow to source
3) 6.0→end of run: flow to waste

8.2. Typical Mass Spectrometry Operating Conditions (Note 12.2.)

| | |
|---|---|
| Ionization Mode: | ESI |
| Polarity: | Positive |
| Scan Type: | MRM |
| Resolution: | Q1 – unit, Q3 – unit |
| Curtain Gas (CUR): | 12 psi |
| Collision Gas (CAD): | 4 psi |
| Temperature (TEM): | 425 °C |
| Ion Source Gas 1 (GS1): | 40 psi |
| Ion Source Gas 2 (GS2): | 60 psi |
| Period 1 | |
| Acquisition Time Delay: | 3.0 mins |
| Period Duration: | 3.0 mins |
| Ion Spray Voltage (IS): | 5500 V |

| Compound: | Ion, *m/z* | | Time, ms | Collision Energy, V |
|---|---|---|---|---|
| | Q1 | Q3 | | |
| XDE-175-J | 748.6 | 142.2 | 50 | 37 |
| XDE-175-L | 760.9 | 142.2 | 50 | 37 |
| XDE-175-*N*-demethyl-J | 734.9 | 128.2 | 50 | 31 |
| XDE-175-*N*-demethyl-L | 746.7 | 128.2 | 50 | 33 |
| XDE-175-J IS | 757.9 | 146.2 | 50 | 37 |
| XDE-175-L IS | 769.9 | 146.2 | 50 | 37 |

| | | | | |
|---|---|---|---|---|
| XDE-175-N-Demethyl-J IS | 739.9 | 128.2 | 50 | 33 |
| XDE-175-N-Demethyl-L IS | 751.7 | 128.2 | 50 | 33 |

8.3. Typical Mass Spectra

Typical mass spectra and product ion spectra of XDE-175, its metabolites and stable isotope internal standards are presented in Figures 1-16.

8.4. Typical Calibration Curve

Typical calibration curves for the determination of XDE-175 and its metabolites in water are shown in Figures 17-20.

8.5. Typical Chromatograms

Typical chromatograms of a 0.025-ng/mL calibration standard, a control surface water sample, a control surface water sample fortified at 0.05 µg/L (limit of quantitation), and a control surface water sample fortified at 50 µg/L (1000 times the limit of quantitation) are presented in Figures 21-24. Typical chromatograms generated using the confirmatory HPLC column are presented in Figures 25-28.

9. DETERMINATION OF RECOVERY OF XDE-175 AND ITS METABOLITES IN WATER

9.1. Method Validation Prior to Field Sample Analysis

Unless otherwise specified, a sample set should contain, at the minimum, the following samples:

At least one reagent blank
At least one control
At least one control fortified at the limit of detection
At least two controls fortified at the limit of quantitation
At least two controls fortified at a higher concentration

9.2. Sample Preparation

No preparation is required.

Note: Due to its low water solubility, XDE-175 is readily adsorbed from water samples onto glass or plastic containers. Consequently, field water samples must be collected in the glass sample vial that will be used for sample extraction, and the entire sample should be extracted as described in Section 9.3. The exact sample volume or mass will need to be determined at sampling or by difference following analysis. The samples should be stored in a freezer in the dark prior to analysis.

9.3. Sample Analysis for XDE-175 and Metabolites in Water

9.3.1. For control and recovery samples, measure 10-mL portions of sample into 40-mL glass vials. Remove the field samples from the freezer and allow to thaw in the dark.

9.3.2. Add the required volume of the appropriate fortification solution to the recovery samples (Section 7.1.2.).

9.3.3. Add 10 mL of acetonitrile and 100 µL of the 0.1-µg/mL mixed XDE-175 and metabolite stable isotope standard (Section 7.2.3.) to each sample.

