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 a Thermo Scientific™ Open Accela™ autosampler coupled to a Thermo Scientific LTQ Orbitrap XL™ mass spectrometer followed by analysis with Thermo Scientific SIEVE™ software (version 2.0). Thermo Scientific Mass Frontier software version 7.0 was used for determination of fragments and fragmentation pathways.

Results: Differential analysis software identified a number of differences between the two samples. High-resolution, accurate-mass (HR/AM) data was effectively used for elucidation of impurities in combination with 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 U.S. 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.

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 (HR/AM data), -MS^2, and differential analysis software for the study of drug impurities when analyzing multiply charged compounds.

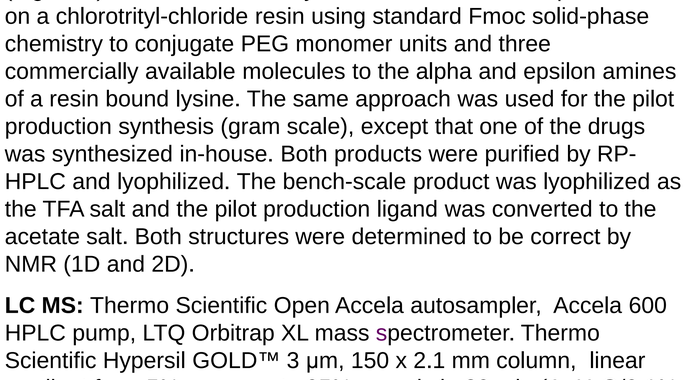
Methods

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 (Figure 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).

LC MS: 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 software version 7.0 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

Structural Elucidation from Differential Analysis

Label-free differential analysis (SIEVE 2.0 software) was used for determination of low-level impurities. Classic alignment and framing was used 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 allowed 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 10 ppm (typical for HR/AM data), 'Frame Retention Time Width' set to 0.75 min. and 'Intensity Threshold' to 10,000 (Figure 2).

Tabulated results after filtering (130 frames, 31 components) are shown in Figure 3. The column 'Compound MW' provides the deconvoluted molecular weight, which is very useful for a first pass check at common LC-MS adducts (Mass Spectrometry Adduct Calculator[3]). No common adducts found. '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.

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 2 . SIEVE software version 2.0 showing alignment of chromatographic peaks as a first step in the workflow. Data files used in the analysis are also shown.

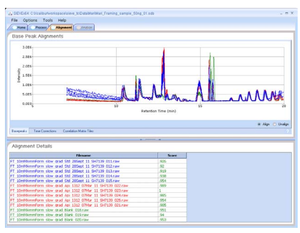
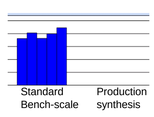
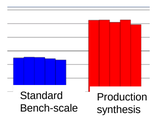


FIGURE 3. SIEVE software results after filtering and grouping isotopes. A 'Ratio of Prod/Std' <1 means that component is more abundant in the bench-scale synthesis (or standard) than in the pilot production synthesis. A 'Ratio of Prod/Std' >2 means the component is more abundant in the pilot production synthesis.

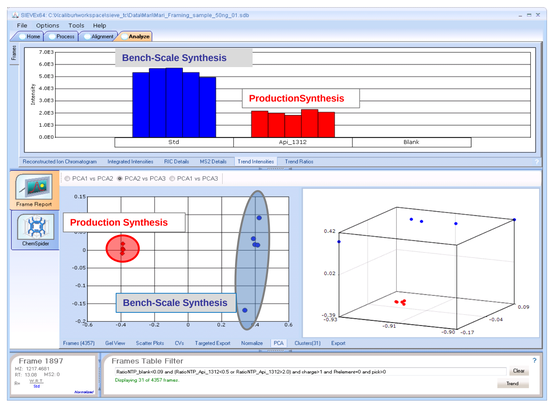
#	ID	*m/z*	Ret. Time	Compd. MW	Ratio Prod/Std	P -Value
1	766	870.6523	12.96	2611.9387	0.00	1.35E-05
2	941	653.4914	12.94	2611.9372	0.00	1.15E-04
3	2091	853.0143	12.85	2558.0202	0.00	3.51E-06
4	2433	653.2404	12.96	2608.9323	0.00	4.71E-04
5	2584	1316.968	12.96	2633.9234	0.00	1.68E-05
6	3030	1060.399	12.09	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





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 Figure 4).

