Impurity Analysis of Selective High Affinity Ligands: Comparison of Bench-Scale vs. Production Syntheses by Label Free Differential Analysis

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Overview

Purpose: Impurity analysis using LC-MS and differential analysis software to determine impurities between two synthetic pathways for a drug with potential in treating Non-Hodgkin's Lymphoma.

Methods: A bench-scale synthesis of the drug is compared to the first batch of a pilot production synthesis. LC-MS (positive mode) using an Open Accela Autosampler coupled to a Thermo Scientific LTQ Orbitrap XL mass spectrometer followed by SIEVE™ 2.0 software analysis. Thermo Scientific Mass Frontier 7.0 software used for determination of fragments and fragmentation pathways.

Results: High resolution spectra and ion trap MS^n data was used to elucidate an abundant impurity present in both products as a chromium chelate to the DOTA structure. Differential analysis software identified a number of differences between the two samples. The extracted ion chromatograms were reconstructed from the HRAM data and elucidation of impurities was performed by ion trap MS^n and Mass Frontier™ software.

Introduction

Products from two syntheses (bench- vs. pilot production-scale) for a novel therapeutic against Non-Hodgkin's Lymphoma (NHL) are analyzed. NHL is a cancer of the lymphatic system where tumor cells spread into the bloodstream and can lodge in almost any organ. It affects 500,000 people yearly in the US. Selective High Affinity Ligands (SHALs) were designed to specifically bind structurally (nanomolar to picomolar Kd's) to a unique region on HLA-DR10 at the same binding site of the antibody that induces cell signaling and apoptosis. The individual drugs making up the ligands are cytotoxic to all tumor and normal cell lines. However, when linked together into the SHAL, they are only cytotoxic to the tumor cells expressing the HLA-DR10 on the cell surface and exhibit minimal uptake by organs like the kidney and liver. One of these novel therapeutics cures mice carrying the human lymphoma (existing drugs only slow progression)[1]. Treatments such as radioimmunotherapy that target tumor cells expressing HLA-DR10 have shown considerable success against NHL. SHALs were designed to mimic antibody (Ab) targeting behavior while decreasing size by 50-100 times.

Impurity analysis of drugs is a complex problem because the impurities may arise from the starting materials used to generate the products, from incomplete synthesis reactions or leachables from reaction vessels or packing material. Guidelines require that drug impurities be identified when present above concentration limits of 0.05 and 0.10%, depending on daily dose. Analysis of impurities by MS is less straightforward if the molecules are large and create multiply charged ions.

The goal of this work is to demonstrate a complete workflow using LC-MS, -MS^2 and differential analysis software for the study of drug impurities when analyzing multiply charged compounds.

Methods

Synthesis of tridentate ligand: A tridentate ligand (SH7144, MW_{mono} 2322.94), composed of three recognition elements covalently held by lysine and miniPEG linkers was synthesized (Fig. 1). The bench-scale synthesis of SH7144 was performed on a chlorotrityl-chloride resin using standard Fmoc solid phase chemistry to conjugate PEG monomer units and three commercially available molecules to the alpha and epsilon amines of a resin bound lysine. The same approach was used for the pilot production synthesis (gram scale), except that one of the drugs was synthesized in-house. Both products were purified by RP-HPLC and lyophilized. The bench scale product was lyophilized as the TFA salt and the pilot production ligand was converted to the acetate salt. Both structures were determined to be correct by NMR (1D and 2D).

Liquid Chromatography Mass Spectrometry: Thermo Scientific Open Accela autosampler, Accela 600 HPLC™ pump, LTQ Orbitrap XL™ mass spectrometer. Thermo Scientific Hypersil GOLD 3 µm, 150 x 2.1 mm column, linear gradient from 5% aqueous to 95% organic in 20 min (A=H_2O/0.1% Formic Acid, 10mM Ammonium Formate, B= Acetonitrile/0.1% FA).

Data Analysis: Classic Recursive Base Peak Framing algorithm (SIEVE 2.0 software) was used in order to analyze +2 and +3 charged spectra. A trend analysis comparing both products and solvent blanks (five replicate runs each) was performed. Mass Frontier 7.0 software was used for structure elucidation in combination with ion trap MS^n spectra.

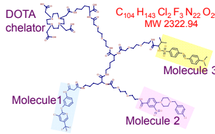


FIGURE 1. SH7144 tridentate ligand: three small molecules linked together by PEGs and lysines. DOTA chelator added to accommodate a radioisotope agent.