9.3.4. Cap and vortex mix for approximately 10 seconds.

9.3.5. Transfer an aliquot of the sample to a 96-deep well plate.

9.3.6. Add approximately 1 mL of each calibration standard (Section 7.3.1.) to empty wells of the 96-well plate and cap.

9.3.7. Chromatograph the samples and standard using the conditions given in Section 8, injecting the calibration standards evenly spaced throughout the run.

9.3.8. For sample extracts which contain XDE-175 and metabolite concentrations > 10 ng/mL (equivalent to >20 µg/L), dilute with acetonitrile:water (80:20) containing 0.5 ng/mL mixed XDE-175 and metabolite stable isotope standard (Section 7.2.4.). Determine the suitability of the chromatographic system using the following criteria:

a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

b. Peak resolution: Determine visually that sufficient resolution has been achieved for the analyte relative to any background interferences.

c. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 21-24 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for the 0.025-ng/mL calibration standard (equivalent to 0.05 µg/L of XDE-175 and or metabolites in the water sample).

## 10. CALCULATIONS

### 10.1. Determination of Isotopic Crossover

In this assay, the analyte and internal standard are quantitated using MS/MS transitions characteristic of each compound. When using stable-isotope labeled internal standards, there is a possibility that isotopic contributions will occur between the transitions used for quantitation of the unlabeled and labeled compounds. This isotopic overlap between the analyte and the internal standard can be determined empirically by

analyzing standard solutions of each compound and should be addressed for accurate determination of concentrations.

10.1.1. To determine the isotopic crossover for XDE-175 and its metabolites and their respective stable isotopes, inject a 0.5-ng/mL mixed XDE-175 and metabolite standard and a 0.5-ng/mL mixed XDE-175 stable isotope standard and determine the peak areas for the analyte and internal standard as indicated below. For example, to determine the contribution of the unlabeled XDE-175-J to the stable isotope labeled XDE-175-J internal standard:

| | |
|---|---|
| XDE-175-J | *m/z* Q1/Q3 748.6/142.2 |
| XDE-175-J IS | *m/z* Q1/Q3 757.9/146.2 |

To determine the contribution of the unlabeled XDE-175-J to the labeled XDE-175-J internal standard:

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area of internal standard transition}}{\text{peak area of analyte transition}}$$

$$\text{Crossover Factor (analyte}\rightarrow\text{ISTD)} = \frac{\text{peak area at } m/z \text{ 757.9/146.2}}{\text{peak area at } m/z \text{ 748.6/142.2}}$$

In a similar manner, to determine the contribution of the labeled XDE-175-J stable isotope to the unlabeled XDE-175-J:

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{\text{peak area of analyte transition}}{\text{peak area of internal standard transition}}$$

$$\text{Crossover Factor (ISTD}\rightarrow\text{analyte)} = \frac{\text{peak area at } m/z \text{ 748.6/142.2}}{\text{peak area at } m/z \text{ 757.9/146.2}}$$

During method development, no significant mass spectral isotopic crossover was observed and therefore no correction of the measured quantitation ratio was performed. If isotopic crossover is encountered it should be assessed and the respective quantitation ratios corrected for accurate determination of concentrations (13.1, 13.2).

10.2. Calculation of Standard Calibration Curve for XDE-175 and its Metabolites

10.2.1. Inject a series of calibration standards (Section 7.3.) using the conditions described in Section 8 and determine the peak areas for XDE-175, its metabolites and internal standards as indicated below:

| | |
|---|---|
| XDE-175-J | *m/z* Q1/Q3 748.6/142.2 |
| XDE-175-L | *m/z* Q1/Q3 760.9/142.2 |

| | |
|---|---|
| XDE-175-*N*-demethyl-J | *m/z* Q1/Q3 734.9/128.2 |
| XDE-175-*N*-demethyl-L | *m/z* Q1/Q3 746.7/128.2 |
| XDE-175-J IS | *m/z* Q1/Q3 757.9/146.2 |
| XDE-175-L IS | *m/z* Q1/Q3 769.9/146.2 |
| XDE-175-*N*-demethyl-J IS | *m/z* Q1/Q3 739.9/128.2 |
| XDE-175-*N*-demethyl-L IS | *m/z* Q1/Q3 751.7/128.2 |

