Direct Analysis of Multicomponent Adjuvants by HPLC with Charged Aerosol Detection

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Overview

Purpose: To develop fast and sensitive HPLC methods suitable to measure the purity of immunological adjuvant formulations.

Methods: Adjuvant mixtures and individual components were analyzed by ultra-high performance liquid chromatography (UHPLC) employing octylsilyl (C8) and perfluorophenyl (PFP) stationary phases on either sub-2 micron porous silica or 2.6 micron solid core silica particles. Analytes were detected by both charged aerosol detection and photodiode array detection.

Results: Charged aerosol detection is shown to be suitable for measuring components including triterpenoid saponins, cholesterol, phospholipids, and surfactants with sensitivity often superior to that obtained by UV absorbance detection.

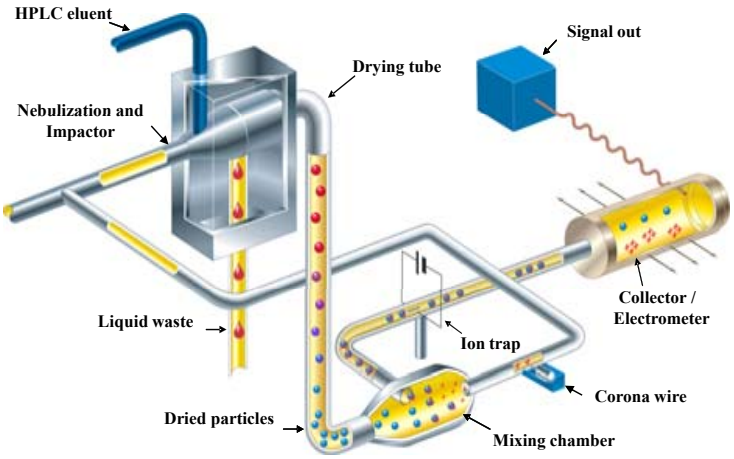
Introduction

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 United States. Complicating such analysis, many adjuvants under investigation contain components that are not readily analyzed by traditional HPLC with UV detection. These include various mixtures of lipids, fatty acids, and glycosides that lack suitable UV chromophores. In this work, the lack of a detectable chromophore in several adjuvant compounds and degradation products was overcome by using HPLC with charged aerosol detection, a detector that can measure any non-volatile compound.

Adjuvant mixtures and individual components were analyzed by UHPLC employing octylsilyl (C8) and perfluorophenyl (PFP) stationary phases on either sub-2 micron porous silica or 2.6 micron solid core silica particles. Analytes were detected by both charged aerosol detection and diode array detection.

The charged aerosol detector is a sensitive, mass-based detector, especially well-suited for the determination of any nonvolatile analyte independent of chemical characteristics. As shown in Figure 1, the detector nebulizes the mobile phase to create aerosol droplets. The mobile phase evaporates in the drying tube, leaving 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 and it is simpler to operate than a mass spectrometer.

FIGURE 1. Charged Aerosol Detector and Principle of Operation



Methods

Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system with a Diode Array Detector DAD 3000 RS and a Corona™ ultra RS™ Charged Aerosol Detector:
Nebulizer Temperature: 25 °C
Power function: 1.00
Data collection rate: 20 Hz

Reagents: HPLC- or LCMS-grade or better

Data Analysis

CDS: Thermo Scientific Dionex Chromeleon™ Chromatography Data System 7.1 SR1

AbISCO-100

Column:	Thermo Scientific Hypersil GOLD™ PFP 1.9 µm, 2.1 × 100 mm
Column Temp:	45 °C
Flow Rate:	0.47 mL/min
Injection Vol.:	10 µL
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; 7, 80; 12, 80.

Sample Prep.:
ABISCO-100™ (ISCONOVA, Uppsala Sweden) was diluted 5-fold with Milli-Q® water and transferred to a glass HPLC autosampler vial.

AddaVax

Column:	Thermo Scientific Accucore™ C8 2.6 µm, 4.6 × 150 mm
Column Temp:	40 °C
Flow Rate:	0.80 mL/min
Injection Vol.:	10 µL
Mobile Phase A:	1 mM ammonium acetate in water
Mobile Phase B:	2-propanol, Fisher Scientific™ Optima™2 LC/MS
Gradient: Time, %B:	0, 65; 3, 65; 13, 90; 18, 90; 18.1, 65; 30, 65.

Sample Prep.:
AddaVax™ (InvivoGen, San Diego, CA) was diluted 10-fold with Milli-Q water and transferred to a glass HPLC autosampler vial.