Results

High Abundant Impurity in Both Synthetic Preparations

An abundant impurity that closely elutes with the main compound was detected in all LC-MS spectra (Fig. 2a). Chromium (Cr^{3+}) was determined to form a stable complex with the DOTA macrocycle. Fig. 2b shows the FT MS spectra of the main compounds (mass accuracy of **$[M+2H]^{2+}$** 3 ppm, resolution ~40k). Stainless steel contains between 11-20% Cr, therefore contamination is presumed to be from contact with ss during synthesis and/or purification. The standard 57Co load method[2] was used to determine that 50 to 60% of the DOTA structure remains in free state. In other words, 40 to 50% of the DOTAs were occupied with an unidentified metal. Consequentially, the specific activity of the ligand with a radioactive metal (90Y, 111In, 67Cu, or 68Ga) would be reduced if using this SHAL for radioimmunotherapy or PET imaging. From the US FDA standpoint, this should be an acceptable contaminant for this therapeutic because the presence of Cr in the DOTA chelator would not be expected to have an effect on the main function of the drug, which kills tumor cells by specific binding to a protein. That is, unless unexpected molecule rearrangements might result from Cr coordination in the macrocycle.

Evidence confirming structure of abundant contaminant: 1) the MS^2 spectrum of **$[M+2H+Cr]^{2+}$** (Fig. 3b) displays three fragment peaks, *m/z* 731.4, 1011.2, 1390.8, containing Cr (determined by mass difference with the corresponding fragment peaks in the MS^2 of **$[M+2H]^{2+}$,** Fig. 3a). **2)** Fragment peak at *m/z* 1648.5, on the other hand, is the same in both MS^2 of **$[M+2H]^{2+}$** and **$[M+2H+Cr]^{2+}$.** This peak indicates loss of the DOTA-containing 'arm': 2322.94 – 674.34 = 1648.6, with associated loss of Cr. **3)** the distinct isotopic pattern of the contaminant reflects the addition of a Cr atom to the molecule as shown in Fig. 3d and per chemical formula simulation in QualBrowser (not shown). **4)** An identical synthetic ligand (SH7133), which lacks the DOTA chelator ring (has a free amine instead), does not display a co-eluting contaminant with the parent drug (data not shown). Note: There is a mass discrepancy of 0.9697 in the theoretical calculations when compared with 3ppm accurate mass spectra that has not been explained (below Fig.3cd).

FIGURE 2. a) LC trace displays co-eluting compound at 12.77 min. b) FT MS spectrum of parent drug $[M+2H]^{2+}$ at *m/z* 1163.480 and Cr contaminant $[M+2H+Cr]^{2+}$ at *m/z* 1189.934.

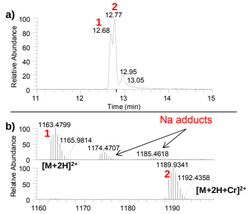


FIGURE 3 a, b. Ion trap MS^2 spectra of a) $[M+2H]^{2+}$ and b) $[M+2H+Cr]^{2+}$ contaminant. Fragment at *m/z* 1648.5 is the loss of the DOTA-containing arm.

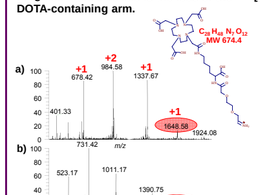
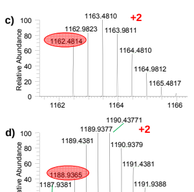


FIGURE 3 c, d. c) FT MS spectrum of $[M+2H]^{2+}$, and d) FT MS spectrum of $[M+2H+Cr]^{2+}$ contaminant.



Low Level Impurities

Label-free differential analysis (SIEVE 2.0 software) was used for determination of low level impurities. The signal detection algorithm chosen was classic alignment and framing since the samples studied are multiply charged by +ESI. Analyzed groups were: a bench-scale synthesis of SH7144 (also called standard), the pilot production-scale synthesis of SH7144, and solvent blanks (50:50 acetonitrile/H_2O/2.5%DMSO). The standard bench-scale synthesis group was set as the control group and the pilot production synthesis and solvent blanks were selected as the trend points. This was performed to allow for background removal. The retention time window for the analysis was from 7 to 20 min. Other parameter settings include: 'Frame *m/z* Width' set to 10ppm (typical for HRAM data), 'Frame Retention Time Width' set to 0.75 min. and 'Intensity Threshold' to 10,000.

First step of the differential analysis is chromatographic alignment (Fig. 4) followed by component detection and identification.

Filtering of the data includes removal of signal from blank samples, filtering on CV within groups of samples to retain reproducible data, removal of signals that go both up or down, and removal of charges less than +2. Tabulated results after filtering (130 frames, 31 components) are shown in Fig. 5.