10.2.2. For each standard, calculate the XDE-175 quantitation ratio.

For example, using the data for XDE-175-J from injection no. 13, Figure 17:

$$\text{Quantitation Ratio} = \frac{\text{peak area of quantitation ion}}{\text{peak area of internal standard ion}}$$

$$\text{Quantitation Ratio} = \frac{\text{XDE-175-J peak area}}{\text{XDE-175-J IS stable isotope internal standard peak area}}$$

$$\text{Quantitation Ratio} = \frac{9456}{166322}$$

$$\text{Quantitation Ratio} = 0.057$$

10.2.3. Prepare a standard curve by plotting the concentration of the analytes on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis), as shown in Figures 17-20. Using linear regression analysis (13.3.) with a 1/x weighting (13.4.), determine the equation for the curve with respect to the abscissa.

For example, using the XDE-175-J data from Figure 17:

$$X = \left(\frac{Y - \text{intercept}}{\text{slope}}\right)$$

$$\text{XDE-175-J conc. (ng/mL)} = \left(\frac{\text{XDE-175-J quantitation ratio} - \text{intercept}}{\text{slope}}\right)$$

$$\text{XDE-175-J conc. (ng/mL)} = \left(\frac{\text{XDE-175-J quantitation ratio} - (0.0004)}{2.2948}\right)$$

10.3. Calculation of Percent Recovery for XDE-175 and its Metabolites

10.3.1. Determine the gross concentration in each recovery sample by substituting the quantitation ratio obtained into the above equation and solving for the concentration.

For example, using the data for XDE-175-J data from injection no. 8, Figure 17:

$$\text{XDE-175-J conc. (gross ng/mL)} = \left(\frac{\text{XDE-175-J quantitation ratio} - (0.0004)}{2.2948}\right)$$

$$\text{XDE-175-J conc. (gross ng/mL)} = \left(\frac{0.052 - (0.0004)}{2.2948}\right)$$

$$\text{XDE-175-J conc. (gross)} = 0.022 \text{ ng/mL}$$

Convert the concentration of ng/mL of XDE-175-J found in the final sample extract prepared for analysis to µg/L of XDE-175-J in the water sample as follows:

$$\text{XDE-175-J conc. (gross µg/L)} = 0.022 \text{ ng/mL} \times \frac{20 \text{ mL}}{10 \text{ mL}}$$

$$\text{XDE-175-J conc. (gross)} = 0.045 \text{ ng/mL or } 0.045 \text{ µg/L}$$

10.3.2. Determine the net concentration in each recovery sample by subtracting the concentration found at the retention time of each analyte in the untreated control sample from that of the gross analyte concentration in the recovery sample.

For example, using the data for XDE-175-J from Figure 17:

$$\text{XDE-175-J conc. (net µg/L)} = \text{XDE-175-J conc. (gross µg/L)} - \text{XDE-175-J conc. (control µg/L)}$$

$$\text{XDE-175-J conc. (net µg/L)} = 0.045 \text{ µg/L} - 0.0000 \text{ µg/L}$$

$$\text{XDE-175-J conc. (net)} = 0.045 \text{ µg/L}$$

10.3.3. Determine the percent recovery by dividing the net concentration of each recovery sample by the theoretical concentration added.

$$\text{Recovery} = \frac{\text{conc. found}}{\text{conc. added}} \times 100\%$$

$$\text{Recovery} = \frac{0.045\ \text{ng/mL}}{0.05\ \text{ng/mL}} \times 100\%$$

$$\text{Recovery} = 89\%$$

10.4. Determination of XDE-175 and its Metabolites in Water

10.4.1. Determine the gross concentration of XDE-175 and its metabolites in each sample by substituting the respective quantitation ratio into the equation for the calibration curve and calculating the uncorrected residue result as described in Section 10.3.1.