Squalene-tocopherol-PS80 mixture

Column:	Thermo Scientific Accucore PFP 2.6 µm, 2.1× 100 mm
Column Temp:	45 °C
Flow Rate:	0.50 mL/min
Injection Vol.:	1 or 3 µL
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.08% formic acid in 2-propanol, Optima 2 LC/MS
Gradient: Time, %B:	-5, 30; 0, 30; 13, 95; 18, 95

Sample Prep.:
Dissolve 24.3 mg DL-α-tocopherol (Acros Organics, NJ) in 24.3 mL 2-propanol.
Dissolve 48.7 mg squalene in 9.64 mL 2-propanol; dilute 5-fold with 2-propanol.
Combine 214 µL squalene solution, 237 µL DL-α-tocopherol solution, 97 µL polysorbate 80 and 452 µL of 2-propanol.

Synthetic MPLA

Column:	Thermo Scientific Accucore C8 2.6 µm, 4.6 × 150 mm
Column Temp:	40 °C
Flow Rate:	0.80 mL/min
Injection Vol.:	10 µL
Mobile Phase A:	1 mM ammonium acetate in water
Mobile Phase B:	2-propanol, Optima 2 LC/MS
Gradient: Time, %B:	0, 65; 3, 65; 13, 90; 18, 90; 18.1, 65; 30, 65.

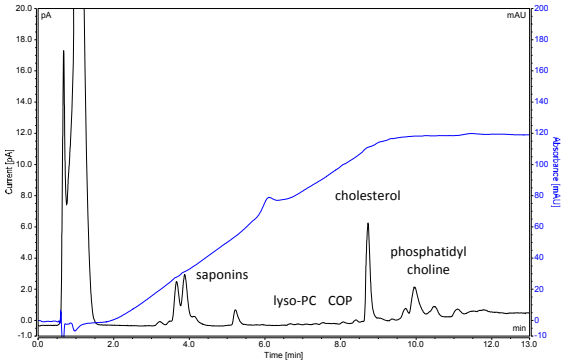
Sample Prep.:
Synthetic MPLA (Avanti Polar Lipids, GA) was dissolved in chloroform:methanol:water (CMW) 80:20:4 at a concentration of about 1 mg/mL and transferred to a glass HPLC autosampler vial.

Results

AbISCO-100

AbISCO-100 is a suspension of purified saponins from *Quillaja saponaria*, cholesterol from sheep wool and egg phosphatidyl choline in phosphate buffered saline[1]. As seen in Figure 2, all components elute within 12 min from the Hypersil GOLD PFP column with good resolution. All components and several degradation products including cholesterol oxidation products (COP) and lyso-PC are detected by the charged aerosol detector, whereas some, such as DPPC, show poor response by UV detection.

FIGURE 2. Charged aerosol response is significantly better than UV response for several components of AbISCO-100 separated by HPLC.



Performance

Calibration curves for the three major components of AbISCO (analyzed in duplicate) are presented in Figure 3. The data were fit to a quadratic equation, yielding coefficients of determination, R^2, greater than 0.999 for all three analytes.

Table 1 presents a summary of the method's performance, including precision of retention time and peak area, the coefficient of determination, and the limits of detection for the three major components of AbISCO.

Figure 3. Calibration data for analysis of AbISCO by HPLC-charged aerosol detection.

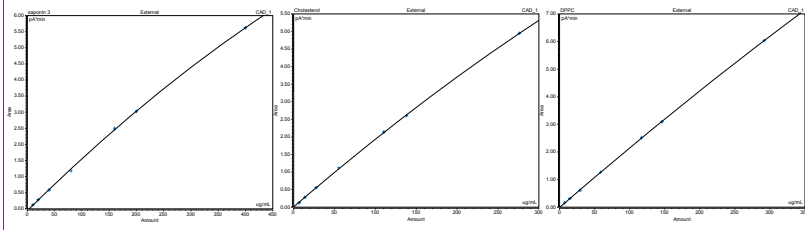


Table 1. Method performance for analysis of AbISCO by HPLC-charged aerosol detection.

Component	Amount (µg/mL)	Ret. Time[1] (%RSD)	Peak Area[1] (%RSD)	LOD[2] µg/mL	R^2*
Saponins	78.2	0.06	0.36	13.0	0.9998
Cholesterol	56.8	0.07	0.39	6.4	0.9999
DPPC	60.2	0.07	1.27	4.4	1.0000

1 for n = 10 replicates

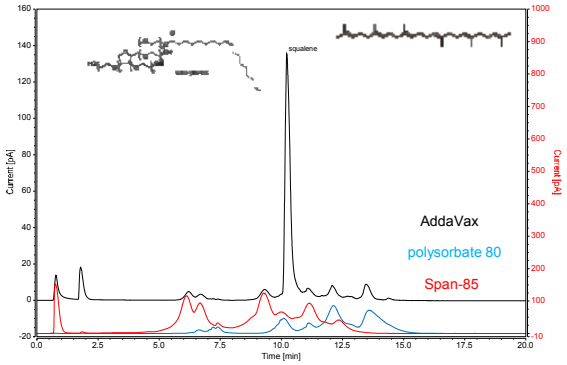
2 Hubaux-Vos method

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

AddaVax

AddaVax, shown in Figure 4, is prepared by emulsification of sorbitan trioleate (SPAN™ 85) in squalene oil (5% v/v) and polysorbate 80 (0.5% w/v) in sodium citrate buffer (10 mM pH 6.5)[2].

