

Short communication

Enhancement of an analytical method for the determination of squalene in anthrax vaccine adsorbed formulations

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Abstract

Specific lots of anthrax vaccine adsorbed administered to members of the U.S. Armed Forces have been alleged to contain squalene, a chemical purported to be associated with illnesses of Gulf War veterans. A method of enhanced sensitivity for determining squalene in anthrax vaccine adsorbed using high-performance liquid chromatography with photodiode array detection has been developed, validated, and applied to 44 bottles of 38 lots of anthrax vaccine. In 43 bottles of 37 lots, no squalene was detected within a detection limit of 1 ng/0.5 ml dose (2 parts-per-billion). One lot, FAV008, was found to contain trace amounts of squalene at 7, 9, and 1 $\mu\text{g l}^{-1}$, levels considerably below normal human plasma levels (290 $\mu\text{g l}^{-1}$). The overall results of this investigation provide direct evidence for the absence of squalene in nearly all of anthrax vaccine preparations tested. © 2006 Elsevier B.V. All rights reserved.

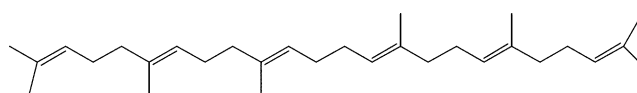
Keywords: Squalene; Anthrax vaccines; Gulf War syndrome; Reverse-phase liquid chromatography; Method validation; Detection limits

1. Introduction

In attempts to explain health problems among some veterans of the Persian Gulf War, a few people have speculated that a vaccine adjuvant, squalene, may have caused an autoimmune disease in veterans. An article in the lay press [1] alleges that the Department of Defense possibly used “an illicit and secret anthrax vaccine on its own troops”. No physical evidence has been offered to support the allegations.

The allegations prompted military officials responsible for the health of military personnel to reassure them that no squalene-containing adjuvant was added to anthrax vaccine. In 2002, we reported a validated methodology for the determination of squalene in anthrax vaccine adsorbed preparations that has a lower detection limit of 140 $\mu\text{g l}^{-1}$ [2]. This method has been applied successfully to seventeen lots of anthrax vaccine adsorbed [2]. At the Army’s request, a new method of enhanced sensitivity and capable of detecting 1 ng of squalene/0.5 ml dose (2 $\mu\text{g l}^{-1}$) was developed, validated, and applied to 44 bottles of 38 lots of anthrax vaccine adsorbed, including those that had been assayed

previously using the lower-sensitivity method. A detection limit of 2 $\mu\text{g l}^{-1}$ has been achieved by taking a larger vaccine sample, adding a pre-concentration step, and injecting a larger volume for chromatographic assay. The structure of squalene is shown below.



SQUALENE
(2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene)
 $\text{C}_{30}\text{H}_{50}$
Mol. Wt. = 410.70

This communication describes the development and validation of an enhanced-sensitivity method for determining squalene and application of the method to 44 bottles of 38 lots of anthrax vaccine adsorbed received from the Army. This new method exceeds detection limits reported by others [3].

2. Experimental

2.1. Vaccine samples

Thirty (30) bottle no. samples of 24 lots of anthrax vaccine adsorbed (AVA) preparation, manufactured by Michigan

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Table 1
Anthrax vaccine samples received in November 2000

