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Research Paper

Concentration Determination of a Recombinant Vaccine Antigen Adsorbed onto an Alum Adjuvant by Chemiluminescent Nitrogen Detection

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Purpose. A chemiluminescent nitrogen detector (CLND) has been evaluated for determining the concentration of an aluminum-adsorbed recombinant vaccine antigen.
Methods. Quantification of the antigen was based upon several nitrogen-containing compounds used to calibrate the CLND. All calibrants (6.75–400 μg/ml) generated linear standard curves, with slopes being directly proportional to the % nitrogen. The limit of quantification (LOQ) was determined to be 6.75 μg/ml based on the performance of the antigen standard curve, and the limit of detection (LOD) was defined by setting the CLND minimum peak area to 40,000 U. The CLND was capable of analyzing antigen-adjuvant suspensions (adsorbed + unbound antigen) without any sample pretreatment. To measure unbound antigen, the suspension was centrifuged and an aliquot of supernatant removed for analysis; the difference between these two measurements was the amount of adsorbed antigen.
Results. The adjuvant exhibited no significant matrix effect. Samples were analyzed in triplicate with observed relative standard deviation values ranging from 0.065% to 10.0%. The most accurate concentrations of the antigen were recovered relative to the antigen itself and to glycine as standards.
Conclusion. This methodology provides a direct measurement of the concentration of a vaccine antigen adsorbed onto an aluminum adjuvant.

KEY WORDS: adjuvant; adsorbate vaccine; alum; chemiluminescent nitrogen detection; nitrogen content.

INTRODUCTION

Vaccine antigens may be formulated with aluminum-containing adjuvants for enhancing immune responses (1–10). The physical and adsorption characteristics of aluminum adjuvants have been extensively investigated (1–18). Antigen-adjuvant binding (adsorption) properties have been generally evaluated by conducting desorption studies, which have included aging (5) and treatment with surfactants (13), pH (14), or interstitial fluid (15,17–19). In these studies, the desorbed antigen was quantified by using bicinchoninic acid (BCA) (11–15,18) and Lowry (17) colorimetric protein assays. These colorimetric assays are unable to directly quantify the adsorbed antigen due to the turbidity caused by the adjuvant suspension; therefore the adjuvanted antigen is centrifuged, and the nonadsorbed and desorbed antigen in the supernatant is quantified. ELISA also has been used (16), especially after exposure of the adjuvanted antigen to interstitial fluid (18).

The Kjeldahl Nitrogen method is among the most commonly used other methods of protein determination in complex matrices. The Kjeldahl method has been applied to proteins in food products: lactalbumin in whey (20), proteins in oilseed meals (21), diary products (22–23), and wild fruits (24). However, the use of mercuric oxide catalyst and the need to perform multiple manipulations make this method cumbersome and time-consuming. To decrease the lengthy analysis time, the digestion method has been recently modified by using microwave digestion (25).

A chemiluminescent method has been developed as an alternative to the traditional Kjeldahl method. A chemiluminescent nitrogen detector (CLND) coupled to a high-performance liquid chromatograph (HPLC-CLND) as well as a stand-alone CLND unit are commercially available. The operational principle of the CLND in either configuration (stand-alone or coupled to an HPLC) is similar. The sample is introduced into a combustion tube packed with an oxidative catalyst under an Argon carrier gas. In the combustion tube, high-temperature oxidation (800–900°C) occurs, during which all chemically bound nitrogen is converted to nitric oxide (NO). The NO is then transferred into the chemiluminescense detector, where it is combined with ozone (O_3) to form excited nitrogen dioxide (NO_2*). Rapid decay of the NO_2* produces light in the 590–2900 nm range, which is amplified by a photomultiplier tube (PMT) and detected.

$$NO + O_3 \rightarrow NO_2^* + O_2 \rightarrow NO_2 + O_2 + h\gamma \quad (1)$$

The peak area is obtained by integrating the PMT signal, which is directly proportional to the nitrogen content. By generating a calibration curve using known standards, the unknown analyte concentration can be determined.