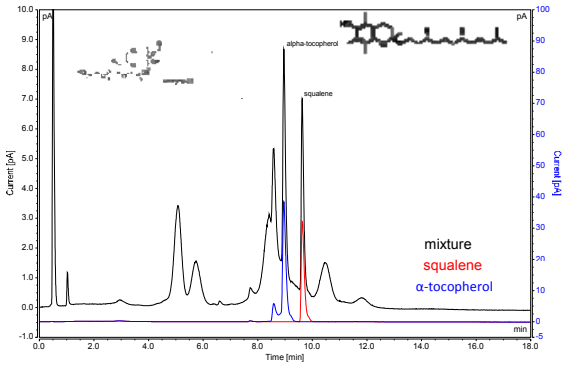
FIGURE 4. Chromatograms of AddaVax and emulsifiers by HPLC-charged aerosol detection.



Squalene-based mixture

Squalene is mixed with other components in several other adjuvants. The chromatogram shown in Fig 5 illustrates the results obtained with a mixture of squalene, DL-α-tocopherol and polysorbate 80 (PS 80).

FIGURE 5. HPLC-charged aerosol detection chromatogram of a mixture of α-tocopherol, squalene and PS 80.



sMPLA

Synthetic monophosphoryl lipid A, an analog of bacterial lipopolysaccharide (LPS), has low toxicity while specifically activating TLR4 but not TLR2. sMPLA contains six acyl groups. Purity analysis is used to quantify contaminants, degradation products, and variants differing in acyl chain number, length, and phosphorylation[3]. Figure 6 compares chromatograms obtained from analysis of sMPLA by inverse gradient HPLC with detection by UV absorbance at 220 nm or charged aerosol detection.

FIGURE 6. Purity analysis of sMPLA by HPLC-UV-charged aerosol detection.

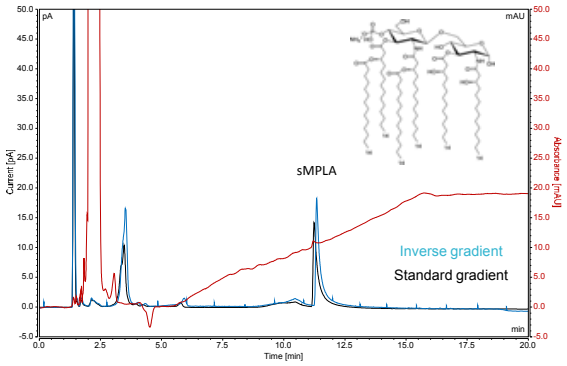


Table 2. Summary of immunologic adjuvant components investigated in this study. (+) annotation indicates applicable detector.

Component	UV (220 nm)	Charged Aerosol
Saponins	+	+
Cholesterol	+	+
Diphosphatidyl choline (DPPC)		+
Mixed phosphatidyl cholines		+
Lyso-phosphatidyl choline		+
Sorbitan trioleate (Span 85)		+
Polysorbate 80 (Tween 80)		+
Squalene	+	+
α-Tocopherol	+	+
sMPLA	+	+

Conclusion

- The HPLC method developed to analyze AbISCO is precise, with retention time precision better than 0.1% RSD and peak area precision between 0.4 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

1. Picard, M.D.; Cohane, K.P.; Gierahn, T.M.; Higgins, D.E.; Flechtner, J.B. High-throughput proteomic screening identifies Chlamydia trachomatis antigens that are capable of eliciting T cell and antibody responses that provide protection against vaginal challenge. *Vaccine*. **2012**, 30(29):4387-93.
2. Mbow M.L.; De Gregorio, E.; Valiante, N.M.; Rappuoli, R. New adjuvants for human vaccines. *Curr Opin Immunol*. **2010**, 2(3):411-6.
3. Raetz, C.R.; Garrett, T.A.; Reynolds, C.M. *et al*. Kdo_2-Lipid A of *Escherichia coli*, a defined endotoxin that activates macrophages via TLR-4. *J. Lipid Res*. **2006**, 47:1097-1111.

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