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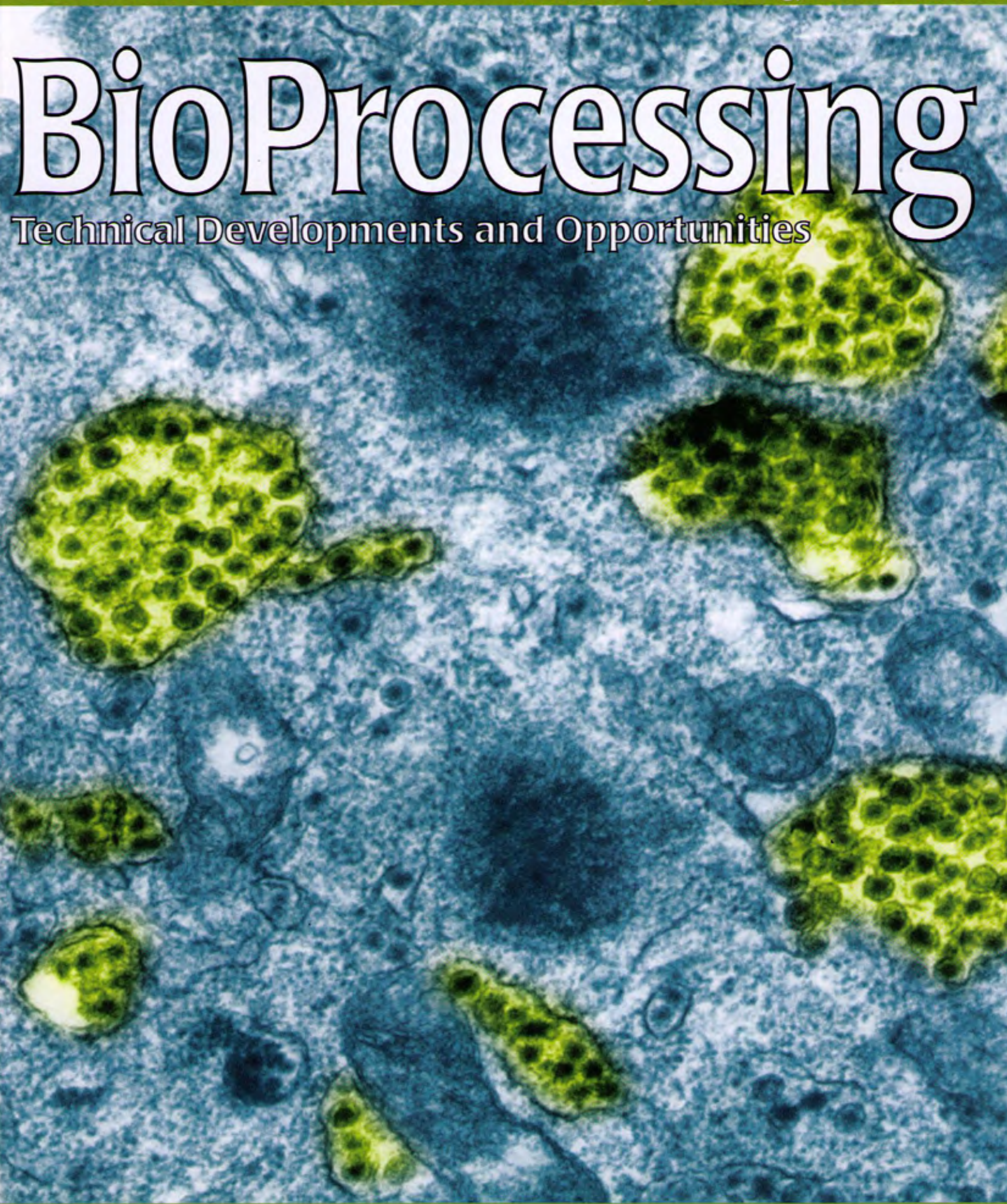

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A Contaminant in the Adenovirus Reference Material

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The Adenovirus Reference Material (ARM) is a purified and well-characterized wild type adenovirus (Ad 5) now available to researchers worldwide. Due to the need for a common reference material, the ARM was produced with the purpose of validating assay methods and internal standards for use in developing recombinant adenovirus for gene therapy. Analysis of ARM by RP-HPLC, however, detected the presence of a contaminant peak with a distinctive A240 local wavelength maximum. The contaminant was found in all of the vials, with some variability in amount between vials. It appears that the contaminant is not associated with the virus and it is unlikely that it will interfere with the use of the ARM as a reference material. The source of the contaminant was probably a leachate or plasticizer from the tubing or containers used during the final processing step.

