Direct Analysis of Multicomponent Vaccine Adjuvants by HPLC with Charged Aerosol Detection

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Key Words
Biotherapeutics, Glycoside, Lipid, Saponin, Universal Detection

Goal

To develop a fast and sensitive HPLC method suitable to measure the strength and purity of immunological adjuvant formulations.

Introduction

A vaccine adjuvant is any substance that helps promote the effectiveness of a vaccine by reducing the amount or frequency of the required dose, by prolonging the duration of immunological memory, or by modulating the involvement of humoral or cellular responses. This functional definition of adjuvants encompasses a very diverse group of substances whose chemical structures and mechanisms of action vary widely. Adjuvants for human or animal vaccines are typically subjected to rigorous standards of analysis including quantification of strength, purity, stability and degradation behavior, even though they are not currently regulated in the same manner as active pharmaceutical ingredients in the US. Complicating such analysis, many adjuvants under investigation contain components that are not readily analyzed by traditional HPLC with UV detection, including triterpenoid glycosides, sterols, fatty acids, and phospholipids that lack suitable UV chromophores.

In this work, the lack of a detectable chromophore in several adjuvant components and degradation products was overcome by using HPLC with charged aerosol detection, a detector that can measure any non-volatile compound. As response is similar for all compounds and independent of chemical structure, charged aerosol detection is able to measure intact adjuvant species along with degradation products and potential impurities, yielding accurate estimates of relative concentration even in the absence of pure primary standards.

The charged aerosol detector is a sensitive, mass-based detector, especially well-suited for the determination of any nonvolatile analyte independent of chemical characteristics. The detector nebulizes the mobile phase to create aerosol droplets. These droplets evaporate in the drying tube to leave dry analyte particles, which become charged in the mixing chamber. The charge is then measured by a highly sensitive electrometer, providing reproducible, nanogram-level sensitivity. This technology has greater sensitivity and precision than evaporative light scattering detection and refractive index detection, is fully gradient compatible, and is simpler to operate than a mass spectrometer.

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Experimental Conditions

Instrument

- Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system including:
  - DGP-3600RS Pump
  - SRD-3600 Solvent Rack with Degasser
  - WPS-3000TRS Thermostatted Analytical Split-Loop Autosampler
  - TCC-3000RS Thermostatted Column Compartment
  - DAD 3000RS Diode Array Detector
- Thermo Scientific™ Dionex™ Corona™ Veo RS™ Charged Aerosol Detector
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 6.8 (SR13)

Conditions		
Column:	Thermo Scientific™ Hypersil GOLD™ PFP 1.9 µm column, 2.1 × 100 mm	
Column Temp:	45 °C	
Flow Rate:	0.50 mL/min	
Injection Vol.:	2 µL	
Sample Temp.:	8 °C	
DAD Detector:	Wavelength:	210 nm
	Response Time:	0.5 s
	Data Collection Rate:	20 Hz
Veo Detector:	Evaporation Temp.:	50 °C
	Power Function:	1.0
	Data Collection Rate:	20 Hz
	Signal Filter:	5 s
Mobile Phase A:	0.1% formic acid in water	
Mobile Phase B:	0.1% formic acid in 10:90 acetonitrile:reagent alcohol	
Gradient:	Time, %B: -5, 35; 0, 35; 8, 90; 13, 90	

Consumables

- Glass autosampler vials, 2 mL, with PTFE septa
- Glass vials, 4 mL, with PTFE septa

Standards

- Cholesterol, >99%, Sigma-Aldrich® C8667
- 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), >99%, Sigma-Aldrich P0763
- 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, (16:0 Lyso-PC), >99%, Avanti® 855675
- Saponins, 25–35%, Sigma-Aldrich S4521

Reagents

- Water, deionized 18.2 MΩ-cm
- Acetonitrile, Optima LC/MS, Fisher A955
- Formic acid, Optima LC/MS, Fisher A117-50
- Reagent alcohol, J.T.Baker® 9229-03

Samples

- AbISCO®-100, PN 20-110-101, ISCONOVA, Uppsala, Sweden

Standard Preparation

Single Component Standards

Prepare diluent by mixing 30 mL of reagent alcohol with 70 mL of deionized water. Prepare a 2 mg/mL solution of purified saponins by transferring 20.0 mg of saponins to a 10 mL glass volumetric flask. Add about 10 mL diluent, swirl gently to dissolve, and bring to volume with diluent. Note that the nominal saponin content varies from 20 to 35%, so this standard allows only an estimate of the saponin content of the adjuvant that is being analyzed.

