Development of physicochemical methods for analysis of pandemic influenza vaccine

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This manuscript was prepared for the Microbiology 8 month Co-op workterm at the Pandemic Influenza Division, Centre for Biologics Evaluation, Biologics and Genetics Therapy Directorate, Health Canada, under the supervision of Dr. Harold Rode, Helen MacDonald-Piquard, Dr. Wilfred Lim, and Dr. Jun Wang.

The utility of a size exclusion high-performance liquid chromatography (SE-HPLC) method, in the identification and quantitation of haemagglutinin (HA) protein in monovalent, inactivated influenza vaccines for the purposes of potency assessment is reported. This method is sufficiently developed to provide a distinct peak separation of HA from other vaccine constituents, and a correlation with HA potency determined by single radial-immunodiffusion (SRID) assay. Sensitivity of the method is demonstrated, with each HA peak accurately titrated to the HA content of the protein load injected. Highly reproducible chromatographic profiles (on a G4000SWxl column) were achieved, demonstrating vaccine protein integrity and stability. Potency assessments, determined by SE-HPLC peak area analysis, provided very good correlation with the quantitative HA protein values, determined by SRID, reported by the manufacturer and by Health Canada's Centre for Biologics Evaluation, Pandemic Influenza Division (PID). Peak resolution was further enhanced by expanding to a tandem SE-HPLC system, utilizing a G4000SWxl SE column coupled to a G3000SWxl column. HA was detected in the 11-17 minute (min) elution, collected in 30 second (sec) fractions, with the smallest, most distinct peak width detected to date at the 15.5 min fraction. This SE-HPLC method has considerable potential for widespread use as a physicochemical method for HA identification and quantification, in quality control testing for seasonal and pandemic influenza vaccines, and as a practical alternative for potency measures by the reagent-dependent SRID assay. Increased resolution and further method development will facilitate the collection of separated fractions for further analysis, to correlate immunoactivity to HA type and content.

The influenza virus is a contagious disease of animals and is categorized as a genus of the family *Orthomyxoviridae*. Influenza viruses are classified according to antigenic differences in their nucleoprotein (NP) and matrix protein (MP1), creating types A, B, and C,³ with solely type A strains being associated with pandemics.¹ Type A influenza viruses are further subdivided based on antigenic differences in their main surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). These major surface antigens are the targets of the host immune response, despite the fact that they are subject to rapid and unpredictable antigenic variation.²

Albeit the natural reservoir for all influenza viruses is aquatic birds⁶, types A and B influenza viruses have been found in human isolates during the past decade, and have been responsible for numerous outbreaks around the world³. Of concern is subtype H5N1 due to its ability to cross the species barrier over to humans. There has been a notable increase in the direct transmission of the H5N1 avian influenza virus to humans⁶. As of December 18 2007, the emerging H5N1 virus has infected 340 individuals since 2003, causing 209 deaths in 13 countries (a 61.5% mortality

rate).⁸ With the added possibility of mutation and genetic reassortment that could lead to human-to-human transmission, H5N1 is a strong candidate for a potential influenza pandemic.

Continued surveillance is indispensable to pandemic preparedness plans. The World Health Organization Influenza Surveillance Programme along with other national health authorities, have been able to make recommendations on vaccine composition.¹⁰ To date, vaccines are the main medical intervention for protecting individuals from influenza outbreaks.⁹ In light of an impending pandemic, drug manufacturers are seeking to develop prototype avian/pandemic influenza vaccines.

As a regulatory agency, Health Canada is directly responsible for the assessment of potency, safety and purity of prototype pandemic influenza vaccines. Potency of influenza vaccines is currently determined on the basis of the vaccine HA content and its ability to generate an effective immune response (against the antigen.) The principle underlying the mechanism of action for influenza viruses is the induction of protective antibodies that guard against the HA glycoprotein of the predicted epidemic strain.⁶

At present the SRID method, approved by the European Pharmacopoeia Commission for the evaluation of inactivated influenza vaccines⁵ and used by most regulatory agencies worldwide, is the only method which provides a visual, easily detectable measure of antigen/antibody binding as a measure of potency. A value of 15 µg of HA per strain per dose is used for vaccination.³ The SRID assay requires a reference antigen reagent to be used as a calibrator and specific antibodies against this antigen.³ The interaction of antigen and antibody creates a zone of precipitation in a semi-solid medium, the diameters of which can be directly correlated to the amount of HA present.⁵ The SRID method is very specific and requires little technology; SRID reagents take approximately 2-3 months to produce—which would be too long in the case of a pandemic outbreak. In an emergency situation, alternative estimates of vaccine potency before reagents become available would be of critical importance. It would therefore be valuable to accumulate comparative data between the SRID test and alternative physicochemical techniques, in order to validate more unconventional approaches towards potency testing.¹¹ Among them are high resolution chromatographic techniques.