Introduction

The Adenovirus Reference Material (ARM, ATCC VR-1516) was conceptualized, developed, produced, and characterized to fulfill the need for a reference testing reagent. The ARM is available to all researchers and producers of recombinant adenovirus (rAd) in gene therapy investigations, and especially for its use in a standardized comparison to vectors.[1,2] During the characterization phase of the ARM project, a contaminant was detected using RP-HPLC. The contaminant had a UV spectrum that was distinct from viral proteins and peptides that had been routinely detected. This contaminant, or perhaps a similar one, had been observed during previous recombinant adenovirus vector preparations by some of the members working on the ARM.

The purpose of this paper is to create awareness of the contaminant and its properties, provide an understanding of the apparently minor impact that the contaminant has on the reference material, and provide awareness of the potential for this type of contamination to occur during the final processing steps of gene therapy rAd or other biological products.

Methods and Materials

All of the information concerning the wild type Ad5 ARM's development, production, and characterization can be found on the Williamsburg BioProcessing Foundation's website at: http://www.wilbio.com. This material was produced, purified, and vialed by Introgen Therapeutics, Inc. in Houston, Texas. Briefly, HEK 293 cells were grown in a CellCube™ 4x100 Bioreactor, and then infected with a plaque-purified Ad5 Wild Type bank provided by Canji, Inc. in San Diego, California.

The infected cell lysate was concentrated, diafiltered, and treated with Benzonase to digest contaminating nucleic acids. The virus was purified by anion exchange chromatography, and then concentrated and diafiltered into the ARM formulation buffer (20 mM Tris, at pH 8.0, at room temperature, 2.5% glycerol, 25 mM NaCl). Finally, the material was filtered through a 0.22 mm filter and frozen at less than -60 °C. Later, this frozen material was thawed, filtered, and vialed.

RP- HPLC Method 1: Method 1 was based on a modified method described by Lehmberg, et al., and was used during the ARM characterization to determine the virus particle concentration.[3] It was also based on the relative hexon content, and utilizes a Jupiter C4 column (150 x 2 mM; 300Å pore) run at 40 °C. The initial injection was first followed by equilibration and then by a two-part gradient. Solvent A was 0.1% trifluoroacetic acid (TFA), and Solvent B was 0.1% TFA in acetonitrile. The sample injection was followed by a ten minute gradient from 20% to 40% Solvent B, and then by a 40 minute gradient from 40% to 60% Solvent B. Column regeneration used an abbreviated gradient of 20 minutes with 20%-60% Solvent B, and followed by equilibration at 20% Solvent B. The flow rate was 0.2 mL/min. A solution of 1% Tween™-20 was added to the samples to give them a final concentration of 0.048%.

RP-HPLC Method 2: Method 2 was based on a modification of a method described by Vellekamp, et al., and was used in the characterization of the ARM for determination of pVIII (precursor to protein VIII; 31K on SDS-PAGE) content as a marker for empty capsid contami-

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ARM Vial	A260	A280	A260/A280	calculated particle/ml (10E11)
Vial A (low))	0.155	0.114	1.36	5.68
Vial B (low)	0.151	0.109	1.39	5.54
Vial C (low)	0.154	0.112	1.38	5.65
Vial D (high)	0.203	0.148	1.37	7.44

Table 1. Absorbance data in 0.1% SDS and virus particle determination of four ARM vials. Samples were diluted 3-fold with ARM Buffer prior to SDS addition. Vials A-C had the low-level of contaminant and Vial D had the high-level of contaminant.

nation.[*] The method utilized a Jupiter C4 column (150 x 2 mm; 300Å pore) run at 50 °C, with injection first followed by equilibration and then by a three-part gradient. Solvent A was 0.1% trifluoroacetic acid (TFA), and Solvent B2 was 0.1% TFA in acetonitrile/aqueous (90:10, v/v). Sample injection was followed by a ten minute gradient from 25% to 40% Solvent B2, and then by a 15 minute gradient from 40% to 44% Solvent B2 and a 15 minute gradient from 44% to 60% Solvent B2. Column regeneration was 2 minutes at 100% B2 followed by equilibration at 25% Solvent B. Flow rate was 0.2 mL/min. Some samples were evaluated in the presence and absence of a detergent {0.5% octyl-ß-D-glucopyranoside (Sigma)} which was seen to have no effect on the chromatography of the contaminant.