Prepare a 2 mg/mL solution of cholesterol by transferring 20.0 mg of cholesterol to 10 mL glass volumetric flask. Add about 10 mL reagent alcohol, swirl gently to dissolve, and bring to volume with reagent alcohol.

Do likewise for DPPC and Lyso-PC. Standards can be stored at 4–8 °C for one month.

Combined Standard

Prepare a combined standard containing 400 µg/mL each of saponin solution, cholesterol and DPPC, and 100 µg/mL Lyso-PC, by pipetting 1.0 mL each of saponin solution, cholesterol and DPPC single component standards and 0.25 mL of Lyso-PC single component standard into a 5 mL glass volumetric flask. Mix and bring to volume with diluent. Standard can be stored at 4–8 °C for one month.

Calibration Standards

Prepare calibration standards at 400, 200, 160, 80, 40, 20, 10, 3, and 1.5 µg/mL by diluting appropriate volumes of the combined standard with diluent solution. It is convenient to use 4 mL glass vials with PTFE septa. Prepare fresh for each analysis. The dilution scheme used for this work is shown in Table 1 below.

Sample Preparation

Pipette 100 µL of AbISCO-100 into a 2 mL glass autosampler vial, add 400 µL of deionized water and gently mix.

Results and Discussion

Chromatography

For this work a standard mixture was prepared containing semi-purified saponins from the Chilean soapbark tree (*Quillaja saponaria Molina*), cholesterol, and DPPC. This standard approximates the composition of several vaccine adjuvants currently under development. A common degradation product resulting from hydrolysis of DPPC was also included, namely 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PC). This standard mixture was analyzed to determine method performance for the adjuvant components.

As seen in Figure 1, all components elute within 12 min from the Hypersil GOLD PFP column with good resolution. The charged aerosol detector is able to detect all components and several degradation products. The main degradation product, Lyso-PC, elutes at 6.4 min. A few lesser impurities or degradation products elute between Lyso-PC and cholesterol. Some of these may be cholesterol oxidation products. One advantage of charged aerosol detection is that the amount of impurities can be estimated with good accuracy even if authentic standards are not available, especially when the inverse gradient approach is used.[1] For example, the peak eluting immediately before cholesterol has a peak area equal to 4.1% of the cholesterol peak and so represents an impurity level of 3.2 µg/mL.

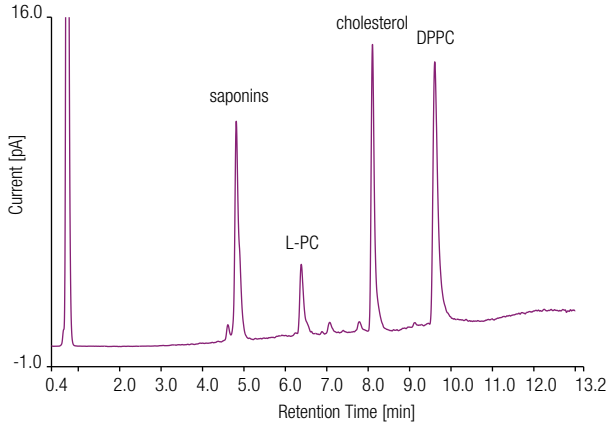

Figure 1: Chromatogram of a synthetic adjuvant standard obtained by reversed phase HPLC with charged aerosol detection.