A relatively recent publication has explored two-dimensional high-performance liquid chromatography (2D-HPLC), coupling size exclusion HPLC with reverse phase HPLC, to characterize influenza vaccine constituents.³ The assessment found that vaccines made by different manufacturers had significantly different profiles, while different vaccine lots within the same manufacturer demonstrated consistent results. This research is of particular interest to pandemic preparedness strategies due to the fact that HA, along with some other viral proteins, was found to separate into a particular peak on the chromatograms. The amount of HA found in the peak correlated fairly well with the amount of HA in the original vaccine preparation.

The Pandemic Influenza Division has adapted methods used by García-Cañas et al., 2007, in the part of physicochemical methods for the characterization and measurement of potency of the pandemic flu vaccines. A novel procedure, requiring no sample pre-treatment, through two size exclusion columns in series was developed. The potential of this SE-HPLC system to quantitate HA was determined with comparative methods such as SRID and protein assays.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical reagent grade and used as received. Sodium chloride crystals were from EMD, Dithiothreitol (DTT) solution and sodium dodecyl sulphate (SDS) were from Sigma Aldrich, and sodium phosphate dibasic anhydrous was from Fisher. Seakem™ ME Agarose was obtained from Cambrex, Zwittergent™ 3-14 Detergent was from Calbiochem, and D-PBS was purchased from

Wisent. Coomassie® Brilliant Blue G-250 was purchased from Bio-Rad, and acetic acid and methanol were from OmniSolv. Micro BCA™ Protein Assay Kit was purchased from Pierce. Distilled water was purified with Milli-Q Synthesis System.

Samples

Monovalent split virus pool vaccine lots from 2004 were used, as well as 2005 and 2006 lots of fragmented filtered monovalent lots of influenza virus vaccines. The virus strains used to produce 2004, 2005 and 2006 monovalent lots were A/New Caledonia/20/99. No sample treatment was performed prior to chromatographic analysis, except when testing the effects of heat and DTT to sample separation. Samples were heated in a water bath to appropriate temperature, and then placed in HPLC autosampler set at room temperature. 12 mM DTT was added prior to injection of sample when testing effects of DTT.

Single Radial Immuno Diffusion (SRID)

SRID tests for determination of hemagglutinin in influenza monovalent bulks were performed according to the procedure described by Health Canada's Biologics and Genetic Therapies Directorate (BGTD) standard operating procedure for: SINGLE RADIAL IMMUNODIFFUSION (SRD) TEST FOR INFLUENZA POTENCY. An agarose gel containing a predetermined amount of anti-serum was cast in a gel mould (Canus). Lyophilized hemagglutinin antigen reagents from A/New Caledonia/20/99 IVR-116 with a content of 54 µg HA/vial as well as lyophilized anti-hemagglutinin sheep serum A/New Caledonia/20/99 with a content of 6 µL/mL of agarose, prepared and standardized by the Center for Biologics Evaluation and Research were used. The reference antigen and monovalent bulks were diluted to predetermined concentrations and were treated with 1% Zwittergent (to disrupt virions) for 30 min. at room temperature. Treated antigen and bulks were then inoculated into holes punched into the solidified gel and left to diffuse at least 18 hrs, overnight at room temperature. Gel was then dried onto Gelbond Film (Lonza) and dried with Whatman filter paper and paper towels. Once dry, gel was stained with Coomassie brilliant blue G-250 in order to ascertain presence of antibody/antigen aggregates. The diameters of the resulting precipitation zone were measured using CorelDRAW Version 12.0.0.458© 2003. Hemagglutinin content was then calculated by the statistical analysis program CombiStats, version 3.0© 2004, EDQM/Council of Europe.

Chromatographic Separations

Instrumentation

The Prostar HPLC System (Varian) was equipped with the following: Prostar 335 Diode Array Detector, covering wavelengths 190-195nm; Prostar 410 Autosampler containing a 250µL-loop; Degassit, Four Channel 10mL/min;

Metatherm Column Heater, 110V; and Pump 230. Data acquisition and integration was performed with Star Workstation Windows XP system (Varian). Chromatograms were viewed in Interactive Graphics as 1-D plots, retention time (x) vs. peak amplitude (y). Peak area was measured on all accounts.