Centrifugation: Certain samples of ARM (50 mL) were centrifuged for 90 minutes at 10,000 RPM in an Eppendorf microfuge with a swinging bucket rotor. The supernatant was reserved, and the pellet was resuspended in 50 mL of 0.1% TFA. Both samples were analyzed by RP-HPLC.

Results and Discussion

Contaminant Peak Detected by RP-HPLC: Samples of ARM were analyzed by two RP-HPLC methods. Both methods detected an early eluting peak that had a different UV spectrum than the other virus proteins. RP-HPLC Method 1 showed this peak eluting in the same position as a minor virus peptide tha was routinely observed in other purified rAd preparations (Fig. 1A, B, page 59). RP-HPLC Method 2 showed partial resolution such that the contaminant peak eluted just prior to the virus peptide (Fig. 2A, B, page 60). The spectrum of the contaminant peak showed a clear local UV maximum at approximately 240 nm, which was not present in the virus peptide (Figs. 1C, 2C) or other virus protein peaks. It was noted that the precise position of the contaminant peak, relative to the early eluting virus peptides, could vary with different HPLC hardware.

Contaminant Amount Varies with Sample Vial: Preliminary evidence indicates that the amount of this contaminant in the ARM could vary from vial to vial. This poses an obvious concern for its use as a reference material. To address this concern, the amount of the contaminant has been evaluated by RP-HPLC, in multiple vials from all four lots of ARM, at three different laboratories (Schering Plough Research Institute, Berlex Laboratories, and Onyx Pharmaceuticals, Inc.). As an example, the results of the evaluation of ten vials from lot #001503, each chromatographed in duplicate using RP-HPLC Method 2, are shown in Figure 3 (page 60). The peak areas for both the contaminant and the peptide show good reproducibility despite the fact that these peaks are not completely resolved. The amount of virus peptide remains essentially constant between vials (as are other virus proteins; data not shown) while the amount of contaminant varies by a factor of approximately 2.7. Expressed differently, the contaminant peak is equivalent to approximately 3%-8% of total peak area at 214 nm for the RP-HPLC of the total virus proteins. Similar results were observed in vials from the other lots evaluated by RP-HPLC (data not shown).

One vial of lot #001503 has a strikingly higher amount of contaminant. The contaminant peak area is reproducibly about 28-fold higher than the average of the ten vials shown in Figure 3. This high-level of contaminant has only been detected in one vial. All other vials show the lower levels of contaminant as seen in Figure 3. We discuss the source of the variation below.

The high-level contaminant vial is approximately 50% of the total peak area at 214 nm for the RP-HPLC of the total virus proteins. We have evaluated the RP-HPLC profile of this vial at 260 nm,

Sample	Lot#	contaminant peak area (AU*min x 10E3)	
		injection 1	injection 2
formulation buffer		0	0
column eluate hold		0	0
concentrated bulk		0	0
diluted prefiltered bulk		0	0
thawed bulk	1503	0	0
thawed bulk	1504	0	0
thawed bulk	1505	0	0
thawed bulk	1506	0	0
filtered bulk	1503	716	730
filtered bulk	1504	645	651
filtered bulk	1505	683	685
filtered bulk	1506	416	425

Table 2. Determination of contaminant amount by RP-HPLC of intermediate processing samples of ARM. Injections were 20 µl using RP-HPLC Method 2.