Receipt date	Bottle no.	Amount	Lot no.	Expiration date	
30 November 2000	WR282908	BP20742	4 5-ml vials	Lot FAV001	15 August 1991
30 November 2000	WR282908	BP20751	2 5-ml vials	Lot FAV002	20 August 1991
30 November 2000	WR282908	BP20760	2 5-ml vials	Lot FAV003	18 October 1991
30 November 2000	WR282908	BP20779	2 5-ml vials	Lot FAV004	27 November 1991
30 November 2000	WR282908	BP20788	2 5-ml vials	Lot FAV005	21 December 1991
30 November 2000	WR282908	BP20797	4 5-ml vials	Lot FAV006	9 January 1992
30 November 2000	WR282908	BP20804	2 5-ml vials	Lot FAV007	1 February 1992
30 November 2000	WR282908	BP20813	1 5-ml vial	Lot FAV008	28 June 1997
30 November 2000	WR282908	BP20822	2 5-ml vials	Lot FAV008	6 January 1993
30 November 2000	WR282908	BP20831	9 5-ml vials	Lot FAV008	5 August 2000
30 November 2000	WR282908	BP20840	1 5-ml vial	Lot 12	19 October 1982
30 November 2000	WR282908	BP20859	1 9-ml vial	Lot 12	23 April 1983
30 November 2000	WR282908	BP20868	2 5-ml vials	Lot FAV012	3 September 1994
30 November 2000	WR282908	BP20877	1 9-ml vial	Lot 13	8 August 1981
30 November 2000	WR282908	BP20886	2 5-ml vials	Lot FAV016	28 July 1998
30 November 2000	WR282908	BP20895	2 5-ml vials	Lot 18	4 February 1989
30 November 2000	WR282908	BP20902	2 5-ml vials	Lot FAV018	10 October 1995
30 November 2000	WR282908	BP20911	2 5-ml vials	Lot FAV018	11 October 1997
30 November 2000	WR282908	BP20920	2 5-ml vials	Lot FAV018	1 June 1995
30 November 2000	WR282908	BP20939	1 5-ml vial	Lot 19	5 October 1991
30 November 2000	WR282908	BP20948	1 5-ml vial	Lot FAV022	29 June 1996
30 November 2000	WR282908	BP20957	5 5-ml vials	Lot FAV030	23 February 1999
30 November 2000	WR282908	BP20966	46 5-ml vials	Lot FAV031	6 October 2000
30 November 2000	WR282908	BP20975	4 5-ml vials	Lot FAV032	1 February 1999
30 November 2000	WR282908	BP20984	2 5-ml vials	Lot FAV032	23 October 1997
30 November 2000	WR282908	BP20993	2 5-ml vials	Lot FAV034	23 February 1999
30 November 2000	WR282908	BP21007	2 5-ml vials	Lot FAV038	15 January 2000
30 November 2000	WR282908	BP21016	2 5-ml vials	Lot FAV044	14 May 2000
30 November 2000	WR282908	BP21025	2 5-ml vials	Lot FAV047	8 September 2001
30 November 2000	WR282908	BP21034	2 5-ml vials	Lot FAV048B	13 April 2002

Department of Public Health (Lasing, Michigan) or by BioPort Corporation (Lansing, Michigan) were received from the Walter Reed Army Institute for Research (Washington, DC). An additional 14 bottle no. samples of 14 lots of AVA produced by the above manufacturers were received on 8 December 2000. A listing of these samples/lots appears in Tables 1 and 2. All samples were stored at $4 \pm 1^\circ\text{C}$ when not in use. We also evaluated samples of other pharmaceutical preparations for squalene, including isophane insulin suspension, human insulin, lidocaine

solution, and sodium and potassium chloride pharmaceutical solutions.

According to the AVA package insert, anthrax vaccine adsorbed is an aqueous suspension of the immunogen protein adsorbed on aluminum hydroxide. The final product contains no more than 2.4 mg aluminum hydroxide (equivalent to 0.83 mg aluminum) per 0.5-ml dose. Formaldehyde, in a final concentration not to exceed 0.02%, and benzethonium chloride, 0.0025%, are added as preservatives.

Table 2
Anthrax vaccine samples received in December 2000

Receipt date	Bottle no.	Amount	Lot no.	Expiration date
8 December 2000	BP21329	1 5-ml vial	FAV009	17 July 1997
8 December 2000	BP21338	1 5-ml vial	FAV012	12 August 1997
8 December 2000	BP21347	1 5-ml vial	FAV017	6 February 1999
8 December 2000	BP21356	1 5-ml vial	FAV018	11 October 1997
8 December 2000	BP21365	1 5-ml vial	FAV019	6 February 1999
8 December 2000	BP21374	1 5-ml vial	FAV020	6 February 1999
8 December 2000	BP21383	1 5-ml vial	FAV022	29 June 1996
8 December 2000	BP21392	1 5-ml vial	FAV024	22 April 2000
8 December 2000	BP21409	1 5-ml vial	FAV033	27 August 1999
8 December 2000	BP21418	1 5-ml vial	FAV036	16 March 1999
8 December 2000	BP21427	1 5-ml vial	FAV037	25 February 2000
8 December 2000	BP21436	1 5-ml vial	FAV038	15 January 2000
8 December 2000	BP21445	1 5-ml vial	FAV041	5 April 2000
8 December 2000	BP21454	1 5-ml vial	FAV043	12 March 2000