Separation Conditions

The samples were stored at 4°C prior to use. Once aliquoted into HPLC vials, samples were placed in a temperature-controlled autosampler tray set at 15°C. The analytical chromatographic columns used in this investigation were the following: Tosoh TSK G4000SWxl, pore size 450Å, 7.8mm (ID) x 30cm (L), Tosoh TSK G3000SWxl, pore size 250Å, 7.8mm (ID) x 30cm (L), Tosoh TSK G2000SWxl, pore size 125Å, 7.8mm (ID) x 30cm (L), and Tosoh TSK G3000SW, pore size 250Å, 7.5mm (ID) x 60cm (L). Performance qualification of columns was assured by standard SE chromatograms of the following samples: Thyroglobulin (Thermo), Ferritin (Sigma), Ovalbumin (Sigma), γ -globulin (Sigma), p-Aminobenzoic acid FW 137.14 (Sigma). Separation was carried out at room temperature. The mobile phase was isocratic and consisted of the following: 0.1M Na₂HPO₄, 0.2M NaCl, 0.1% SDS, and was adjusted to pH 7 with 4N NaOH (Sigma), except when samples were tested at pH 5. Elution was carried out at a flow rate of 1.0 mL/min. Detection wavelength was set at 214nm.

Protein Analysis

MicroBCA (bicinchoninic acids) protein assay kit was used (Pierce). The absorbance of the samples, standards of bovine serum albumin (BSA), and controls (sample diluent, PBS) was measured at 562nm on a Multiskan Spectrum spectrophotometer (Thermo). Total protein was quantified as BSA equivalents, based on a standard curve of 0, 0.5, 1, 2.5, 5, 10, 20, 40, 200 μ g/mL of BSA. This method uses BCA as the detection reagent for Cu⁺¹, which is formed when Cu⁺² is reduced by protein in an alkaline environment. It was chosen for its compatibility with the samples, as well as a working range of 2-40 μ g/mL available as a microplate procedure, which permitted all samples to be read simultaneously. Results were analysed by the SkanIt Software 2.2, and compiled in Microsoft Excel (XP).

RESULTS AND INTERPRETATIONS

The initial set of experiments investigated any correlation of HA content in the sample vaccine with peaks obtained by SE-HPLC.

SRID

At present, SRID is the established analytical method for the measurement of HA content in vaccines. **Table 1** illustrates SRID results for 10 monovalent lots (Vaccines A-J) from 2004.

SE-HPLC Selectivity

Initial steps utilized SE-HPLC to determine if the components of the vaccine could be reproducibly separated and if any of the peaks correlated with the HA content of the vaccine. The applicability of the SE-HPLC setup was first assessed using several relational assessments. SE-HPLC's ability to produce chromatograms in which each peak could be accurately titrated to the HA content of the sample is demonstrated in **Figure 1** (the load responsiveness of the system). Peaks are labelled 1-8 and the designated putative HA peak is Peak 3 (see **Figure 1**). The assay linearity was determined by plotting the putative HA peak area, having a retention time (RT) of approximately 9 min. (25 μ L injection RT: 9.033), as function of the amount of sample (μ L) injected (**Figure 2**). The standard curve illustrated in **Figure 2** shows that peak area is proportional to the amount of HA injected into the SE-HPLC system. Adequate linearity was demonstrated by a correlation coefficient of > 0.99.

The applicability of the SE-HPLC setup for HA quantification was further assessed. Several standard curves were taken from various peaks of sample K chromatographic profile to calculate the amount of HA present in the SE-HPLC injection. These values were then compared with the manufacturer's Certificate of Analysis (CoA) (**Tables 2 to 5**). This suggests that SE-HPLC is distinct enough to be used for peak separation and potency assessment, irrespective of the location of the putative HA peak. The data suggests that each peak can be accurately titrated to the HA content of the protein load injected, appearing in **Figures 3 to 6**. Sample K was used for each injection on the G4000SWxl column.

Table 1: Compilation of PID SRID Results. All lots were tested in triplicate (with the exception of Vaccine D done in quadruplet). Averages of triplicate potencies were taken and appear below.