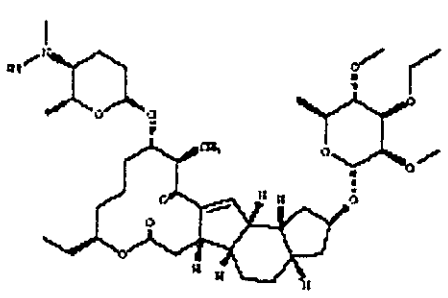
10.4.2. For those samples that require correction for the method procedural recovery, use the average recovery of all the recovery samples at or above the limit of quantitation, as described in Section 9.1, from a given sample set to correct for method efficiency. For example, continuing with the data from Figure 17 and the average recovery from Table 2 for the samples analyzed on 09-May-2005:

$$\text{XDE-175-J conc. (corrected µg/L)} = \text{XDE-175-J conc. (gross µg/L)} \times \left(\frac{100}{\text{Average\% Recovery}}\right)$$

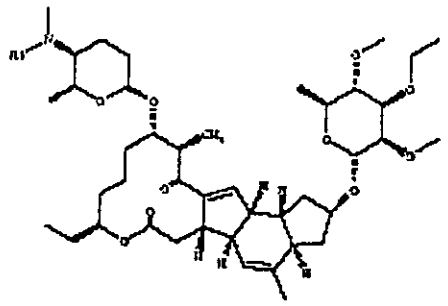
$$\text{XDE-175-J conc. (corrected µg/L)} = 0.045\ \mu g/g \times \frac{100}{94}$$

$$\text{XDE-175-J conc. (corrected)} = 0.048\ \mu g/L$$

Table 1. Identity and Structure of XDE-175, its Metabolites and Stable Isotope Internal Standards



XDE-175-J, R1 = $CH_3$
XDE-175-N-Demethyl-J, R1 = H



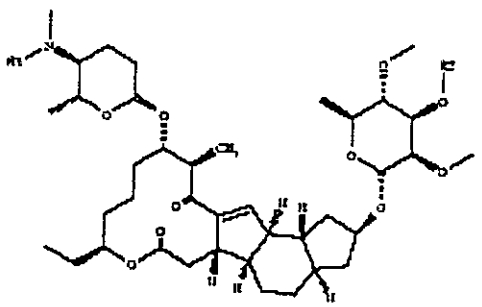
XDE-175-L, R1 = $CH_3$
XDE-175-N-Demethyl-L, R1 = H

| Common Name of Compound |
|---|
| XDE-175-J<br><br>Molecular Formula: $C_{42}H_{69}NO_{10}$<br>Formula Weight: 748.011<br>Nominal Mass: 747.5<br>CAS Registry Number: 187166-40-1<br>CAS Name: 1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione, 2-[(6-deoxy-3-O-ethyl-2,4-di-O methyl-a-L-mannopyranosyl)oxy]-13-[[(2R,5S,6R)-5-(dimethylamino)tetrahydro-6-methyl 2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,4,5,5a,5b,6,9,10,11,12,13,14,16a,16b-hexadecahydro 14-methyl-, (2R,3aR,5aR,5bS,9S,13S,14R,16aS,16bR) |
| XDE-175-L<br><br>Molecular Formula: $C_{43}H_{71}NO_{10}$<br>Formula Weight: 760.022<br>Nominal Mass: 759.5<br>CAS Registry Number: 187166-15-0<br>CAS Name: 1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione, 2-[(6-deoxy-3-O-ethyl-2,4-di-O-methyl-a-L-mannopyranosyl)oxy]-13-[[(2R,5S,6R)-5-(dimethylamino)tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-4,14-dimethyl-, (2S,3aR,5aS,5bS,9S,13S,14R,16aS,16bS) |