FIGURE 4. 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.



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 Figure 3 for MS^2 analysis. The fragmentation spectra from those were then compared to the MS^2 of the parent and abundant compounds to look for similarities.

Figure 5 shows the comparison for MS^2 of *m/z* 745.97 vs. *m/z* 1163.48 ($[M+2H]^{2+}$, parent compound) in the production-scale sample. SIEVE software results indicated this component was more abundant in the synthetic product. It displays one common fragment with the parent compound. Its fragmentation pathway was elucidated using the "Fragments and Mechanisms" feature in Mass Frontier software. SIEVE software provided the deconvoluted MW of 745.97 as 2235.8944 amu, which was 87.0492 difference from the parent compound. A likely pathway is the loss of 1558.54 amu, structure shown in Figure. 5. ChemSpider was used to elucidate the difference between parent compound and impurity (ongoing work).

FIGURE 5. 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.

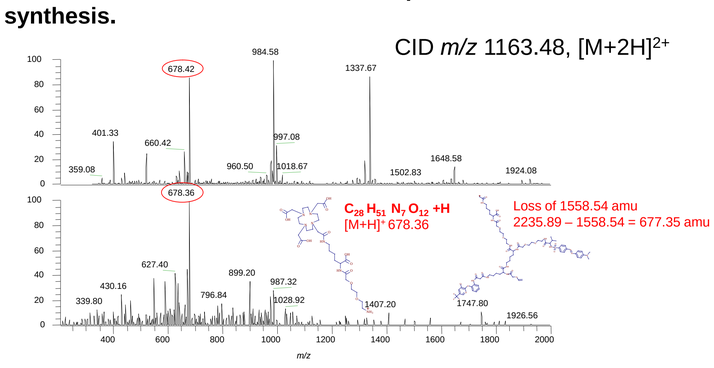
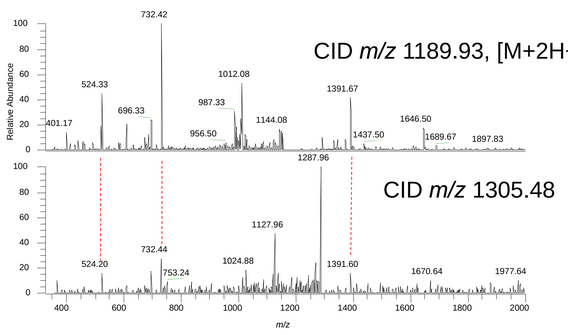


Figure 6 shows the MS^2 of *m/z* 1305.48 compared to the MS^2 fragmentation of *m/z* 1189.93 in the bench-scale sample as SIEVE software results indicate this component is more abundant in that synthetic product. *m/z* 1189.93 is the $[M+H]^{2+}$ of a major impurity in SH-7144 that is associated with the DOTA moiety and co-elutes with the parent compound (work not shown).

The *m/z* 1305.48 component has three peaks in common with the $[M+2H+X]^{2+}$ adduct but not with the $[M+2H]^{2+}$ parent compound of SH7144 (data not shown). The common peaks with the $[M+2H+X]^{2+}$ adduct are: *m/z* 524.2, 732.4, 1391.6 (Fig. 6). *m/z* 732.4 and 1391.6 are fragments that contain the DOTA macrocycle with an undetermined metal or contaminant. The *m/z* 1305.48 fragmentation also displays unique fragments at: *m/z* 753.2, 1220.24 and 1287.96, whose corresponding structures are yet to be determined.

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 solubility of the two synthetic products. More stringent purification steps and/or quality control of the supplied DOTA chelating ring might have to be considered.

FIGURE 6. Ion trap CID for $[M+2H+X]^{2+}$ *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.
- Two compounds identified by SIEVE software to be more abundant in the bench-scale standard or the pilot production synthesis were further analyzed by ion trap MS^2.
- Ion trap fragmentation patterns and Mass Frontier software linked the different component in the pilot scale synthesis to SH-7144 and the component mostly in the bench-scale synthesis was related to an impurity of the DOTA macrocycle ring.
- SIEVE software analysis offers both fast and easy interpretation of results, therefore is ideally suited for impurity analysis of drugs. It handles multiply charged spectra especially 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.

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