Sample	Year 2004 (μ g/mL)
Vaccine A	343.4
Vaccine B	620.61
Vaccine C	769.74
Vaccine D	636.56
Vaccine E	492.1
Vaccine F	423.46
Vaccine G	518.38
Vaccine H	505.55
Vaccine I	503.93
Vaccine J	520.73

Figure 1: Dilution Series of Sample K on the G4000SWxl Column, demonstrating SE-HPLC system's ability to respond to different sample volumes. Injection volumes were as follows: 5µL, 10µL, 15µL, 20µL, and 25µL.

Figure 2: Standard Curve Made from Dilution Series of Sample K on the G4000SWxl Column Chromatogram above (Figure 1), using peak at retention time 9.034 min. in order to demonstrate the linear relationship between amount of HA injected and peak intensity.

Figure 3: Standard Curve demonstrating [HA] as a Function of its Peak Area for Peak at Retention Time of 9 min.

Figure 4: Standard Curve Demonstrating [HA] as a Function of its Peak Area for Peak at Retention Time of 9.5 min.

Table 2: Peak at Retention Time of 9 min., Used to Make Standard Curve.

μL Injected	μg HA from CoA	HPLC Peak Area	Calculated HA from curve	Total μg HA/mL monovalent bulk
0	0	0	0	0
5	2.59	21110808	2.67	533.36
10	5.18	43316052	5.44	544.25
20	10.36	86807664	10.88	545.95
25	12.95	108923920	13.64	545.74
30	15.54	129919312	16.27	542.26

Total μg HA/mL according to CoA: 518Average of total μg HA/mL according to curve : 541.9116**Table 3: Peak at Retention Time of 9.5 min., Used to Make Standard Curve.**

μL Injected	μg HA from CoA	HPLC Peak Area	Calculated HA from curve	Total μg HA/mL monovalent bulk
0	0	0	0	0
5	2.59	not incl.	not incl.	not incl.
10	5.18	39360104	5.57	557.01
20	10.36	61320296	8.71	435.37
25	12.95	98828096	14.07	562.62
30	15.54	105186200	14.97	499.127

Total μg HA/mL according to CoA: 518Average of total μg HA/mL according to curve : 513.5305**Table 4: Peak at Retention Time of 12.5 min., Used to Make Standard Curve.**

μL Injected	μg HA from CoA	HPLC Peak Area	Calculated HA from curve	Total μg HA/mL monovalent bulk
0	0	0	0	0
5	2.59	not incl.	not incl.	not incl.
10	5.18	13015581	6.01	600.78
20	10.36	21908086	10.45	522.70
25	12.95	31578930	15.29	611.58
30	15.54	30783012	14.89	496.38

Total μg HA/mL according to CoA: 518Average of total μg HA/mL according to curve : 557.86075

Figure 5: Standard Curve Demonstrating [HA] as a Function of its Peak Area for Peak at Retention Time of 12.5 min.

Figure 6: Standard Curve demonstrating [HA] as a Function of its Peak Area for Peak at Retention Time of 14.5 min.

SE-HPLC Reproducibility

Replicate injections of sample K were performed on different days and peak areas were compared, and the mean and the standard deviation among injections were calculated. Results are expressed in **Table 6** and a qualitative representation of the data is presented in **Figure 7**. **Figure 7** suggests that peak areas may be used to determine potency measures and are consistent and highly reproducible.

Other consistent patterns have been obtained for two 2005 New York strains as well as for two 2005 Jiangsu strains. This suggests that the SE-HPLC is likely to be applicable to different influenza strains, irrespective of their variations in HA, and may be utilized for a predicted H5N1 pandemic strain.

Sample Stability and Manufacturing Consistency

A comparison of potency data from the manufacturer in 2004 and PID results taken in 2007 were performed and demonstrate that on average, 3 year old samples exemplified ~89% of the potency claim by the manufacturer, giving a coefficient of variance of ~11% as seen in **Table 7**. This slight decline in potency may be attributed to many factors such as the possibility of degradation over time as well as aggregation of the proteins which occurs upon storage of any vaccine.

Another point of interest lies in the manufacturing consistency. The HA content of the monovalent bulks produced in 2004, by SRID determination, varied from 411 to 748 µg/ml, while the HA content of 3 lots of the same

Figure 7: Coincident Profiles Vaccine K Taken at Different Times during June 2006. Dates included: June 8/06, June 9/06, June 13/06, June 15/06 (correspond to Table 6).

Table 5: Peak at Retention Time of 14.5 min., Used to Make Standard Curve.