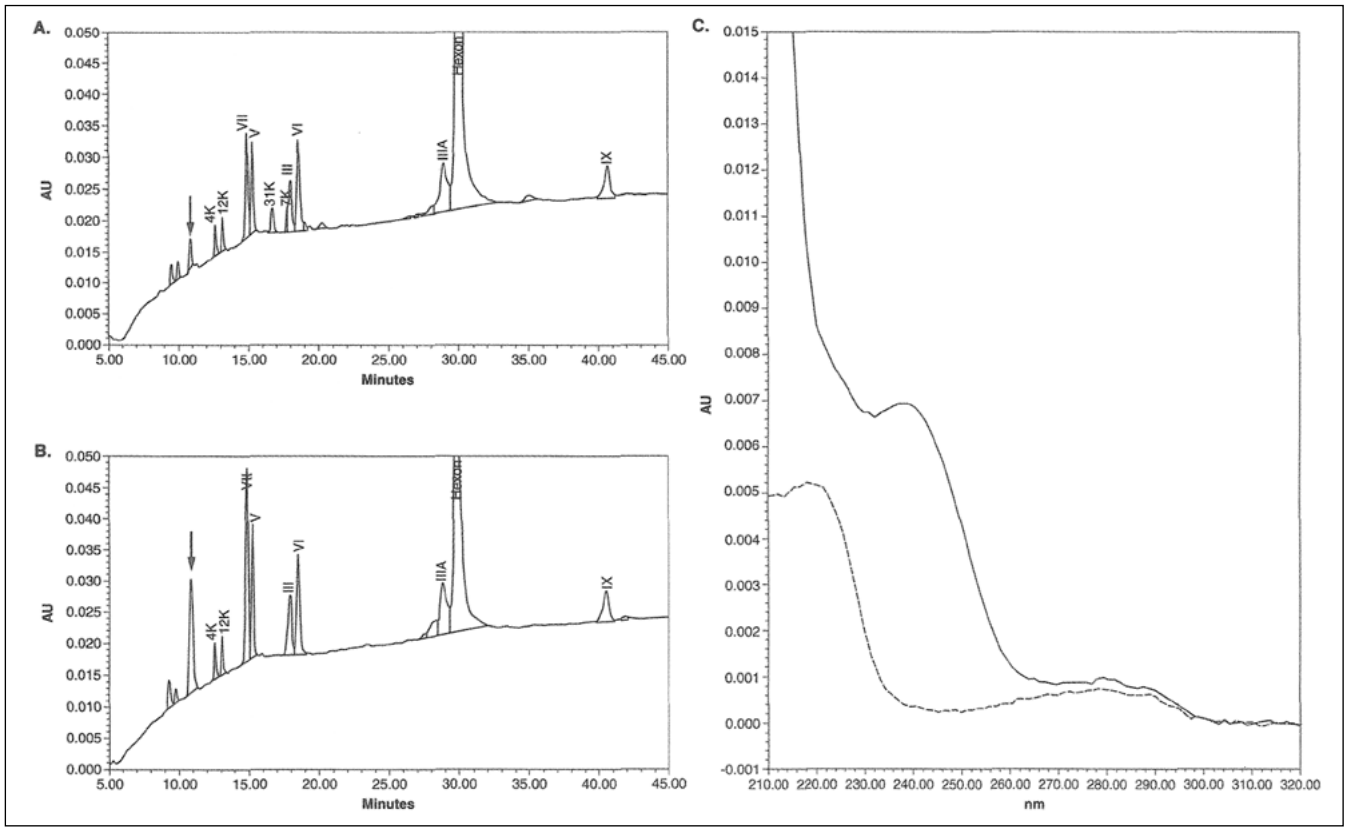

Figure 1. RP-HPLC analysis of control rAd and ARM by Method 1. (A) Control sample of column-purified rAd at 1.16 x 1012 particles/ml diluted 1:10 (50 µl injection). Arrow indicates the viral peptide peak position. (B) ARM sample (10 µl injection) has a contaminant peak area at 214 nm of 7.5% including viral peptide peak. Arrow indicates contaminant peak position. (C) Comparative UV spectra of the viral peptide peak from (A) (dashed) and contaminant peak from (B) (solid).

another minor contaminant peak found to elute about 1.5 minutes earlier than the major contaminant. Together, the two contaminant peaks contributed about 80% of the total RP-HPLC peak area at 260 nm in this vial.

Effect of the Contaminant on Virus Particle Determination by A260 in SDS: The contaminant's ability to substantially contribute to the A260 value raises concerns about the possibility that it might produce significant positive bias in A260 measurements made for particle concentration. The vial containing the high-level contaminant is useful in addressing this question. Samples from four vials of ARM, three with the low level and one with the high level of contaminant, is diluted three-fold prior to addition of the 1% SDS. Then the A260 is measured as in the ARM A260 SDS protocol. Table 1 shows the virus particle concentrations of the three low-level contaminant samples ranging from 5.5 to 5.7 x 10^{11} particles/mL. This is consistent with the established value of the ARM at 5.8 x 10^{11} particles/mL as determined by the combined results of 16 different laboratories. The high-level contaminant sample gives a clearly different value of 7.4 x 10^{11} particles/mL, due to the contribution of the contaminate to the A260 measurement. This value is approximately 30% higher than the one measured for the ARM with the low level of contaminant. Since the high-level contaminant sample has about 28-fold more contaminant than the average for the low-level contaminant samples, it is estimated that the contaminant only inflates the calculated particle concentration by about 1%.

