Everolimus-d_4: An Internal Standard for Quantitation of Everolimus and Related Immunosuppressants by LCMS

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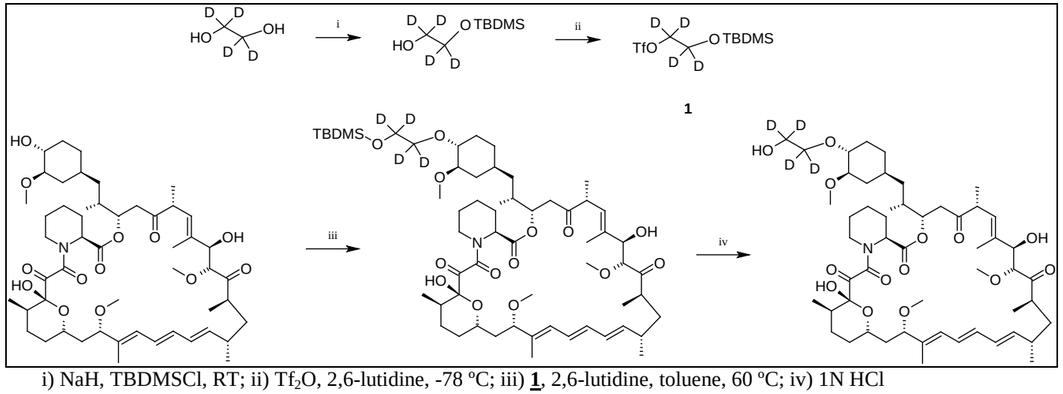
Everloimus-d_4 was synthesized and certified for use as an internal standard in LCMS applications. Use of the standard in LCMS applications was demonstrated by quantitation of an everolimus control sample against a calibration curve.

Synthesis

The synthesis was developed from rapamycin by analogy to published procedures for native Everolimus.[1]



i) NaH, TBDMSCl, RT; ii) Tf_2O, 2,6-lutidine, -78 ºC; iii) **1**, 2,6-lutidine, toluene, 60 ºC; iv) 1N HCl

Ethylene-d_4 glycol was monosilylated with t-butyldimethylsilylchloride in the presence of base and converted to the trfilate (1) with triflic anhydride/2,6-lutidine.[1a] Rapamycin was alkylated with 1 in 2,6-lutidine in toluene to give TBDMS protected everoliums-d_4 followed by deprotection with 1N HCl to form the product everolimus-d_4.[1b,c]

Certification of Everolimus-d_4

Everolimus-d_4 was certified for use as a reference material by testing for chromatographic purity, isotopic purity and residual impurities (Table 1). The product is suitable for use as internal standard for analysis of everolimus and other immunosuppressants by mass spectrometry.

Use of Everolimus-d_4 in LCMS Applications

Everolimus-d_4 was used as internal standard to quantitate the concentration of an independently prepared control sample of native everolimus to an everolimus calibration curve. Both native and labeled everolimus were screened on triple quadruple and QTOF instruments. MSMS of Everolimus- d_4 was performed by infusing to Agilent 6410 QQQ (FR=135V, CE= 65V). The transitions observed for everolimus- d_4 were 984.6 →393.3, 409.3 and 655.4; corresponding to native everolimus 980.6→389.3, 409.3 and 651.4.

Quantitation was performed on a Waters Xevo-G2 QTOF in TOF MS mode. Ions monitored are M+Na^+ (m/z 980.5706 and 984.5958 for native and labeled respectively). The extraction window was ± 0.010 Da of theoretical exact mass.

Internal standard Spiking Solution: Everolimus-d_4 was prepared at 5 µg/mL in methanol.
Everolimus Control sample: Everolimus sample was prepared at 100 µg/mL in acetonitrile. Native everolimus was procured from Sigma.
Everolimus Calibration Curve: A four point calibration curve of everolimus was prepared with points from 54 to 135 µg/mL in acetonitrile.
Working solutions: Samples were prepared for analysis by adding 1000 µg internal standard solution into 50 µg of each sample and curve point and 700 µL of methanol. The working concentration was 2-3 µg/mL.