µL Injected	µg HA from CoA	HPLC Peak Area	Calculated HA from curve	Total µg HA/mL monovalent bulk
0	0	0	0	0
5	2.59	not incl.	not incl.	not incl.
10	5.18	23753722	4.72	472.41
20	10.36	48762004	9.73	486.29
25	12.95	60510332	12.08	483.02
30	15.54	71088352	14.19	483.04

Total µg HA/mL according to CoA: 518

Average of total µg HA/mL according to curve : 481.1895

strain produced in 2006 varied from 371 to 534 µg/mL (Table 7). This amount of variation in HA content among lots of monovalent bulks is quite typical of biological systems.

In contrast, the SE-HPLC profile is quite consistent among all lots tested, and the respective content correlations demonstrate less variability. Figure 8 illustrates the SE-HPLC system's ability to capture protein integrity as seen by the similar shape of the 2004 and 2006 chromatograms.

Correlation between SE-HPLC and SRID

Potency measure by HA titres of influenza monovalent bulks, as determined by SE-HPLC and those obtained by the established SRID method were studied and compared to the values presented in the manufacturer's CoA. HA

concentration can be estimated by SE-HPLC using the following calculation concept:

$$\frac{[A]_{HA}}{[A]_{Total}} \propto \frac{[HA]_{CoA} \cdot [Total\ protein]_{CoA}}{[Total\ protein]_{CoA}} \propto [HA]_{HPLC}$$

As previously mentioned, SRID is the established method for HA content determination, ie. potency testing, and was the confirmatory method for HA quantification. Thus, the above gross calculation of HA content using SE-HPLC should also be comparable to SRID results. As shown in Table 8, the HPLC data on average gave potency values ~106% of those supplied by the manufacturer on the CoA, and ~119% of the SRID potency results.

Table 6: Results from Chromatograms Taken from 20µL Injections of Sample K on Different Days. The dates were the following: June 8/07, June 9/07, June 13/07, and June 15/07. Data represents the peak area over total area of chromatogram.

Peak Name	June 8,2007	June 9,2007	June 13,2007	June 15,2007	MEAN	STD DEV.
Peak # 1	5.4019	5.5295	6.6084	6.1828	5.931	0.567
Peak # 2	17.1499	17.0613	13.5871	21.879	17.419	3.405
Peak # 3	33.651	33.6539	36.5349	31.4994	33.835	2.066
Peak # 4	8.27	8.1902	6.2294	8.5577	7.812	1.067
Peak # 5	3.4886	3.3259	1.8227	3.527	3.041	0.817
Peak # 6	10.6054	10.753	8.9807	9.9625	10.075	0.806
Peak # 7	0.2476	0.2456	0.2334	0.4813	0.302	0.120
Peak # 8	20.9126	20.9354	26.0035	17.9103	21.440	3.357
Peak # 9	0.2731	0.3053			0.289	0.023

Note: Peak # 3 (bold) is putative HA peak

Table 7: Table illustrating potency for the same monovalent lots provided in 2004 and again in 2006. Potencies were obtained from manufacturer CoA and PID SRID results.

Year	Sample Names	CoA Potency (µg/mL)	Range Specificat. from CoA	PID SRID Results (µg/mL)	Age of sample during SRID (yrs)	Std Dev from Range	SRID% of label claim with 3yr old samples	Sample Names	CoA Potency (µg/mL)
2004	Vaccine A	411	report	343.400	3		83.552	N/A	N/A
	Vaccine B	574		620.610	3		108.120	Vaccine B'	534
	Vaccine C	741		769.740	3		103.879	Vaccine C'	411
	Vaccine D	718		636.560	3		88.657	Vaccine D'	371
	Vaccine E	604		492.098	3		81.473	N/A	N/A
	Vaccine F	449		423.460	3		94.312	N/A	N/A
	Vaccine G	619		518.381	3		83.745	N/A	N/A
	Vaccine H	555		505.548	3		91.090	N/A	N/A
	Vaccine I	632		503.926	3		79.735	N/A	N/A
	Vaccine J	649		520.731	3		80.236	N/A	N/A
S.D.		104.669	S.D.	118.33		S.D.	9.966	S.D.	84.949
						Mean	89.480		
						Coefficient of Var	11.138		

These data show that there is relatively good correlation between results obtained for the determination of HA content, by SRID and HPLC methods. Interestingly, the SRID data (derived from the CoA testing, at production), and the HPLC results were in closer agreement than comparisons made at times post-manufacture, for consistency testing. It is not yet clear in the studies if the HPLC method is unable to separate any degradation products, which may compromise HA content calculations.