2.2. Squalene reference standard

The squalene reference standard, Aldrich Lot no. 04029JY, 99.2% pure, was purchased from Aldrich Chemical Company (Milwaukee, WI). The certificate of analysis stated that the chemical was a colorless liquid, its infrared spectrum conformed to the structure as illustrated on page 33B of Edition 1, Volume I of “The Aldrich Library of FT-IR Spectra”, and its purity, 99.2%, was based on gas–liquid chromatographic analysis. The material was used without further purification.

To further characterize this squalene reference standard, its principal component was verified as squalene by ^1H NMR, ^{13}C NMR and mass spectrometry. Because the reference standard is labeled “air sensitive”, the bottle was opened only under a blanket of argon; the bottle content also was covered with the same gas.

All transfers were performed using pipets. Stock solutions were prepared by transferring aliquots to argon-purged volumetric flasks, dissolving and diluting to volume with 2-propanol (Mallinckrodt, Chrom AR). Stock standards were further diluted with 2-propanol or hexane (Mallinckrodt, Chrom AR) to prepare working standards.

2.3. Sample preparation

All analyses were performed in triplicate. The vaccine preparations were thoroughly shaken by hand to ensure a uniform material for sampling. A 2-ml glass syringe with needle was purged with argon gas and 1.0 ml argon was introduced into the sample bottle using the needle and syringe. The bottle was shaken by hand again and followed by removal of 1.00 ml of the vaccine using the syringe and transferred to a 4-ml glass vial. To this vial was added 1.00 ml of HPLC grade hexane and the vial immediately capped. The vial was inverted several times and vigorously agitated for 5 min using a Vortex-Genie 2 mixer (Van Waters and Rogers) set at maximum speed. The mixture was separated into two layers by centrifugation for 3 min at approximately 2800 rpm. The top layer was transferred to a tapered centrifuge tube annealed to a 50-ml round-bottomed flask and rotary evaporated to dryness at room temperature. The residue was dissolved in 100 μl of hexane for HPLC analysis.

2.4. Spiked standard (AS): squalene spiked in anthrax vaccine adsorbed

A standard curve for squalene-spiked vaccine preparation (AS curve) was prepared. Anthrax vaccine adsorbed, Lot FAV044, 1.00 ml per aliquot, was spiked with squalene solutions ranging from 2.5 to 39.3 ng/ml 2-propanol. Each 1.0-ml aliquot of squalene-spiked vaccine standards was extracted with 1.00 ml of hexane (see Section 2.3 for extraction procedure), the hexane layer was transferred to a round-bottomed flask, and the solution was reduced to dryness at room temperature using a rotary evaporator. The residue was re-dissolved in 100- μl hexane, and 40 μl was injected into the chromatograph. Triplicates of each AS concentration were prepared and 40 μl of each AS solu-

tion was injected into the chromatograph, placing 0.98–15.7 ng squalene on column.

2.5. HPLC conditions

HPLC was performed at ambient temperature (20–23 °C) using a Waters Millennium system that included the Model 600 pump, 717+ autoinjector, and 996 PDA detector. The stationary phase was a Metachem C8, 5 μm , 3.0 mm \times 250 mm column. The mobile phase was methanol (100%, Mallinckrodt Chrom AR) and was run at a flow rate of 0.5 ml/min. The analytical wavelength was 203 nm and UV spectral data were acquired from 197 to 300 nm. For each chromatographic run, the injection volume was 40 μl . Under the above conditions, squalene eluted at 4.9 min.

3. Results

3.1. Spiked standard (AS): squalene spiked in anthrax vaccine adsorbed

3.1.1. Spiking matrix

The developed extraction and assay method was applied to AVA Lot FAV044. The results showed this Lot was squalene-free [4] so it can serve as the sample matrix for spiking experiments for method validation. Overlay chromatographic profiles of squalene in hexane (40 $\mu\text{g l}^{-1}$), hexane extract from a 40 $\mu\text{g l}^{-1}$ squalene-spiked Lot FAV044, and unspiked Lot FAV044 are shown in Fig. 1.