Chromatograms and method details are provided in Figures 1&2 and Tables 2&3. Spectra of triple quadruple product ion scan are provided in Figure 4.

Results

The internal standard proved suitable for use in quantitative applications. The calibration curve was linear with r^2=0.9979. Concentration of the control sample was 106.27 µg/mL with 1.54 %RSD.

Table 1: Certification of Everolimus-d_4

Analytical Test	Method	Results	
Chromatographic Purity by HPLC/PDA Analysis	SP10-0102	99.3%	
Identity by LC/MS Analysis	SP10-0107	Consistent with Structure	
Isotopic Purity by LC/MS SIM Analysis	SP10-0107	0.00% D_0 vs D_4	
		0.00% D_0	2.51% D_3
		0.03% D_1	96.70% D_4
		0.76% D_2	
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure	
Residual Solvent Analysis by GC/FID Headspace	AM1087 [1]	4.91%	
Residual Water Analysis by Karl Fischer Coulometry	USP <921>, SP10-0103	0.46%	
Purity Factor[2]		94.0%	

[1] *Validated analytical method*
[2] *Purity Factor = (100 - wt% residual solvent - wt% residual water) x Chromatographic Purity/100*

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Figure 1: Extracted Ion Chromatogram of Everolimus and Everolimus-d_4

