

Influence of elemental impurities in aluminum hydroxide adjuvant on the stability of inactivated Japanese Encephalitis vaccine, IXIARO®



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ABSTRACT

Aluminum hydroxide is a critical raw material in the production of many vaccines. It is used as an adjuvant in the formulation of the final bulk vaccine, and for this it must meet the specifications of the European Pharmacopeia Monograph. We investigated whether vaccine stability was affected by the presence of trace amounts of elemental impurities in commercially available aluminum hydroxide. The content of residual elemental impurities in commercially available aluminum hydroxide was determined by selective and sensitive inductively coupled-plasma mass spectrometry and inductively coupled plasma atomic emission spectroscopy. We found significant differences between different suppliers, but also between different lots from the same supplier. Inactivated Japanese encephalitis vaccine, IXIARO®, was used to study the effect of residual metals in aluminum hydroxide on antigen stability. We propose that antigen degradation occurred via a pathway involving the metal-catalyzed, auto-oxidation of a process-related impurity (sulfite). Thus, sulfite auto-oxidation resulted in antigen degradation when residual Cu was present at elevated concentrations in aluminum hydroxide.

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1. Introduction

Aluminum compounds are the only adjuvants used widely for human vaccines, and they are the most common adjuvants in veterinary vaccines [1]. Aluminum hydroxide and aluminum phosphate adjuvants are generally prepared by exposing an aqueous solution of aluminum ions to alkaline conditions in a controlled chemical environment resulting in precipitation of the insoluble hydroxide. Various soluble aluminum salts can be used to produce aluminum hydroxide. Each manufacturer utilizes proprietary raw materials and has standardized the individual production process to achieve reproducible adjuvant quality. Processing conditions, including temperature, stirring speed and reagent concentrations, significantly influence the biophysical and biochemical properties of this type of mineral adjuvants, and they are considered trade secrets of individual manufacturers.

Solubilized (heavy) metal anions present at low concentrations in aluminum-containing raw materials may co-precipitate during

adjuvant preparation. These residual metals may change the characteristics of aluminum hydroxide to differ from those of “pure” aluminum hydroxide. After the initial precipitation step, all downstream processing steps only remove water-soluble impurities; thus, they will not remove metals either adsorbed or embedded into the aluminum hydroxide gel matrix.

Methods for determining the presence of residual metals in aluminum hydroxide gels are specified in guidelines from European agencies (Ph. Eur. 2.4.8) and the United States Pharmacopoeia (USP 231). These methods are based on the precipitation of a large group of heavy metals (including Pb, Hg, Bi, As, Sn, Cd, Ag, Cu, Mo, and Se) with thioacetamide. The total quantity is semi-quantitatively determined against a lead (Pb) standard with the maximum amount specified as ≤ 20 ppm. More specific methods for Fe and Arsenic contents are covered by the guidelines; these are specified as ≤ 15 ppm and ≤ 1 ppm, respectively. All aluminum hydroxide lots examined in this study met these safety specifications. It is well known that residual metal impurities can catalyze a variety of complex degradation reactions in biopharmaceuticals, such as oxidation and proteolytic degradation [2,3]. This investigational study aimed at investigating in more detail antigen stability related to the presence of trace amounts of elemental impurities in commercially available aluminum hydroxide adjuvants. We

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used the Japanese encephalitis (JE) vaccine, IXIARO[®], as a model antigen to represent a highly purified, inactivated viral antigen [4]. Furthermore the residual trace amounts of metals in commercially available aluminum hydroxide preparations obtained from three different suppliers was characterized based on sensitive and selective inductively coupled-plasma mass spectrometry (ICP-MS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES). Based on our results, we propose a potential antigen degradation pathway driven by metal-catalyzed sulfite auto-oxidation.