3.1.2. Linearity

A standard curve for squalene spiked into the vaccine preparation (AS) was prepared from data generated from solutions described in Section 2.4. Triplicate AVA Lot FAV044 samples were spiked with squalene concentrations ranging from 2.5 to 39.3 ng/1.00 ml, extracted, concentrated, and chromatographed. The overall results show respectable linearity ($r^2 = 0.9983$) over this concentration range for the derived regression equation, $y = 4763x - 878$.

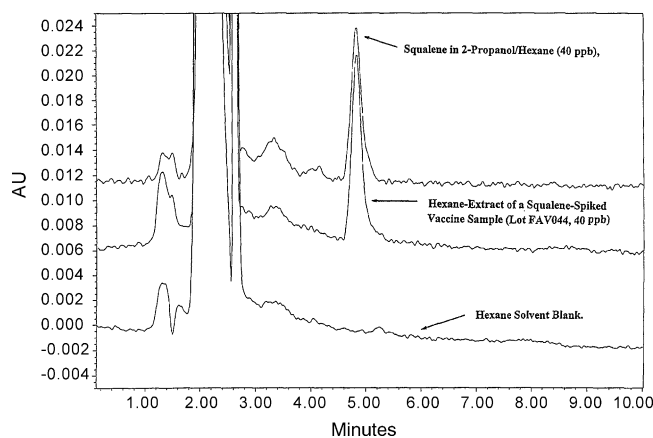


Fig. 1. Overlay chromatographic profiles of squalene in 2-propanol/hexane (40 ppb), hexane extract from a 40-ppb squalene-spiked Lot FAV044, and hexane blank.

Table 3
Precision and accuracy results for squalene spiked into anthrax vaccine adsorbed (Lot FAV044)

Sample I.D.	Squalene Conc. (ng ml ⁻¹)	Peak area	Calculated squalene (ng ml ⁻¹)	Percent accuracy	Average percent accuracy	S.D.	Precision (%R.S.D.)
AS0-A	0	0					
AS0-B	0	0			–		
AS1-A	2.5	12989	2.8	112			
AS1-B	2.5	11449	2.4	96.0			
AS1-C	2.5	9982	2.1	84.0	97.3	14.4	13.1
AS2-A	4.9	24592	5.4	110			
AS2-B	4.9	22778	5.0	102			
AS2-C	4.9	22073	4.8	98	103	5.9	5.6
AS3-A	9.8	48010	10.7	109			
AS3-B	9.8	48588	10.9	111			
AS3-C	9.8	44733	9.1	99.6	107	5.7	4.4
AS4-A	19.7	91444	20.6	104			
AS4-B	19.7	89803	20.2	103			
AS4-C	19.7	88604	19.9	101	103	1.5	1.6
AS5-A	39.3	185377	41.9	107			
AS5-B	39.3	193619	43.8	111			
AS5-C	39.3	186472	42.2	107	108	2.1	2.4
AS5-D	39.3	239468	40.6	103			
AS5-E	39.3	205252	34.5	87.8			

3.1.3. Accuracy and precision

The squalene-spiked anthrax vaccine standards showed a mean accuracy value of 97.3% at 2.5 µg l⁻¹ and 108% at 40 µg l⁻¹. The precision of the data, expressed as %R.S.D., ranges from 13.1% at 2.5 µg l⁻¹ to 2.4% at 40 µg l⁻¹. The values, tabulated in Table 3, are acceptable, especially when such low concentrations are considered.

3.1.4. Recovery

Recovery was based on comparing the respective peak areas of the spiked vaccine extracts to those of the neat standards. These comparative values are shown in Table 4.

The squalene peak area representing a 1.00-ml extract, evaporated to dryness, and reconstituted to 100 µl (*a*) was compared to the corresponding neat standard concentration (*b*) treated in the same manner for each concentration level. Squalene extraction efficiency (*c*) was based on the *a/b* ratio

times 100. The mean extraction efficiency values ranged from 96 to 100%.