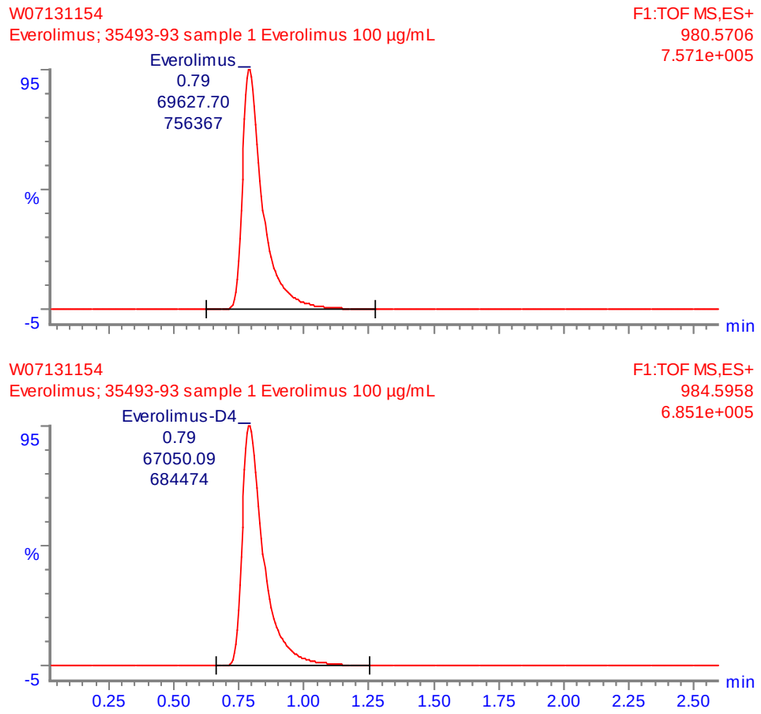


Figure 2: Calibration Curve of Native Everolimus

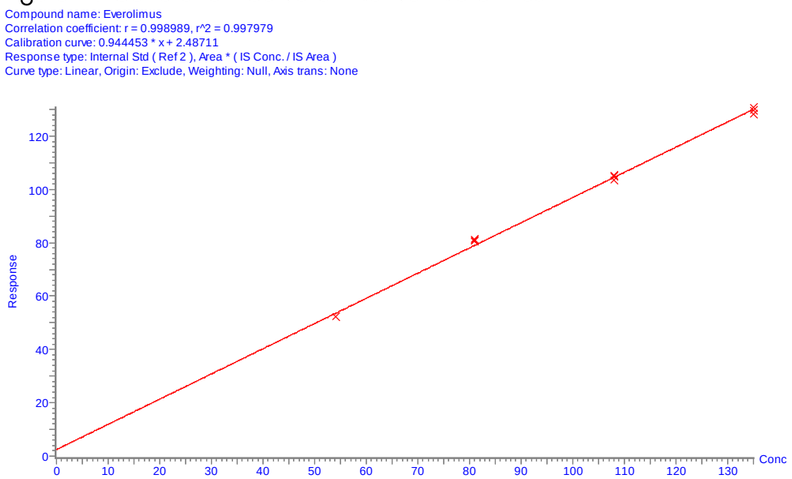


Table 2: LC Conditions

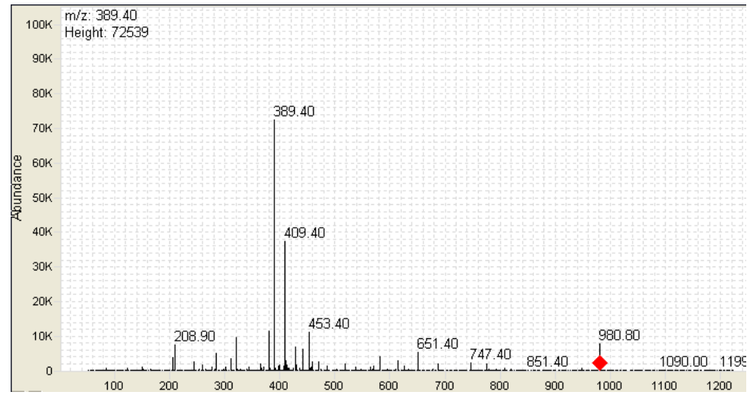
Column	Waters Xselect CSH C18 3.5uM, 2.1*10mm Guard Column			
Column Temperature	35.0 C			
Solvent A	Water with 0.1% formic acid			
Solvent B	Methanol with 0.1% formic acid			
Flow Rate	0.400 mL/min			
Injection Volume	5 µL with needle wash			
Gradient	Time(min)	%A	%B	Curve
	Initial	30	70	
	0.2	30	70	6
	0.6	0.1	99.9	6
	1.0	0.1	99.9	6
	1.2	30	70	6
	5	30	70	6

Table 3: MS Detection Conditions

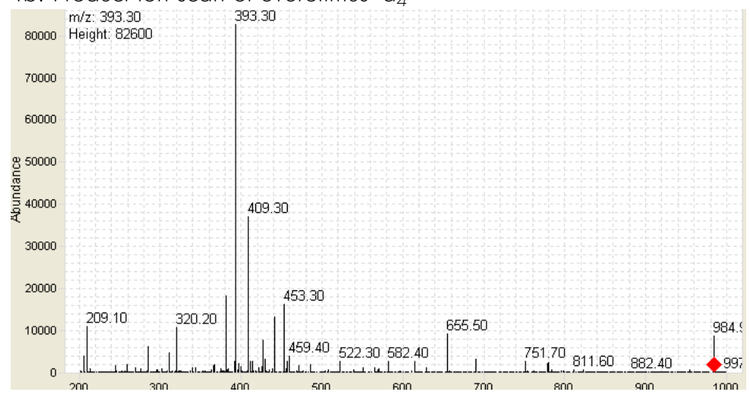
Acquisition mass range:	400 Da to 1500 Da.
Calibration mass range:	430.995 Da to 1450.119 Da
Analyser	Sensitivity Mode
Ion Source	ES+
Capillary	3.5 kV
Sampling Cone	90 V
Extraction Cone	4 V
Source Temperature	130°C
Desolvation Temperature	450°C
Cone Gas Flow	10.0 L/Hr
Desolvation Gas Flow	1200.0 L/Hr
Collision Energy	6 V
Lock Mass	556.277100 (LeuEnk Positive MS)
Scan Time	0.300 sec
Interscan Time	0.014 sec
Data Format	Centroid
Scans to Average	3.0

Figure 4: MSMS of Everolimus- d_4

4a: Product ion scan of everolimus



4b: Product ion scan of everolimus- d_4



Instrument: Agilent 6410 Triplequad.

References

1. a) J. Org. Chem. 1986, 51, 3390-3391; b) US5665772; c)US2010/0094408.