This calculation concept has also been proven for 2005 monovalent lots of vaccine K, with data provided by Dr. Cynthia Allen, PID, CBE, BGTD, Health Canada, Feb.14, 2007.

The MicroBCA protein assay was used as a confirmatory method for the quantification of total protein content of the monovalent bulks and the results appear in **Table 9**. On average, there appears to be a systemic increase in the MicroBCA results, as compared to those provided by the manufacturer (**Figure 9**). However, this is a sufficiently reasonable trend for several reasons. The samples are 3 years old and are subject to degradation and aggregation in addition to the lack in consistent homogeneity in many biological substances such as vaccines. Of greater importance is the fact that the manufacturer used a different analytical method, Kjeldahl nitrogen analysis.⁷ Whereas the BCA assay measures the number of peptide bonds accessible

Figure 8: Juxtaposition of 3 Chromatograms Illustrating 20µL Injections of the Same Samples from 2004 and 2006 on the G4000SWxl Column to Show Manufacturing Consistency. (a) Sample B overlayed onto sample B', (b) Sample C overlayed onto sample C', (c) Sample D overlayed onto sample D'.

Table 8: Comparison of Potency Values of 10 Vaccine Monovalent Lots from 2004 Determined by SE-HPLC, SRID and Provided by CoA.

HPLC RESULTS								SRID RESULTS				
Vaccine	[HA] µgHA/mL	TI Protein in 1mL (µg/mL)	Ratio of HA TI Prot	% HA Protein	Mean	Calculated [HA]	% of CoA	% of SRID	[HA]	Calculated	% of CoA	
					HA peak area TI Area	(HA peak)/100*TI Prot (TI Area)	[HA] µgHA/mL	[HA] µgHA/mL	µgHA mL	% HA TI Protein	[HA] µgHA/mL	
A	411	1018	0.404	40.4	42.705	434.737	105.775	126.598	343.4	33.733	83.552	
B	574	1927	0.298	29.8	39.659	764.229	133.141	123.142	620.61	32.206	108.120	
C	741	2350	0.315	31.5	29.954	703.919	94.9958	91.449	769.74	32.755	103.879	
D	718	2072	0.347	34.7	40.863	846.681	117.922	133.009	636.56	30.722	88.657	
E	604	1755	0.344	34.4	33.79	593.015	98.1813	120.508	492.098	28.04	81.473	
F	449	1266	0.355	35.5	32.001	405.133	90.2301	95.672	423.46	33.449	94.312	
G	619	1584	0.391	39.1	45.395	719.057	116.164	138.712	518.381	32.726	83.745	
H	555	1506	0.369	36.9	39.605	596.451	107.469	117.981	505.548	33.569	91.090	
I	632	1625	0.389	38.9	39.153	636.236	100.67	126.256	503.926	31.011	79.735	
J	649	1788	0.363	36.3	33.524	599.409	92.3589	115.109	520.731	29.124	80.236	
Means							105.691	118.843	Mean			89.480
Difference							5.691	18.843	Difference			-10.520

N.B. All "Total Protein" data was obtained from CoA (column 3)

Legend:

- White: All results not highlighted are from ID Biomedical Certificate of Analysis (**CoA**)
- Yellow: Results and calculations taken from Pandemic Influenza Division **SRID** data
- Green: Results and calculations taken from Pandemic Influenza Division **HPLC** data

to the colour reagent, the Kjeldahl method quantifies the total nitrogen of both proteinaceous and non-proteinaceous compounds. It is known that interfering substances can influence the results obtained by the MicroBCA method and these are suspected causes of increased total protein values.

Optimization methods

PID's current approach to quantifying HA using SE-HPLC has proven feasible.

However, current efforts are focused on the ability to improve HA peak resolution on the SE-HPLC system since the G4000SWxl column yielded an insufficiently resolved HA peak, making it difficult for future quantification that would require isolation and collection of the HA peak. Several variables were assessed including: mesh size of the column beads; connecting several SE-HPLC columns together in series (tandem SE-HPLC), and alterations in the temperature and pH of the elution buffer to try to induce conformational changes in the HA molecule. **Table 10** illustrates the various manipulations made in order to optimize HA peak resolution. The most significant of these manipulations were the changes in column pore size as well as the use of two-dimension SE chromatography (using two columns in series). **Figures 10 and 11** are included to

Table 9: Comparison Between Total Protein Content of 10 2004 Monovalent Lots from MicroBCA Assay and CoA.