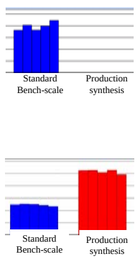
The column 'Compound MW' provides the deconvoluted molecular weight, which is very useful for a first pass check at common LC-MS adducts. 'Ratios of Prod/Std' (column filled yellow) of <1 represent *m/z* peaks found in higher abundance in the bench-scale synthesis than in the production-scale and ratios >2 represent *m/z* values that occur in higher abundance in the production-scale sample.

Extracted ion chromatograms using data from column labeled '*m/z*' from the components table were then reconstructed with FT MS data acquired at 60k and 100k resolving power (measured at *m/z* 400). The abundance of the components in one synthesis vs. the other was confirmed this way (data not shown). The elucidation of differences between the two preparations, by comparing LC traces, would not have been possible without prior differential analysis by SIEVE software.

FIGURE 4 . SIEVE 2.0 software for low level impurity analysis showing alignment of chromatographic peaks as a first step in the workflow and datafiles used in the analysis.

FIGURE 5. SIEVE software results after filtering and grouping isotopes. A 'Ratio of Prod/Std' <1 means that component is more abundant in the bench-scale synthesis (or standard) than in the pilot production synthesis. A 'Ratio of Prod/Std' >2 means the component is more abundant in the pilot production synthesis.

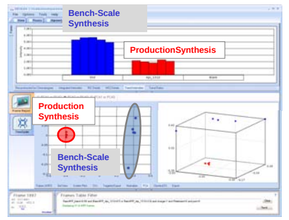
#	ID	*m/z*	Ret. Time	Compd. MW	Ratio Prod/Std	P -Value
1	766	870.6523	12.96	2611.9387	0.00	1.35E-05
2	941	653.4914	12.94	2611.9372	0.00	1.15E-04
3	2091	853.0143	12.85	2558.0202	0.00	3.51E-06
4	2433	653.2404	12.96	2608.9323	0.00	4.71E-04
5	2584	1316.968	12.96	2633.9234	0.00	1.68E-05
6	3030	1060.399	12.09	2119.7862	0.00	8.26E-06
7	3172	640.012	12.89	2558.0202	0.00	6.00E-04
8	3174	1279.017	12.88	2558.0194	0.00	1.42E-04
9	4315	1316.465	12.94	2630.9161	0.00	3.77E-05
10	2580	780.6531	12.64	2339.9399	0.11	2.70E-06
11	2910	889.3698	12.44	1776.7251	0.122	9.69E-10
12	1897	1217.468	13.08	2433.9266	0.383	3.99E-07
13	1135	1181.926	12.18	2363.8407	0.42	7.89E-07
14	1882	1140.466	12.73	2280.922	0.00	0.002
15	1628	1171.407	12.58	2342.801	0.00	400.147
16	2147	1144.902	12.26	2289.794	0.00	641.79
17	762	760.6471	12.7	2280.92	0.00	0.005
18	508	1305.475	12.94	2610.937	0.00	0
19	445	1166.922	12.79	2333.831	0.00	0.002
20	2912	1119.447	12.19	2236.88	0.00	470.395
21	4172	874.3188	13.01	2619.935	0.00	0.116
22	105	1194.435	12.91	2386.8548	2.094	3.54E-08
23	730	1205.426	12.93	2408.8377	2.24	1.19E-09
24	259	581.742	12.76	2324.9384	2.354	1.78E-04
25	113	797.6251	12.93	2389.8534	2.63	1.18E-08
26	425	597.72	12.93	2386.8508	3.333	3.52E-07
27	1449	1144.908	12.75	2289.8043	14.126	2.37E-05
28	1671	1151.915	12.79	2303.8185	22.184	2.93E-06
29	2257	745.9706	12.71	2235.8944	29.022	2.70E-06
30	2671	750.6437	12.7	2250.9102	36.76	6.39E-06
31	3253	768.6151	12.77	2303.8205	41.381	1.56E-05



Low Level Impurities (continued)

A Principal Component Analysis (PCA) plot reveals that, as expected, the two synthetic products are very similar to each other and very different from the solvent blank (data not shown). However, differences between the two synthetic products are also evident (shown in Fig. 6).

FIGURE 6. Principal Component Analysis results window. *m/z* 1217.468 appears in greater abundance in the standard bench-scale synthesis than in the pilot production one.