The UV spectra, in 0.1% SDS, of the above four ARM samples is determined. As shown in Figure 4, the three low-level contaminant sample spectra are superimposable, while spectrum for the high-level contaminant sample is distinctly different at the lower wavelengths. Also shown is the result of subtracting the spectrum of a low-level contaminant sample from the high-level contaminant sample. This resulting spectrum, which represents the contaminant, shows the local wavelength maximum at about 240 nm, which is also seen in the spectrum of the contaminant peak on RP-HPLC. Comparison of the above sample spectra in the absence of SDS also shows a very similar subtraction spectrum to the one obtained with the sample in the presence of SDS (data not shown). The A260/A280 ratio of the high-level contaminant sample is not significantly different from the value determined from multiple samples of ARM (1.37 in the presence of 0.1% SDS.).

Centrifugation Separates the Contaminant from the Virus: Samples of different ARM vials, including the one with the high-level contaminant, are centrifuged for 90 minutes at 10,000 RPM in an Eppendorf microfuge to pel-

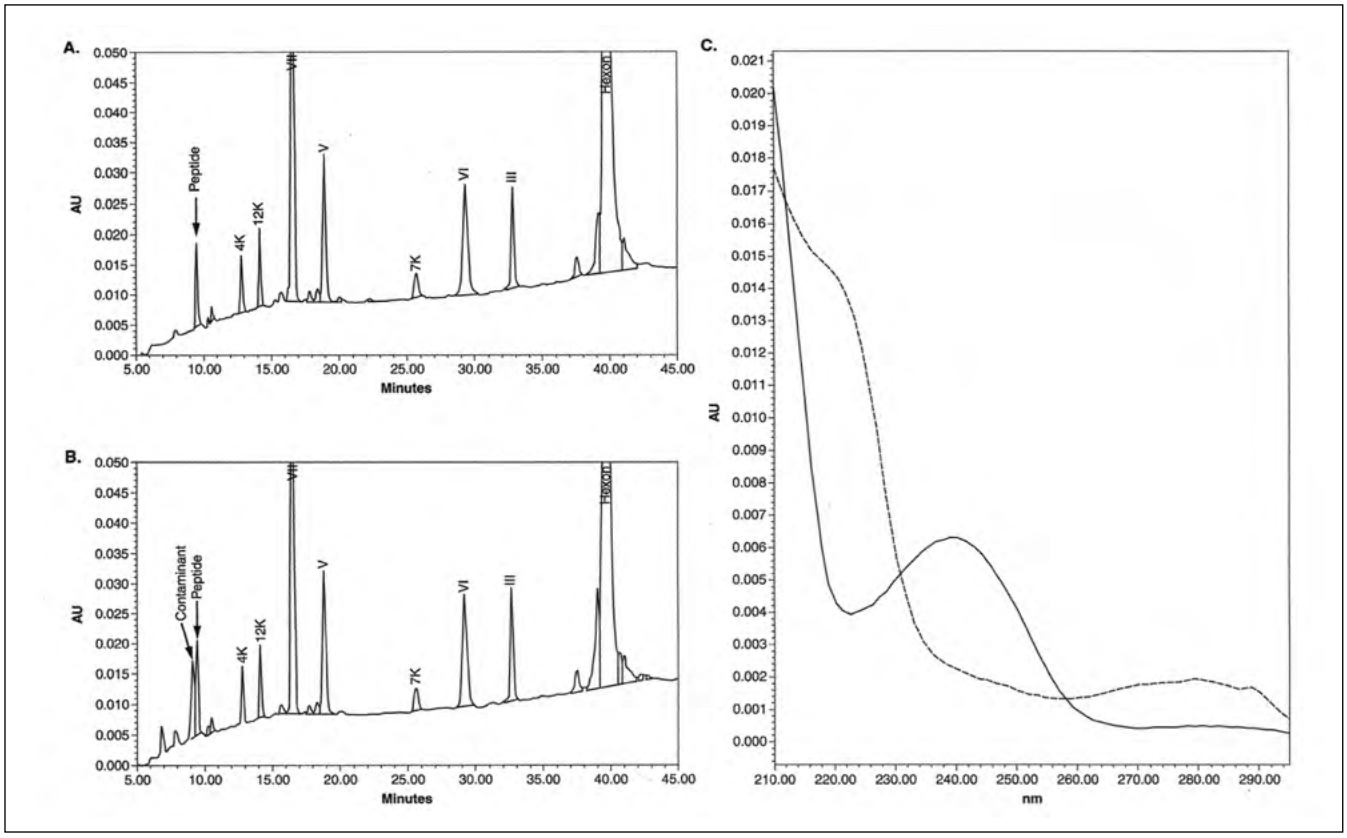

Figure 2. RP-HPLC analysis of control rAd and ARM by Method 2. (A) Control sample of a highly-purified rAd at 5.0 x 1011 particles/ml (20 µl injection). Arrow indicates the viral peptide peak position. (B) ARM sample (20 µl injection) has a contaminant peak area at 214 nm of 3%. Arrows indicate the contaminant and the viral peptide peak positions. (C) Comparative UV spectra of the viral peptide peak (dashed) and contaminant peak (solid), both from (B).

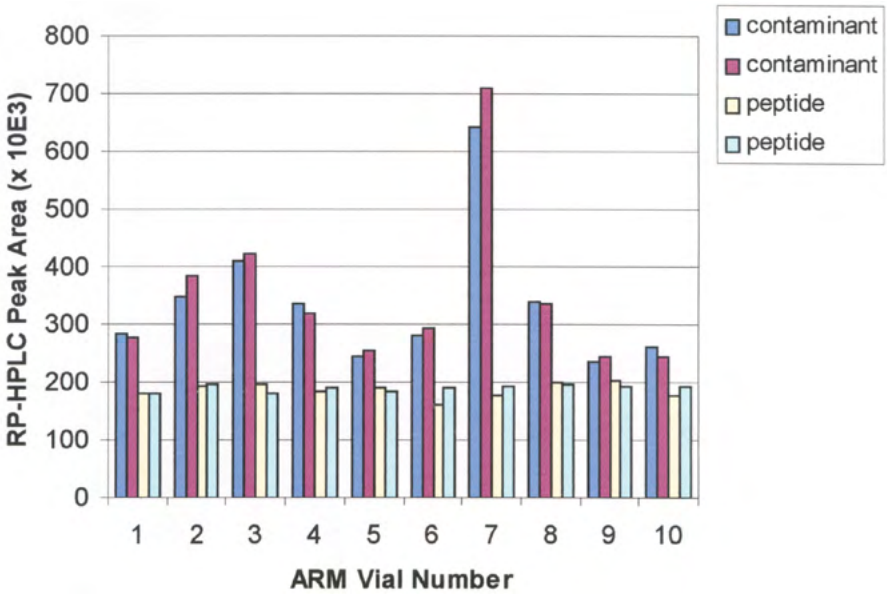