A real adjuvant sample was also analyzed. AbISCO-100 is a suspension of purified saponins from *Quillaja saponaria Molina*, cholesterol from sheep wool, and egg phosphatidyl choline in phosphate buffered saline.[2,3] As seen in Figure 2, the real adjuvant sample exhibits a more complex elution profile than the standard as a result of differences in the saponin and phospholipid content. The saponins comprise a group of structurally

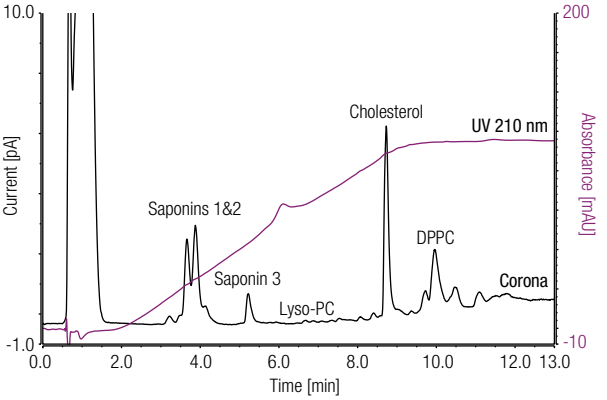

Figure 2: Chromatogram of AbISCO adjuvant obtained by reversed phase HPLC with either charged aerosol detection (black trace) or UV detection at 210 nm (blue trace).

Table 1: Calibration Standard Preparation.

Target Concentration (µg/mL)		Volume of Combined Standard (µL)	Volume of Diluent (µL)	Final Volume (µL)
Saponins, Cholesterol, DPPC	Lyso-PC			
400	100		n/a	n/a
200	50	1000	1000	2000
160	40	400	600	1000
80	20	200	800	1000
40	10	100	900	1000
20	5	50	950	1000
10	2	25	975	1000
3	0.75	15	1985	2000
1.5	0.38	15	3985	4000

related triterpenoid glycosides that typically show chromatographic differences as a result of both natural variation and differences in the purification process. The egg phosphatidyl choline includes DPPC along with similar phospholipids differing in fatty acid type and position. The UV detector shows poorer sensitivity to the analytes than the charged aerosol detector and a greater baseline shift as a result of the mobile phase gradient.

Performance

To evaluate method precision, ten injections were made of a standard containing 80 µg/mL of each of the four analytes. Table 2 presents a summary of the method's precision of retention time and peak area. Calibration curves for Lyso-PC and the three major components of AbISCO are presented in Figures 3–6. The data were fitted to a quadratic equation, yielding coefficients of determination, R^2, greater than 0.999 for all four analytes. Table 3 presents a summary of the method's calibration performance including the coefficients of determination and the limits of detection for the three major components of AbISCO and the degradation product Lyso-PC.

Table 2: Retention time and peak area precision of method for direct determination of multi-component adjuvants by HPLC with charged aerosol detection.

Analyte	Ret. Time (min)	Amount (µg/mL)	Ret. Time Precision[1] (% RSD)	Peak Area Precision[1] (% RSD)
Saponins	4.8	74.1	0.05	1.5
Lyso-PC	6.4	61.3	0.05	0.87
Cholesterol	8.1	75.7	0.02	1.1
DPPC	9.6	76.5	0.02	0.67

[1] for n = 10 replicates

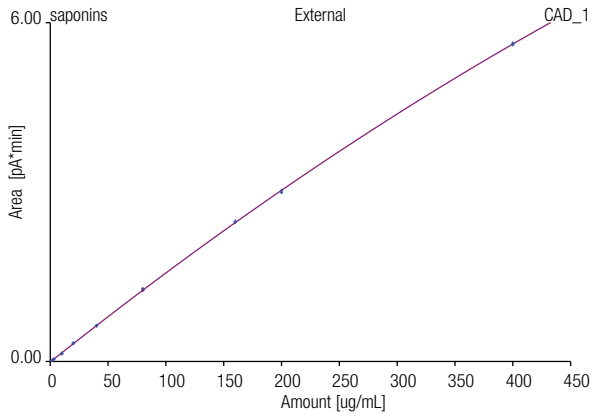

Figure 3: Calibration curve of saponins determined by HPLC with charged aerosol detection.