Vaccine	Total Protein from CoA	Total Protein from MicroBCA	% of CoA Tot. Protein
A	1018	1016.3	99.83
B	1927	2209.65	114.67
C	2350	2947.35	125.42
D	2072	N/A	N/A
E	1755	2312.95	131.79
F	1266	1845.45	145.77
G	1584	2207.4	139.36
H	1506	2046.35	135.88
I	1625	2511	154.52
J	1788	2387.6	133.53
Mean			131.20

Note: N/A—no sample remaining to perform protein assay.

Table 10: The Various Parameter Manipulations and Combinations in Order to Maximize Putative HA Peak Resolution.

Optimization Methods	Various combinations of Methods										
Columns:	----- 2-D HPLC -----										
• TSK G4000SWxl, pore size 450Å	X					X (1 st)	X (1 st)	X (1 st)	X (1 st)	X (1 st)	X (1 st)
• TSK G3000SWxl, pore size 250Å			X		X X		X (2 nd)	X (2 nd)	X (2 nd)	X (2 nd)	X (2 nd)
• TSK G2000SWxl, pore size 125Å		X				X (2 nd)					
• 60cm G3000SWxl, pore size 250Å				X							
Mobile phase properties:											
• pH 7	X	X	X	X	X	X	X		X	X	X
• pH 5								X			
• treat with DTT, 26°C									X		
Sample Treatments											
• Heated to 60°C										X	
• Boiled											X
Putative HA Peak Properties for Sample B											
• Presence of HA in SRID fraction (min)	8, 9, 10	5, 6	4. 5 -7	N/A	N/A	N/A	15.5	N/A	N/A	N/A	N/A
• Retention Time of putative HA peak on chromatogram (min)	9.17	6. 37	6.36	10.81	UN	13.52	15.32	N/A	N/A	N/A	N/A
• HA peak area/Tl. area	47.23	5. 68	25.36	25.99	UN	28.69	28.07	N/A	N/A	N/A	N/A
• Peak width (sec)	85.6	0.0	40.3	71.5	UN	0.0	45.7	N/A	N/A	N/A	N/A
• Injection date	Aug. 1/07	Aug.15/07	Aug. 29/07	Oct. 2007	Sept. 2007	Sept. 2007	Sept. 2007	N/A	N/A	N/A	N/A
Putative HA Peak Properties for Sample L											
• Presence of HA in SRID fraction (min)	N/A	N/A	N/A	13-16	N/A	N/A	15.5-16	N/A	N/A	N/A	N/A
• Retention Time of putative HA peak on chromatogram (min)	9.70	N/A	6.29	10.79	N/A	13.58	15.04	14.08	14.99	13.97	13.64
• HA peak area/Tl. Area	48.51	N/A	30.85	28.53	N/A	23.49	31.52	15.31	28.69	15.95	17.00
• Peak width	48.6	N/A	37.6	73.8	N/A	0.0	51.2	58.2	54.8	81.0	0.0
• Injection date	June 29/07	N/A	Aug. 29/07	Oct. 2007	N/A	Sept. 2007	Oct. 15/07	Oct. 15/07	Oct. 10/07	Sept. 25/07	Sept. 26/07

illustrate the differences in sample chromatographic profiles run on different columns.

The data suggests that by changing separation conditions, such as column resolution range, HA (which has a molecular mass of ~83kDa when uncleaved⁴ and in its monomeric form) elutes at different times and for different durations during a run. This was directly confirmed by collecting fractions from the sample injections and confirming the presence of HA in the alleged HA peaks using SRID.

The putative HA peak retention times for column G4000SWxl, G2000SWxl, G3000SWxl, and G4000SWxl-G3000SWxl are 9.17min, 6.37min, 6.36min, and 15.32min, respectively. Columns G4000SWxl-G3000SWxl in series were evaluated as being the optimal SE-HPLC setup, taking

into account location of putative HA peak, width of HA peak, and chromatographic profile.

G4000SWxl's putative HA peak resolution was poor and contained an unresolved shoulder peak, as seen in **Figure 10(a)**. In addition, the width of the peak was very wide as proven by SRID, which contained detectable amounts of HA in fractions eluting at minutes 8, 9, and 10.

Column G2000SWxl demonstrated a more narrow HA peak width (with a SRID detection from minutes 5 and 6), however its retention time of 6.36min was far too close to the elution of high molecular mass components, due to its proximity to the first spike on the chromatographic profile in **Figure 10(b)**. This suggests the possibility of contaminating proteins in the putative HA peak. It appears that neither of

Figure 9: Comparing Total protein Content as per MicroBCA assay and CoA Results of 2004 Monovalent Lots.