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The CLND is useful for measuring the amount of chemically-bound nitrogen in a sample of unknown concentration. All nitrogen-containing species will contribute to the signal, hence there is no specificity toward mixtures of compounds containing nitrogen unless they are separated as with HPLC-CLND. The HPLC-CLND has been used for the analysis of peptides (26–27) and food-grade proteins (28) as well as determining the mass-balance of small nitrogen-containing molecules undergoing forced degradation (29) and characterization of combinatorial organic molecule libraries (30). Although the HPLC-CLND can provide specific and sensitive detection, samples must be soluble for injection; thus, adsorbed antigens with aluminum adjuvants cannot be analyzed using this configuration.

MATERIALS AND METHODS

Chemiluminescent nitrogen analysis was performed with a TN-110 elemental analyzer composed of the following components: automatic boat controller (ABC), TN-110 main module and nitrogen detector (COSA Instrument Corporation, Norwood, NJ, USA). The analysis parameters for the CLND are listed in Table I.

E.M. Sergeant Pulp and Chemical Co. (Clifton, NJ, USA) supplied the aluminum-containing adjuvant (2% Alhydrogel bulk solution). The standards L-histidine, L-arginine, and glycine were purchased from Fluka BioChemika (St. Louis, MO, USA) and L-tryptophan and bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, MO, USA). The purified recombinant vaccine antigen (reference material) in addition to aluminum-absorbed antigen were obtained from the Process R&D group at Shire Biologics. All standards were dissolved with buffer (10 mM sodium phosphate-10 mM sodium chloride, pH 7.2) to a stock solution of 400 μg/ml. Serial dilutions were then prepared from this stock solution to concentrations of 200, 100, 50, 25, 12.5, and 6.75 μg/ml. The 2% (w/v) bulk solution of Alhydrogel was diluted to 0.10, 0.14, 0.18, 0.22 and 0.26% (w/v) using the sodium phosphate-sodium chloride buffer. For total antigen determination, the vial containing antigen-adjuvant suspension was homogenized using a bench top vortex mixer, and an aliquot of the homogenized suspension was directly loaded onto the ABC. Unbound antigen was measured by centrifugation of the homogenous suspension, after which an aliquot of the supernatant was removed for analysis. Centrifugation was conducted on a bench-top centrifuge at 13,000 × *g* for 10 min. A 50-μl gas-tight syringe was used to deliver a 20-μl aliquot (in triplicate) onto the sample boat contained in the ABC. Each injection required six minutes to analyze, and all samples were analyzed in triplicate.

Table I. CLND Analysis Parameters

Detector settings			
Sensitivity			High
Integration delay (s)			0
Slope start			200
Slope end			20
End threshold			0.1
Detection time			5
Scan rate			5
Blank			0
D/dt gain			10
Normal end			ON
Timer end (s)			300
Base correct			ON
Minimum area			40,000
Base line (%)			25
Interval (s)			999
Gas settings			
Ar/O2 flow (ml/min)			400
Ar time (s)			5
O2 flow (ml/min)			300
O2 time (s)			600
O3 flow (ml/min)			133
Heater settings			
Temp 1 (°C)			800
Temp 2 (°C)			900
ABC program settings			
Position 1	120	Time (s)	40
Position 2	150	Time (s)	15
Position 3	0	Time (s)	0
End time (s)	120	Cool time (s)	30

RESULTS AND DISCUSSION

Calibration Curves

Several amino acids and proteins (including the vaccine antigen) were investigated as standards to calibrate the CLND. All calibration curves exhibited a linear response over the concentration range evaluated. For this study, all regression lines were set to an intercept of zero. The properties and regression analysis, precision and accuracy of each standard are listed in Tables II and III, respectively. Although manual injections are performed, precision is very good. Even though 10% relative standard deviation (RSD) is observed with the tryptophan standard (lowest % nitrogen), all other % RSD's were ≤5.2% and recoveries ranged from 85.3–105.3%. A direct correlation exists between % nitrogen and the slopes of each standard (excluding the vaccine antigen). A plot of slope vs. % nitrogen results in a regression line defined by the equation $y = 87.005x + 62.488$ with a correlation coefficient 0.998 Based on this linear relationship, it is possible to determine the slope value of any nitrogen-containing molecule, provided the % nitrogen is known.

The vaccine antigen did not fit well into the model, although linearity was demonstrated. A slope value of 1768 was experimentally determined; however a slope value of 1568 resulted from the regression equation. Quantification of the antigen was based upon the experimental slope value.