2. Materials and methods

2.1. Analysis of metals in aluminum hydroxide

Residual metals were analyzed with ICP-MS (Agilent 7700× ICP-MS in high-helium-collision-cell mode) at the Medical Laboratory, Bremen (Germany). In short, samples of aluminum hydroxide in suspension were digested with concentrated HNO₃ in a microwave-assisted high-pressure vessel. The clear solution was then further diluted and analyzed. Residual concentration of heavy metals are reported in ppm (1 ppm corresponds to 1 µg/mL) if not otherwise stated. The presence and content of the following metals were determined: Al, Fe, Ni, Cu, Co, Cr, Ag, Cd, W, Pb, V, Rb, Mo. Depending on the analyte, the limit of quantification (LOQ) was 0.005–0.025 ppm as confirmed by spike-recovery experiments.

In addition, a semi-quantitative, 70-element scan was performed by Environmental Scientifics Group Ltd. (ESG, UK) with a combination of ICP-MS (Agilent 7500ce) and ICP-AES (Perkin Elmer Optima 4300DV), both calibrated with certified standards. The element scan is a screening method with limited sensitivity and accuracy. At levels an order of magnitude above the LOD for an element, the results are estimated to have an uncertainty of ±50%.

2.2. Aluminum hydroxide

For this investigational study, we selected aluminum hydroxide adjuvants produced from three different manufacturers (Table 1): Chemtrade (US; formerly General Chemical), SPI Pharma (France), and Brenntag Biosector (Denmark). Aluminum hydroxide produced by Brenntag Biosector under the trade name “Alhydrogel[®] 2%” was the approved adjuvant used in IXIARO[®]. In addition, Brenntag Biosector offered a second grade of Alhydrogel[®] to be used for this investigative study. Compared to the standard product with a specified ammonium content of ≤50 ppm, this “improved” Alhydrogel[®] underwent more wash cycles with water after the alkaline precipitation step to further reduce the residual ammonium. Three lots of the “improved” Alhydrogel[®] were evaluated to determine whether more wash cycles could also reduce the residual metal impurities. For the aluminum hydroxide samples obtained from Chemtrade and SPI Pharma, only a few primary metal impurities (Cu, Ni, Fe) were analyzed in the preliminary evaluation. All lots of aluminum hydroxide used in this study met the specifications of the European Pharmacopeia Monograph 1664, as stated in the corresponding certificates of analysis.

2.3. Manufacturing process of IXIARO[®]

IXIARO[®], the commercially manufactured JE vaccine (in Australia and New Zealand available as JESPECT[®]), is produced by Valneva Scotland, Ltd. This vaccine is a highly purified, formaldehyde-inactivated, whole JE virus (JEV), which is adsorbed onto an aluminum hydroxide adjuvant. The JEV was the attenuated SA₁₄-14-2 strain, which was cultivated in vero cells and purified by ultrafiltration, protamine-sulfate treatment, sucrose

gradient density centrifugation followed by inactivation. Following the inactivation period, the residual formaldehyde was neutralized with sodium metabisulfite. The neutralized, inactivated virus (NIV) was adjusted with PBS to a desired protein concentration of 12 µg/mL. The final drug product bulk vaccine (FBV) was prepared by adsorption of the antigen to Alhydrogel[®] to a final concentration of 0.5 mg/mL Al³⁺. More details of the manufacturing process were described previously [6]. Note that the current commercial vaccine does not contain any preservatives. Aliquots from NIV and FBV obtained from commercial manufacturing lots were used to prepare the exploratory vaccine formulations used in this study.

2.4. Antigen desorption from aluminum hydroxide

Inactivated JEV antigen was desorbed from aluminum hydroxide in the presence of 0.8 M potassium phosphate buffer (pH 8) containing BSA (50 µg/mL). After incubation at 25 °C for 1 h, aluminum hydroxide particles were removed by centrifugation, and the supernatant containing the antigen was analyzed with ELISA. The average antigen recovery under routine conditions was determined as 75 ± 10%.

2.5. Inactivated JEV ELISA (polyclonal based)

The ELISA method utilizes polyclonal sheep anti-JEV coating antibody to capture inactivated JE virus. The primary detection antibody was a polyclonal rabbit anti-JEV antibody, and the secondary antibody was a donkey anti-rabbit antibody conjugated to HRP. A six-point standard curve, ranging from 0.05 to 0.8 antigen units (AU)/mL was prepared from an inactivated JEV reference standard and used to calculate antigen content in the samples.