Figure 10: SE-HPLC analysis of sample B (100µL injection) eluting from 3 different SE columns. Rectangular boxes indicate putative HA peaks. (a) Sample run on TSK G4000SWxl column, pore size 450Å. (b) Sample run on TSK G2000SWxl, pore size 125Å. (c) Sample run on TSK G3000SWxl, pore size 250Å. (d) All three profiles overlayed for comparison.

Figure 11: SE 2-D analysis of sample M4017NC (20µL injection). (a) Sample run on TSK G3000SWxl + G4000SWxl in series. (b) Sample run on TSK G4000SWxl + G3000SWxl in series. (c) A display of (a) in blue, overlaid with profiles generated from columns G4000SWxl (red) and G3000SWxl (green).

these columns were optimal for identifying and isolating the putative HA peak.

Based upon the above analysis, the G3000SWxl is expected to yield more optimal results since HA should elute between the previous two retention times. **Figure 10(c)** demonstrates that the HA peak is better resolved than in the G2000SWxl column. However HA was detected in quite a vast range by SRID, eluting from minutes 4.5-7. In addition, its retention time of 6.37 is unexpectedly low and near that of the G2000SWxl column. It appears that the G3000SWxl gives a pattern that is not easily correlated to those of the G2000SWxl and G4000SWxl. It is speculated that this may be related to secondary interactions that may play a greater role with this particular type of column.

This led to the use of two columns in series, as modeled in **Figure 11**. The use of tandem HPLC was suspected to separate out the HA peak even further. The first column would perform an initial separation, and the second column would further isolate the sorted vaccine sample constituents. SRID results detected HA in minutes 15.5, suggesting the putative HA peak width is the smallest detected to date, and correlates well with the chromatogram retention time of 15.32min. The inversion of columns G4000SWxl and G3000SWxl in series yielded only minimal differences. **Figure 11(b)** illustrates a slightly enhanced resolution immediately prior to the elution of HA at 15.32min, suggesting that some contaminating proteins from the HA

peak have been further separated out, thus facilitating HA identification, isolation, and ultimately quantification.

Recommendations and Future Prospects

The use of a tandem SE-HPLC method, along with the conventional SRID method to quantitate HA has proven effective in identifying the peak containing HA, and in correlating HA content determined by SE-HPLC with SRID potency results. Now that this correlation has been established, more data must be assessed to further validate the method for HA quantification. Of importance will be its ability to assess samples of H5N1, or other identified strains of prototype vaccine lots, for the identification and quantification of HA for a potential pandemic strain. However, in order to estimate the precise amount of HA as a potency measure, sufficient for regulatory purposes, a standard HA preparation (which is unlikely), or alternatively, a measure of the amount of contaminating proteins in the HA peak is required. This will have implications for the actual pandemic vaccine since the amount of contaminating proteins will be unknown. It therefore remains to be investigated whether the ratio of HA: contaminating protein in the peak can be estimated by the use of other separation techniques.

Reverse-phase HPLC (RP-HPLC) for this application has already been explored by Kapteyn in 2006, and more recently by Garcia-Cañas and colleagues. Garcia-Cañas et al.

were able to separate HA from other viral proteins into a single peak with good reproducibility, but were limited by their ability to quantitate the amount of HA present. Although Kapteyn et al. successfully quantified HA, samples tested were not formaldehyde treated. Considering that egg-based influenza vaccines are routinely inactivated with formaldehyde (which introduces irreversible cross-linking of the HA subunits in their manufacturing process), such an analytical method is not directly approved. However, along the line of the Garcia-Cañas et al. study, it appears that RP-HPLC may be used to identify contaminating proteins of the HA peak. To date, preliminary work has begun to investigate the applicability of RP-HPLC to identify the amount of co-eluting proteins in the HA peak.

Capillary electrophoresis (CE) is another physicochemical technique that may be used for contaminating protein identification. Its main advantage over HPLC is its minimal sample preparation time and small amounts of sample and solutions required, making it a competitor for the quick assessment of sample contents, including molecular weight, aggregates, and modified forms of the protein samples. The development of alternative physicochemical methods is imperative for the preparation of an upcoming pandemic and SE-HPLC's ability to identify and potentially quantitate influenza vaccine potency may serve as an invaluable tool to pandemic preparedness strategies.

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