Adjuvant Analysis

Adjuvant suspensions and corresponding supernatants were analyzed to determine the nitrogen contribution. Nitro-

Table II. Nitrogen-Containing Compounds Used as Standards

Standard	Molecular weight (Da)	Number of nitrogen	Percent nitrogen
L-Tryptophan	204	2	13.7
BSA	66,000	780	16.4
Vaccine antigen	70,000	862	17.3
Glycine	75	1	18.7
L-Histidine	155	3	27.1
L-Arginine	174	4	32.2

Table III. Regression Values, Precision and Accuracy Ranges for Standards

Standard	Slope	R^2	Precision range* (%RSD)	Accuracy range[a] (% Recovery)
L-Tryptophan	1267	0.9996	0.73–10.0	88.9–105.3
BSA	1474	0.9996	0.74–3.1	96.9–103.6
Vaccine antigen	1768	0.9994	0.10–2.2	95.7–105.3
Glycine	1706	0.9996	0.065–5.2	85.3–105.4
L-Histidine	2384	0.9996	0.45–4.5	88.9–105.3
L-Arginine	2883	0.9998	0.32–3.6	90.1–102.0

[a] The precision and accuracy ranges are the low and high values for all standard concentrations.

gen was detected in all adjuvant suspensions as well as supernatants (Table IV). Because the chemical composition of the nitrogen-containing species associated with adjuvant is not known, quantification was based on BSA, vaccine antigen and glycine. Differences in nitrogen concentration are clearly observed, although there is closer agreement between those of glycine and the antigen due to the closeness of their slope values. The glycine may be more representative of the level of nitrogen, since it is assumed that the source of the adjuvant nitrogen originates from a one-nitrogen-containing compound(s); however the % nitrogen is not known. The supernatants exhibit nitrogen concentration values that are in close agreement to those of the suspensions. This indicates that the nitrogen source can be extracted or possibly washed off the adjuvant. There is a direct correlation of nitrogen peak-area relative to % adjuvant (suspension: $y = 41160x + 47658$, supernatant: $y = 42269x + 20349$); thus the contribution of nitrogen can be subtracted from the total nitrogen concentration of the adsorbed antigen.

Adsorbed Vaccine Antigen Analysis

Two vaccine antigen (200 μg/ml) formulations were evaluated, an aqueous and an adjuvanted formulation. An aliquot of the aqueous formulation was loaded onto the ABC. For the adjuvanted formulation two measurements were performed: total antigen (bound + unbound) and unbound antigen. The unbound antigen was measured by centrifugation of the suspension and then removing an aliquot of the supernatant for analysis. The antigen concentrations determined for several formulated batches are listed in Table V. Early in development, antigen concentrations were based upon BSA, due to the unavailability of purified antigen reference material. Measured antigen concentration relative to BSA show greater (15–30%) than expected levels. The expected concentration, prior to adsorption, was 200 μg/ml based on A280 measurements (data not shown), for both aqueous and adjuvanted formulations. This proved that the BSA, although of similar molecular weight and % nitrogen as the antigen, was not the most appropriate analytical standard.

Subsequent studies included amino acids containing 1, 2, 3, and 4 nitrogen atoms and the actual vaccine antigen as calibrants. The amino acids spanned a range of % nitrogen from 13.7–32.2 (Table II). The amino acid most comparable to the antigen in % nitrogen was glycine. The antigen concentrations relative to glycine were in closer agreement with the expected levels, with a deviation of only 4–21%. When the vaccine antigen reference material became available, quantification of the formulated antigen exhibited reduced deviations (–2% to 14%) relative to the antigen itself. Obviously, using the antigen reference material would be the ideal standard, pending availability and purity.