2.6. Inactivated JEV ELISA (monoclonal based)

The monoclonal (mAb)-based JEV ELISA assay was similar to the polyclonal JEV ELISA assay format, except a monoclonal anti-JEV antibody (clone 52-2-5) was used for coating. This mAb 52-2-5 was shown to be specific for both active and inactive JEV, and it recognized a neutralizing epitope on domain III of the envelope (E) protein [5]. This neutralizing effect of the mAb gave rise to the notion that the epitope might be important for the antigen to elicit a protective immune response.

2.7. Assessment of vaccine stability based on poly- and monoclonal ELISA data

Any variability in the antigen desorption procedure could change the recovery of antigen, and thus, alter the absolute antigen content determined by ELISA. We used a calculation of the ratio of desorbed antigen measured in monoclonal and polyclonal ELISAs (mono/polyclonal ELISA ratio) as a measure for antigen stability. This approach allowed us to compensate for any variability of recovered antigen after desorption and to have an independent and reproducible measure for stability. The polyclonal ELISA measured the total desorbed antigen, and the monoclonal ELISA measured a structurally intact, specific, neutralizing epitope. A ratio close to 1 corresponded to a high content of intact antigen; a ratio close to 0 corresponded to a low content of intact antigen, which indicated antigen degradation. A broad qualification data set indicated an estimated ratio variability of ±8%, when based on a single measurement.

2.8. Mouse potency assay

To evaluate the neutralizing antibody response, we inoculated female CD-1 mice (10 per group) with a single peritoneal injection

Table 1
Overview of commercially available aluminum hydroxides tested.

Supplier	Trade name	Catalogue number	Aluminum Specification	Number of lots investigated	Quantitative detection of metal ion impurities by ICP-MS and ICP-AES	Semi-quantitative testing for elemental impurities in 70 element scan
Brenntag	Alhydrogel® 2%	843250	Aluminum ^a content 9.0–11.0 mg/mL	10	Cu, Ni, Fe, Co, Cr, Ag, Cd, W, Pb, V, Rb, Mo	2 lots tested
	Alhydrogel® 2% “improved”	n.a.	Aluminum ^a content 9.0–11.0 mg/mL ^a	3		Not tested
Chemtrade	Rehydragel LV	203120070602	Aluminum oxide (Al ₂ O ₃) 1.8–2.2%	11	Cu, Ni, Fe	2 lots tested
SPI Pharma	Aluminum hydroxidewet-gel VAC 20	4800	Aluminum oxide (Al ₂ O ₃) 1.9–2.1%	2	Cu, Ni, Fe	2 lots tested

* Equivalent to 1.8%–2.2% Al₂O₃.

(on day 0) with six different doses of the final vaccine (ranging from 3000 ng to 12.3 ng). Single serum samples were collected on Day 21 and tested with the plaque reduction neutralization test (PRNT) to evaluate seroconversion in each individual mouse. PRNT was carried out on vero cells, challenged with the live virus, JEV SA₁₄-14-2. Potency was expressed in terms of the 50% effective dose (ED50); i.e. the amount of administered vaccine (in ng), which resulted in a 50% seroconversion rate in mice. Higher ED50 values indicated lower vaccine potencies.

2.9. Preparation of exploratory JEV vaccine formulations

Exploratory formulations were prepared to evaluate the influence of metals on the stability of inactivated JEV in solution under accelerated (22 °C) storage conditions. The NIV solution was obtained from the commercial manufacturing process (lot NIV 11A74). The antigen content was adjusted by diluting the concentrated NIV two-fold with PBS. The solutions were adjusted to pH 7 and pH 8, and each was spiked with metal ions Ni(II), Cu(II), and Cr(III) at final concentrations of 0.1, 0.5, and 1 ppm, respectively.