Figure 3. Determination of contaminant from ten vials of ARM. Ten vials of ARM lot #001503 were analyzed by RP-HPLC Method 2. Injections were 20 µl and each sample was run in duplicate. The duplicate values of the contaminant and the viral peptide are plotted as peak area in absorbance units (at 214 nm) x min.

let the virus. Examination of the supernatant with RP-HPLC shows that all of the contaminant is present, but none of the virus proteins. Essentially none of the contaminant remains in the resuspended pellet, but almost all of the virus proteins do. This indicates that there is no apparent association between the contaminant and the virus, allowing purification of the contaminant.

Preliminary Analysis of the Contaminant by LC/MS and GC/MS (data not shown): We prepare samples of purified contaminant for analysis by removing the virus with centrifugation. After background subtraction, the LC/ESI-MS analysis of the contaminated samples shows two major peaks. They are m/z 263.0 and m/z 171.0. Due to the relatively high orifice potential, the m/z 171.0 could be a fragment ion generated from the m/z 263.0. Definite structure could not be proposed from this data without further work. Besides the expected glycerol and Tris from the

buffer, the GC-EI-MS analysis shows evidence of DMSO, $(CH_3)_2$-CH)-$(CH_2)_{12}$-CO_2-CH_3, and various silanes, although the silanes may have bled from the siloxane GC column.

In the previous work of one collaborator, a similar contaminant peak had been found in some adenovirus preparations. In this work, the peak was collected from RP-HPLC and analyzed by GC-MS. Identified compounds was various siloxanes and methyl-benzaldehyde. None of the compounds were consistent with the coating of the GC capillary. The siloxanes could be decomposition products from plasticizers. For example, methyl-benzaldehyde is a widely used solvent found in silicone glues. This contamination has been successfully traced to a container used during the purification.