3.1.5. Limit of detection

Based on a signal-to-noise ratio of ≈3:1, the limit of detection (LOD) of the method is approximately 0.7 ng on column from a 40-µl injection. Chromatograms of squalene in 2-propanol/hexane and in a hexane extract of spiked vaccine (Lot FAV044), both concentrations at this limit of detection, are depicted in Fig. 2. When the LOD is applied to vaccine samples, the 1.00-ml extraction volume, reduced to 100 µl, represents the amount of squalene in 1.00 ml of vaccine. Thus, the LOD is 1.8 ng per 1.00 ml of vaccine (1.8 µg l⁻¹ or 1.8 ppb).

3.1.6. Sample assay results

Except when noted, all reported results are based on a LOD of 1.8 ng squalene per ml of vaccine or 1.8 ppb. Of the 44 bottles

Table 4
Recovery of squalene from spiked anthrax vaccine adsorbed

Sample I.D.	Conc. (ng ml ⁻¹)	Peak area, <i>a</i>	Ave. std. (NS) peak area, <i>b</i>	Extraction efficiency, <i>c</i>	Average extraction efficiency
AS1-A	2.5	12989		109	
AS1-B	2.5	11449	11918	96.0	
AS1-C	2.5	9982		83.8	96.3
AS2-A	4.9	24592		106	
AS2-B	4.9	22778	23148	98.4	
AS2-C	4.9	22073		95.4	99.9
AS3-A	9.8	48010		102	
AS3-B	9.8	48588	47110	103	
AS3-C	9.8	44733		95.0	100
AS4-A	19.7	91444		102	
AS4-B	19.7	89803	89950	99.8	
AS4-C	19.7	88604		98.5	100
AS5-A	39.3	185377		98.3	
AS5-B	39.3	193619	188489	103	
AS5-C	39.3	186472		98.9	100

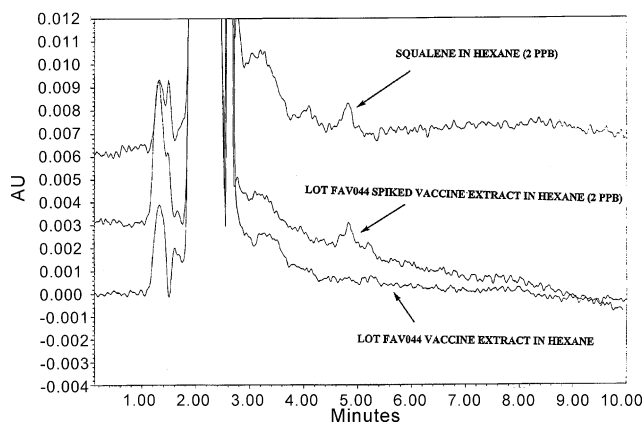


Fig. 2. Chromatograms of squalene in 2-propanol/hexane and in a hexane extract of spiked vaccine (Lot FAV044) at this limit of detection.

of vaccine analyzed, only the three bottles of Lot FAV008 were found to contain trace amounts of squalene at 7, 9, and an estimated $1 \mu\text{g l}^{-1}$. A chromatogram of bottle BP20813 is shown in Fig. 3.

Lot FAV030 and the 14 subsequent Lots contained no detectable squalene (FAV016, -018, -019, -020, -022, -024, -031, -032, -033, -034, -038, -044, -047, and -048B; however, they contain a trace, unknown component that elutes just after squalene, 5.1 min versus 4.9 min for squalene. When the extract of one of these lots was spiked with squalene and chromatographed, the unknown and spiked squalene are about 50% baseline resolved,