The adsorbed FBV was either obtained from commercial-scale manufacturing (FBV 11D87, formulated with Alhydrogel®, Lot 4539) or prepared in the laboratory by adding selected aluminum hydroxide lots to the adjusted NIV solutions to a final concentration of 500 µg Al/mL. All of these formulations contained residual formaldehyde (approx. 1.4 mM) and sulfite (approx. 1.2 mM) at concentrations similar to the commercial product. Metal ion spiking and pH adjustments were performed as described above for NIV preparations. Exploratory formulations with varying concentrations of residual formaldehyde and sulfite were prepared by adding appropriate amounts of stock solutions. Final formulated samples were stored in plastic tube, at normal (2–8 °C) and accelerated conditions (22 °C) for 7 weeks. After storage, the antigen was desorbed and quantified with ELISAs (polyclonal and monoclonal). In all experiments, control samples were prepared without any spiked metals and stored under the same conditions. Working solutions of metal ions (1 mM final concentration) were prepared in bidest. water, followed by sterile filtration through 0.2-µm syringe filters. All metal salts (nickel(II)sulfate hexahydrate, copper(II)chloride dehydrate, chromium(III)chloride hexahydrate) were purchased from Sigma. The formaldehyde stock solution (308 mM) was prepared by diluting 37% formaldehyde (Sigma) with purified water. Sodium metabisulfite (Na₂S₂O₅) stock solution, which contained 400 mM SO₃²⁻, was prepared by dissolving 1.52 mg sodium metabisulfite (Sigma) in 40 mL PBS.

3. Results

3.1. Scan of 70 elements

This preliminary 70 elemental screening provides only rough estimates of whether any metals were present. Due to the high analytical uncertainty of the method, only elements present at concentrations greater than 15-fold of the LOD were considered elevated (Table 2, highlighted numbers). The complex composition of different Aluminum hydroxide samples was evident from the large differences found in residual contents of Ba, Ca, Cr, Fe, Ga, Mg, Na, S, Sr, and Zr. VAC 20. Rehydragel LV had higher levels of Ca (83–120 ppm), Mg (22–72 ppm), and Na (250–310 ppm) compared to Brenntag's Alhydrogel®, which had residual levels within the range of the LOD. The Ba levels measured in Rehydragel LV samples (approx. 1 ppm) were over 200-fold higher than those measured in Brenntag's Alhydrogel® (<0.005 ppm), and slightly higher than those measured in VAC 20 samples (approx. 0.07 ppm). Also, Cr (0.5 ppm) was highest in Brenntag's Alhydrogel® samples. Elevated concentrations of Fe (0.7–4.7 ppm) and Ga (0.6–1.8 ppm) were detected in all samples. Rehydragel LV had notably lower concentrations of sulfur (<5–6.2 ppm) compared to those in VAC20 (390–490 ppm) and Alhydrogel® (310–320 ppm). Varying levels of residual metal impurities might be explained by differences in raw materials used (e.g. sources of aluminum salts, chemicals, water quality) in the production processes of the individual manufacturers. Results indicated that aluminum hydroxide adjuvant is a complex raw material that contains various elemental impurities at different concentrations. Further characterization would require analysis with more sensitive, quantitative methods to confirm the actual concentrations of metal impurities.

3.2. Quantitative determination of residual metals by ICP-MS

Currently, Brenntag supplies the aluminum hydroxide used in the IXIARO® vaccine; thus, it was important to evaluate batch-to-batch variability of residual metal impurities on a large number of lots. To ensure we quantitatively determined a larger number representative elements by sensitive and quantitative ICP-MS (Table 3). Fe and Cr were selected since higher concentration was already detected in the 70 element screen. Other metals were selected for analyses on a basis of their general catalytic properties, such as Ni, Cu, Co, Ag, Cd, W, Pb, V, Rb and Mo. We observed that different Alhydrogel® lots showed large variations in the amounts of residual metals. The most notable metals present were Fe (0.183–5.640 ppm), Cr (0.020–1.874 ppm), and

Table 2
Semi-quantitative analyses of 70 elements in 2% aluminum hydroxide samples determined with ICP-AES and ICP-MS. Concentrations greater than 15-fold of the corresponding LOD are highlighted (Bold). Elements below the limit of detection in all samples are shown at the end of the table.^a