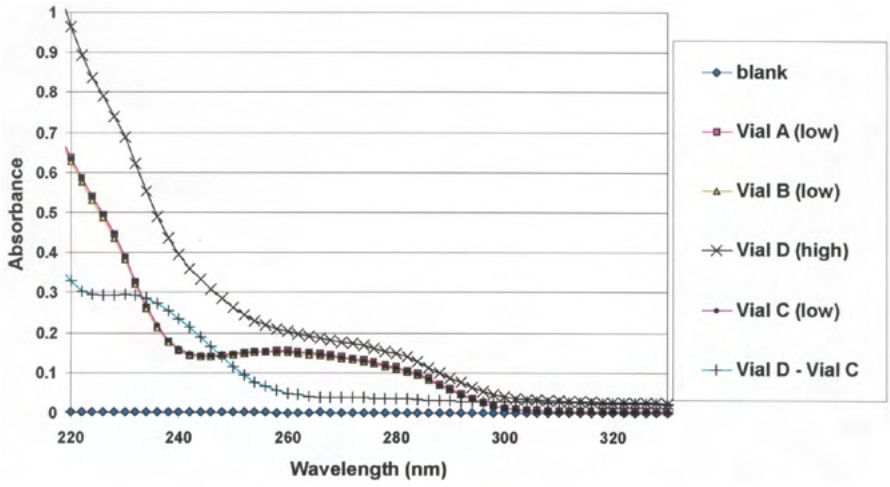

Figure 4. UV spectra of ARM samples in 0.1% SDS. The spectra are of the four samples of ARM used for particle determination by A260 in SDS that were shown in Table 1. Vials A-C are low-level contaminant ARM samples while Vial D is the high-level contaminant sample. The spectrum for Vial D - Vial C is the difference spectrum of a low-level contaminant vial from the high-level contaminant vial, and represents the spectrum of the contaminant.

Evidence That the Contaminant was Introduced During the Final Processing Step: Samples are obtained of intermediate processing steps following the ARM purification with anion exchange chromatography. These samples are chromatographed on RP-HPLC to determine if the contaminant is present. The results (Table 2) show no detectable contaminant in the column eluate hold sample, or in the concentrated bulk sample. The bulk is then divided into four lots and frozen. After thawing, samples from all four lots show no contaminant. However, all lots show similar, yet detectably different, low levels of the contaminant after filtration but prior to vialing.

The above results indicate that some amount of contaminant was probably introduced to the ARM during the final filtration step, and may have been a leachate from the tubing or a container. This would account for the differences seen in the bulk from different lots. But it would not account for the vial-to-vial differences within the same lot. These differences could be due to the introduction of additional contaminant from the tubing used during the fill process.

Probability of a High-level Contaminant ARM Vial: Since a high-level of the contaminant clearly interfered with the determination of virus particle concentration by A260 SDS, it was necessary to estimate what percentage of the vials might have this level. After examining 59 vials from all four lots, only one vial with a high-level of contaminant was detected by RP-HPLC. This leads to an estimate that a vial with a high-level of contaminant will occur less than 2% of the time. Furthermore, another 30 vials were analyzed by 15 laboratories to determine the particle concentration, by A260 / SDS, as part of the ARM characterization protocol. Since no values were similar to, or greater than, that seen with the high-level contaminant vial (7.4×10^{11} particles/mL), we concluded that none of these 30 vials contained the high-level of contaminant. This led to the adjusted estimate that a vial with a high-level contaminant will occur approximately 1% of the time.

If an end-user laboratory obtains a high-level contaminant vial, the presence of the contaminant would likely have no effect on most methods of analysis, such as infectivity or anion exchange HPLC. As seen above, it shows a higher than expected value by the A260 SDS particle determination method. The expected particle concentration for the ARM is 5.8×10^{11} particles/mL, and since the 95% confidence limits predict most readings will fall between 5.6 and 6.0×10^{11} particles/mL, then a vial with a value significantly greater than 6.0×10^{11} particles/mL might contain a high level of the contaminant. The presence of a high level of contaminant might also be supported by comparison of the UV spectrum (in the presence or absence of SDS) with a contaminant-free rAd sample. Confirmation would require analysis by an RP-HPLC method.

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