Table IV. Nitrogen Determination of Adjuvant

Sample	Area	Nitrogen concentration (μg/ml), relative to standard curve of BSA	Antigen	Glycine
Neat				
0.10% Alhydrogel	245,738	8.3	6.9	7.2
0.14% Alhydrogel	366,731	12.4	10.4	10.8
0.18% Alhydrogel	385,774	13.1	10.9	11.4
0.22% Alhydrogel	503,135	17.1	14.2	14.8
0.26% Alhydrogel	589,142	20.0	16.7	17.4
Supernatant				
0.10% Alhydrogel	229,935	7.8	6.5	6.8
0.14% Alhydrogel	335,327	11.4	9.5	9.9
0.22% Alhydrogel	468,818	15.9	13.3	13.8
0.26% Alhydrogel	585,875	19.9	16.6	17.3

Ultraviolet Spectroscopy Measurements

Antigen concentrations of aqueous and supernatant solutions were measured by absorbance at 280 nm (A_{280}). The A_{280} concentrations are compared to CLND determinations (Table VI). The CLND concentrations based on the BSA standard show the greatest deviations (17–24% for aqueous antigen and supernatant) with respect to A_{280}. Nevertheless, very good agreement of CLND concentrations was observed for the aqueous antigen formulations (≤4%) and supernatant (≤8%) relative to the antigen.

Table V. Formulated Antigen Concentration Data

Sample	Area	Antigen concentration (μg/ml), relative to standard curve of BSA	Antigen	Glycine
Aqueous samples				
Formulation 1	1,205,879	241.0	200.8	209.4
Formulation 2	1,211,723	248.1	206.7	215.7
Formulation 3	6,789,688	257.1	214.3	223.5
Formulation 4	6,528,570	262.5	218.8	228.2
Formulation 5	6,397,843	256.2	213.5	222.7
Adjuvanted samples				
Formulation 1	1,352,656	235.3	196.1	215.4
Formulation 2	1,434,726	245.5	204.6	224.2
Formulation 3	7,361,601	251.2	209.3	229.2
Formulation 4	7,118,890	280.1	233.4	254.3
Formulation 5	6,316,664	249.5	207.9	227.7
Supernatant of adjuvanted samples				
Formulation 1	753,169	156.7	130.6	146.1
Formulation 2	798,060	161.0	134.2	149.8
Formulation 3	4,877,544	182.9	152.4	168.9
Formulation 4	4,794,923	179.9	149.9	166.3
Formulation 5	4,986,912	187.5	156.2	172.8

Table VI. Comparison of Concentration Values Obtained by Nitrogen Analysis and A_{280}

Sample	A_{280} (μg/ml)	Antigen concentration (μg/ml), relative to standard curve	
		BSA	Antigen
Aqueous samples			
Formulation 1	201.2	241.0	200.8
Formulation 2	205.6	248.1	206.7
Formulation 3	205.1	257.1	214.3
Supernatant of adjuvanted samples			
Formulation 1	121.1	156.7	130.6
Formulation 2	124.9	161.0	134.2
Formulation 3	139.5	182.9	152.4

CONCLUSIONS

The TN-100 chemiluminescent nitrogen analyzer has been used for the concentration determination of a vaccine antigen adsorbed onto an aluminum-containing adjuvant. During early process and formulation development studies, the antigen concentration was calculated relative to BSA as the calibrant, as antigen reference material was not available. BSA was chosen as the standard on the basis of similar molecular weight and % nitrogen to the vaccine antigen. The CLND was further calibrated against additional standards (amino acids and the antigen). All calibration curves produced linear responses from 6.75 to 400 μg/ml as well as very good precision and accuracy. The amino acid standards expanded the range of quantification as a function of % nitrogen. The % nitrogen of the calibrants was used to determine which amino acid (other than the antigen itself) was appropriate to quantify the antigen. For this antigen, the amino acid glycine was most appropriate as a calibrant.

Suspensions of the antigen-adjuvant were directly injected into the analyzer. The adjuvant did not interfere with the operation of the CLND. Given the background level of nitrogen, analysis of the aluminum enabled the background nitrogen level to be subtracted from the antigen-adjuvant level. Unbound antigen was measured after centrifugation of the suspension and analysis of the supernatant. The accuracy of the CLND was measured against A_{280} determinations for the aqueous and desorbed antigen, which agreed to within 4% and 8%, respectively. Thus measurements were accurate and precise.

The CLND was insensitive to matrix effects, thus substantiating that this methodology can be applied to essentially any antigen-adjuvant formulation, provided that the background nitrogen is accounted. Due to the non-availability of the antigen reference material during early formulation adsorption studies, antigen measurements can be performed relative to a comparable standard of similar % nitrogen. Not only was sensitive analysis achieved but also analysis time was shortened.

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