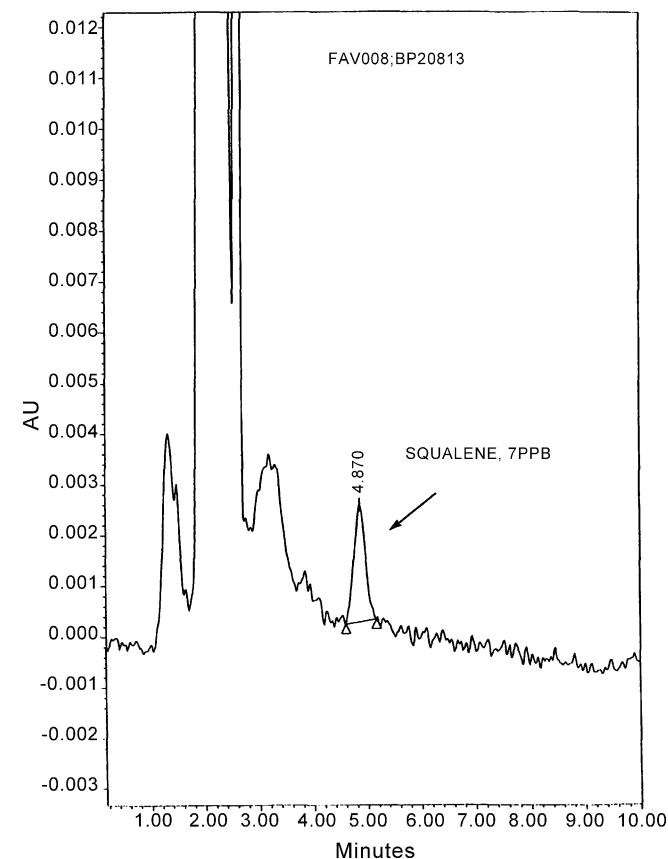


Fig. 3. Chromatogram of Lot FAV008, showing squalene at $7 \mu\text{g l}^{-1}$ (ppb).

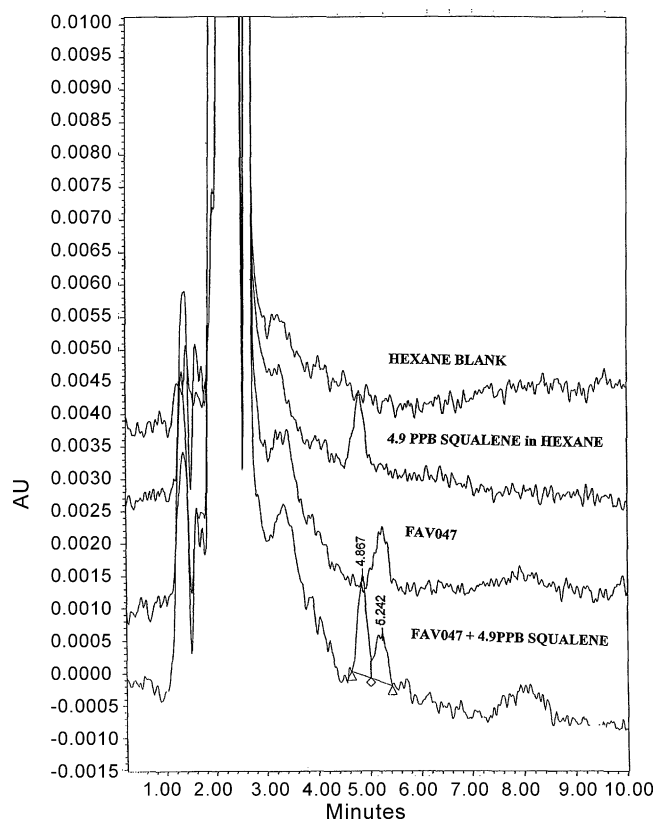


Fig. 4. Overlaid chromatograms for the hexane blank, squalene at $4.9 \mu\text{g l}^{-1}$ (ppb), Lot FAV047, and Lot FAV047 spiked with squalene at $4.9 \mu\text{g l}^{-1}$ (ppb).

showing clearly that the unknown was not squalene. Overlaid chromatograms for the hexane blank, squalene standard ($4.9 \mu\text{g l}^{-1}$), Lot FAV047 and Lot FAV047 spiked with squalene ($4.9 \mu\text{g l}^{-1}$) are shown in Fig. 4.

No squalene or other contaminants were found in Lots FAV036, -037, -041, or -043 nor in isophane insulin suspension, human insulin, lidocaine solution, or sodium and potassium chloride pharmaceutical solutions.

4. Discussion

Because squalene is present in copious amounts on human fingers and hands, laboratory equipment and laboratory surfaces in contact with ungloved hands are contaminated with squalene. For this reason, all sample preparation manipulations must be carried out with gloved hands, and prior to use, all glassware and plasticware are washed with HPLC-grade hexane to remove possible squalene contamination. Additionally, hexane can extract materials from plasticware; when such extracts are of sufficient quantities, they can interfere with the chromatographic determination of squalene.