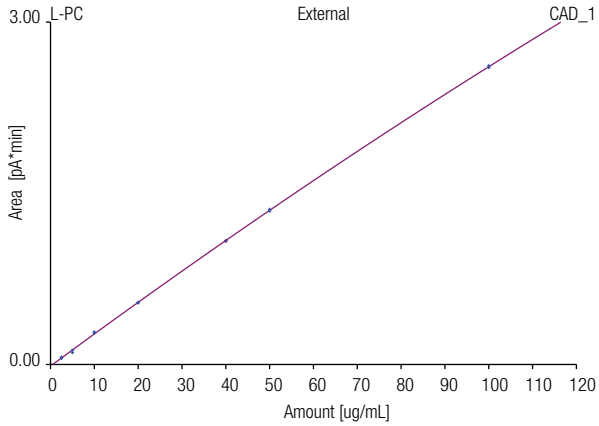

Figure 4: Calibration curve of Lyso-PC determined by HPLC with charged aerosol detection.

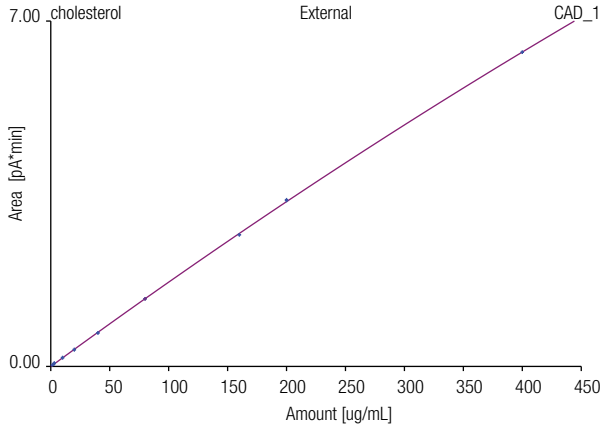

Figure 5: Calibration curve of cholesterol determined by HPLC with charged aerosol detection.

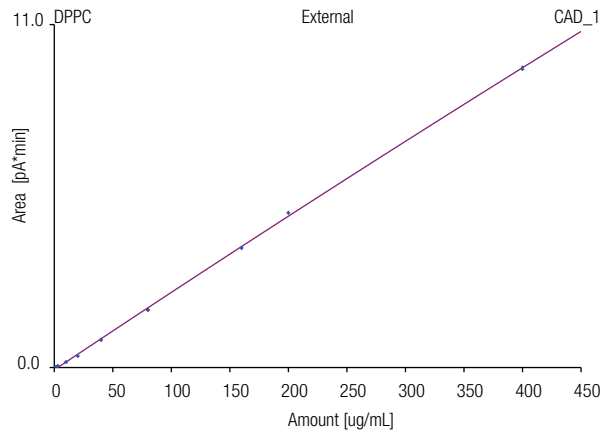

Figure 6: Calibration curve of DPPC determined by HPLC with charged aerosol detection.

Table 3: Calibration range and limits of detection of method for direct determination of multi-component adjuvants by HPLC with charged aerosol detection.

Analyte	Ret. Time (min)	Cal Range (µg/mL)	R^2*	LOD[1] (% RSD)
Saponins	4.8	1.5–400	0.9999	6.3
Lyso-PC	6.4	0.38–100	0.9998	2.3
Cholesterol	8.1	1.5–400	0.9999	6.4
DPPC	9.6	1.5–400	0.9996	16

** 7 levels, in duplicate, quadratic fit with no offset*

[1] Hubaux-Vos method

Conclusion

The HPLC method developed to analyze adjuvants such as AbISCO is precise, with retention time precision better than 0.1% RSD and peak area precision between 0.8 and 1.3% RSD for the major components.

Charged aerosol detection enables sensitive measurement of adjuvant components not amenable to detection by UV absorbance. Detection limits for saponins, cholesterol, and DPPC were in the low µg/mL (ng on-column) range.

By responding uniformly to structurally diverse compounds, charged aerosol detection is able to measure intact adjuvant species along with degradation products and potential impurities, yielding good estimates of relative concentration, even in the absence of pure primary standards.

References and Acknowledgements

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