Structural Elucidation from Differential Analysis

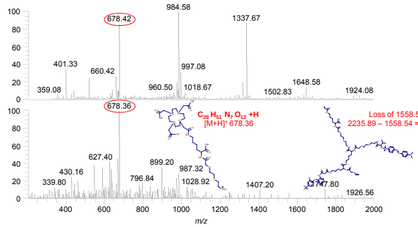
To minimize the likelihood of common adducts of acetonitrile, ammonium, Na+, K+, etc. remaining after differential analysis, the list of components (both *m/z* and deconvoluted species) were checked using a Mass Spectrometry Adduct Calculator[3]. This calculator tool takes into consideration +1, +2, +3 charged species and dimers. Other potential adducts with the LC conditions used would occur in negative mode, mainly those of formic, acetic acid and TFA and were not considered as data were collected in positive ion mode.

None of the common adducts came within a 10ppm window of the components found by SIEVE software, after adjusting compound MW from the table to the appropriate charged species.

Two components, *m/z* values 745.971 and 1305.475, with Ratios (Production/Bench-scale) of 29.02 and 0.00 respectively, were picked from the table for MS^2 analysis. The fragmentation spectra from those were then compared to the MS^2 of the main compound and its Cr chelate to see how they related to the main compounds.

Fig. 7 shows the comparison for MS^2 of *m/z* 745.97 vs. *m/z* 1163.48 in the production-scale sample as SIEVE software results indicates this component is more abundant in that synthetic product. Because it displays one common fragment with **$[M+2H]^{2+}$** they are suspected to be related. SIEVE provides the deconvoluted MW of 745.97 as 2235.89 amu. A likely pathway is the loss of 1558.54 amu, structure shown below.

FIGURE 7. Ion trap CID for **$[M+2H]^{2+}$,** *m/z* 1163.48 (M=SH7144, top trace) and CID of component at *m/z* 745.97 found by SIEVE software to be more abundant in the production scale synthesis.



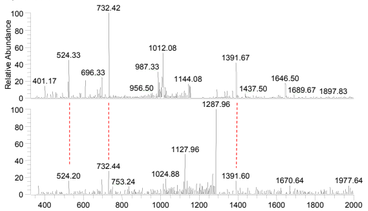
Structural Elucidation from Differential Analysis (continued)

Fig. 8 shows the MS^2 of *m/z* 1305.48 compared to the MS^2 fragmentation of 1189.93 in the bench-scale sample as SIEVE software results indicates this component is more abundant in that synthetic product.

The *m/z* 1305.48 component has a couple of peaks in common with the $[M+2H+Cr]^{2+}$ adduct but not with the $[M+H]^{2+}$ of SH7144 (data not shown). The common peaks with the Cr adduct are: 524.2, 732.4, 1391.6. As Fig. 3 a and b show, *m/z* 732.4 and 1391.6 are fragments that contain the DOTA macrocycle with a Cr adduct. The *m/z* 1305.48 fragmentation also displays unique fragments at: *m/z* 753.2, 1220.24, 1287.96 whose corresponding structures are yet to be identified.

Further work is being done to analyze all the different components identified by SIEVE software, judge their relevance and attempt to quantitate relative levels. These might have significance in explaining the different solubilities of the compounds, even though the crystals are visually similar. The main contaminant was readily identified by HRAM and MS^2 mass spectrometry and this one is probably of greater immediate relevance. More stringent purification steps and/or quality control of the supplied DOTA chelating ring might be considered.

FIGURE 8. Ion trap CID for **$[M+2H+Cr]^{2+}$** *m/z* 1189.93 (M = SH7144, top trace) and CID of component at *m/z* 1305.48 found by SIEVE software to be more abundant in the bench-scale synthesis.



Conclusion

- A tridentate Selective High Affinity Ligand (SHAL) was successfully studied with a hybrid mass spectrometer. High resolution, accurate mass data was used in conjunction with label-free differential analysis to determine differences in the products from two syntheses. Ion trap MS^n data was utilized for structure elucidation of those differences.
- An abundant contaminant present in both synthetic products was measured: high resolution spectra (acquired at 60k or 100k resolving power) allowed measurement of the unique isotopic pattern that results with the addition of a Cr atom to the ligand.
- Ion trap MS^2 confirmed the abundant impurity with Cr is associated solely with the DOTA macrocycle ring.
- SIEVE 2.0 software analysis offers both fast and easy interpretation of results, therefore is ideally suited for impurity analysis of drugs. It handles multiply charged spectra specially well, spectra that are more complex to visualize and compare directly from LC traces.

References

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2) Hok, S., Natarajan, A., Balhorn, R., DeNardo, SJ, DeNardo, GL, and Perkins, J. Synthesis and Radiolabeling of Selective High-Affinity Ligands Designed to Target Non-Hodgkin's Lymphoma and Leukemia. Bioconjugate Chem. 2007, 18, 912-921.
3) Mass Spectrometry Adduct Calculator, Fiehn Lab, UC Davis, modified 2010.