Lot FAV044 proved to be free of squalene at least down to the LOD of 1.8 ng ml^{-1} ; thus, it was used as the matrix for LOD, linearity, accuracy, and recovery studies.

Standard curves produced from squalene extracted from spiked-vaccine preparations (AS) showed good linearity, even to concentrations near the LOD. For the squalene-spiked standards in anthrax vaccine adsorbed, accuracy ranged from 97 to 108%,

and precision ranged from 14.4 to 1.5% over the 2.5–39.9 $\mu\text{g l}^{-1}$ range.

The recovery results for squalene extracted from squalene-spiked AVA preparations are acceptable. The mean recovery percentages for the spikes show an average recovery of >96%.

Except for three samples of Lot FAV008, in which squalene was detected at 9 $\mu\text{g l}^{-1}$ in Lot FAV008;BP20822, 7 $\mu\text{g l}^{-1}$ in Lot FAV008;BP20813, and a small trace (<1 $\mu\text{g l}^{-1}$) in Lot FAV008;BP20831, no squalene was detected in the remaining 41 of 44 bottle no. samples of AVA tested. Although these three bottle no. samples are from the same lot, they differ by bottle number (BP) and expiration dates (Table 2). Identity of the squalene was based on chromatographic retention time and UV spectra. Identity confirmation by LC/MS could not be obtained because of the low amount present. Also, owing to the trace amounts present, they are more likely a result of contamination and not an intended component of the vaccine formulation. Irrespective of the source, this concentration is far below that normally found in human plasma, 290 $\mu\text{g l}^{-1}$ [5].

It has been shown unambiguously that the 5.1-min peak, found in numerous lots of the anthrax vaccine adsorbed, is not squalene. Assuming that its response to the UV detector is the same as squalene per unit weight, a concentration estimate is $\sim 4 \mu\text{g l}^{-1}$. Attempts to identify this unknown by LC/MS were unsuccessful.

An indirect determination of squalene by an anti-squalene antibody test (ASA) in sera of Gulf War veterans has been reported [6] in which sera from participants who received lots FAV020, FAV030, FAV038, FAV041, and FAV043 responded positively in some, but not all. Because the authors suggest the production of ASA may be linked to squalene, the lack of squalene

in these lots, as determined by a direct analytical method, suggests the production of ASA resides in other factors.

Squalene is a component of certain adjuvants in some vaccine preparations licensed outside the United States [7]. Its concentration in these vaccine preparations are reported to be in the 0.2–5% [8,9] range (i.e., 2,000,000–50,000,000 $\mu\text{g l}^{-1}$). If squalene were present in the AVA preparations at these concentrations, its amounts would range from 2 to 50 mg per 1.0-ml dose. These amounts of squalene would be 1–25 million times the LOD of our method and would be very easily detected and measured.

The development and validation of an enhanced method to directly determine squalene and its application to anthrax vaccine adsorbed products produced over a 20-year period (1982–2002) provides strong evidence to assure military personnel that such products are squalene-free.

References

- [1] G. Matsumoto, *The Pentagon's Toxic Secret*, Vanity Fair, 1999, pp. 83–98.
- [2] R.J. Spanggord, B. Wu, M. Sun, P. Lim, W.J. Ellis, *Pharm. Biomed. Anal.* 29 (2002) 183–193.
- [3] H.-T. Lu, Y. Jang, F. Chen, *Chromatographia* 59 (2004) 367–371.
- [4] This Lot has been determined to be squalene-free, SRI Report No. 1008.
- [5] D.S. Goodman, *J. Clin. Invest.* 43 (1964) 1480–1485.
- [6] P.B. Asa, R.B. Wilson, R.F. Garry, *Exp. Mol. Pathol.* 73 (2002) 19–27.
- [7] F.R. Vogel, M.F. Powell, C.R. Alving, *A Compendium of Vaccine Adjuvants and Excipients*, 2nd ed, www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf.
- [8] R. Hjorth, Mammalian vaccines composition comprising squalene or squalene, phospholipid and a surfactant as adjuvant. *Eur. Pat. Appl.* EP745388 A1 4 December 1996, CA 126:65365.
- [9] R. Hjorth, Adjuvants for viral vaccines. *Eur. Pat. Appl.* EP 745387 A2 4 December 1996, C.A. 126:58857.