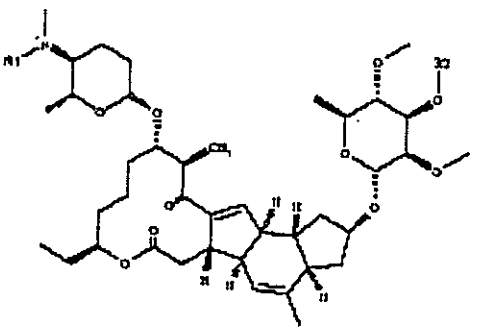
Table 1. (Cont.) Identity and Structure of XDE-175, its Metabolites and Stable Isotope Internal Standards

| |
|---|
| XDE-175-*N*-demethyl-J<br><br>Molecular Formula: $C_{41}H_{67}NO_{10}$<br>Formula Weight: 733.984<br>Nominal Mass: 733.5<br>CAS Registry Number: N/A<br>IUPAC Name: (2R,3aR,5aR,5bS,9S,13S,14R,16aS,16bR)-9-ethyl-14-methyl-13-{[(2S,5S,6R)-6-methyl-5-(methylamino)tetrahydro-2H-pyran-2-yl]oxy}-7,15-dioxo-2,3,3a,4,5,5a,5b,6,7,9,10,11,12,13,14,15,16a,16b-octadecahydro-1H-as-indaceno[3,2-d]oxacyclododecin-2-yl 6-deoxy-3-O-ethyl-2,4-di-O-methyl-beta-L-mannopyranoside |
| XDE-175-*N*-demethyl-L<br><br>Molecular Formula: $C_{42}H_{69}NO_{10}$<br>Formula Weight: 745.995<br>Nominal Mass: 745.5<br>CAS Registry Number: N/A<br>IUPAC Name: (2S,3aR,5aS,5bS,9S,13S,14R,16aS,16bS)-9-ethyl-4,14-dimethyl-13-{[(2S,5S,6R)-6-methyl-5-(methylamino)tetrahydro-2H-pyran-2-yl]oxy}-7,15-dioxo-2,3,3a,5a,5b,6,7,9,10,11,12,13,14,15,16a,16b-hexadecahydro-1H-as-indaceno[3,2-d]oxacyclododecin-2-yl 6-deoxy-3-O-ethyl-2,4-di-O-methyl-beta-L-mannopyranoside |

Table 1. (Cont.) Identity and Structure of XDE-175, its Metabolites and Stable Isotope Internal Standards



XDE-175-J, R1 = $^{13}CD_3$, R2 = $C_2D_5$
XDE-175-*N*-Demethyl-J, R1 = H, R2 = $C_2D_5$



XDE-175-L, R1 = $^{13}CD_3$, R2 = $C_2D_5$
XDE-175-*N*-Demethyl-L, R1 = H, R2 = $C_2D_5$

| Common Name of Internal Standard | |
|---|---|
| XDE-175-J IS | |
| Molecular Formula: | $C_{41}{}^{13}CH_{51}D_8NO_{10}$ |
| Formula Weight: | 757.051 |
| Nominal Mass: | 756.5 |
| CAS Registry Number: | N/A |
| XDE-175-L IS | |
| Molecular Formula: | $C_{42}{}^{13}CH_{43}D_8NO_{10}$ |
| Formula Weight: | 769.062 |
| Nominal Mass: | 768.5 |
| CAS Registry Number: | N/A |
| XDE-175-N-Demethyl-J IS | |
| Molecular Formula: | $C_{41}H_{42}D_5NO_{10}$ |
| Formula Weight: | 739.014 |
| Nominal Mass: | 738.5 |
| CAS Registry Number: | N/A |
| XDE-175-N-Demethyl-L | |
| Molecular Formula: | $C_{42}H_{42}D_5NO_{10}$ |
| Formula Weight: | 751.025 |
| Nominal Mass: | 750.5 |
| CAS Registry Number: | N/A |