Supplier	LOD (ppm)	Brenntag (Alhydrogel®)		Chemtrade (Rehydrogel LV)		SPI Pharma (Aluminum hydroxide wetgel VAC 20)		Instrument
Lot		4712	4746	2329401	2339401	201200198	201300059	
Element		Metal content (ppm)						
Al	5	9500	9300	9700	9800	9700	11000	ICP-AES
As	0.03	<0.03	<0.03	<0.03	0.049	0.59	1.1	ICP-MS
Ba	0.005	<0.005	<0.005	0.88	1.1	0.07	0.087	ICP-AES
Be	0.001	<0.001	<0.001	0.014	0.012	<0.001	0.0012	ICP-MS
Ca	0.2	2.4	<0.2	83	100	89	120	ICP-AES
Cd	0.005	<0.005	<0.005	0.0051	0.011	<0.005	<0.005	ICP-MS
Co	0.002	<0.002	<0.002	0.013	0.013	0.017	0.0098	ICP-MS
Cr	0.03	0.64	0.42	0.051	0.033	0.096	0.072	ICP-AES
Cu	0.04	<0.04	<0.04	<0.04	<0.04	0.28	<0.04	ICP-AES
Fe	0.04	1.0	0.67	1.8	2.2	4.7	3.4	ICP-AES
Ga	0.005	0.58	0.88	1.2	1.2	1.7	1.8	ICP-MS
Gd	0.002	<0.002	<0.002	0.003	<0.002	<0.002	<0.002	ICP-MS
Hf	0.002	<0.002	<0.002	<0.002	<0.002	0.011	0.013	ICP-MS
K	1	<1	<1	<1	1.8	2.7	1.8	ICP-AES
La	0.002	<0.002	0.0099	0.14	<0.002	<0.002	0.72	ICP-MS
Li	0.002	<0.002	<0.002	0.022	0.039	0.0094	0.019	ICP-MS
Lu	0.0002	<0.0002	0.00021	0.0003	<0.0002	<0.0002	<0.0002	ICP-MS
Mg	0.01	<0.01	<0.01	22	26	72	23	ICP-AES
Mn	0.02	<0.02	<0.02	<0.02	0.055	0.033	0.023	ICP-AES
Na	0.2	<0.2	<0.2	270	290	250	310	ICP-AES
Nb	0.001	<0.001	<0.001	<0.001	0.0018	0.0011	0.0011	ICP-MS
Ni	0.02	<0.02	<0.02	<0.02	0.0049	<0.02	<0.02	ICP-MS
P	1	<1	<1	<1	<1	<1	1.9	ICP-AES
Pb	0.01	<0.01	<0.01	<0.01	<0.01	0.21	<0.01	ICP-MS
S	5	320	310	<5	6.2	390	480	ICP-AES
Sb	0.003	<0.003	<0.003	<0.003	<0.003	0.0049	<0.003	ICP-MS
Sn	0.004	0.0059	0.0079	0.015	0.028	0.034	0.048	ICP-MS
Sr	0.03	<0.03	<0.03	1.5	1.6	0.64	0.73	ICP-MS
Ta	0.0004	<0.0004	<0.0004	0.00046	<0.0004	0.00059	<0.0004	ICP-MS
Te	0.04	<0.04	<0.04	<0.04	<0.04	<0.04	0.054	ICP-MS
Ti	0.03	<0.03	<0.03	0.14	0.14	0.4	0.45	ICP-MS
U	0.0005	<0.0005	<0.0005	0.0011	0.00079	0.0064	0.012	ICP-MS
V	0.05	<0.05	0.067	0.11	0.091	0.098	0.072	ICP-AES
W	0.003	<0.003	<0.003	<0.003	0.0034	<0.003	<0.003	ICP-MS
Y	0.001	<0.001	<0.001	0.0031	0.0018	<0.001	<0.001	ICP-MS
Zr	0.0005	0.0027	0.004	0.029	0.035	0.11	0.12	ICP-MS

^a Ag (<0.002), Au (<0.005), B (<0.5), Bi (<0.003), Ce (<0.0005), Cs (<0.0005), Dy (<0.001), Er (<0.005), Eu (<0.005), Ge (<0.03), Hg (<0.005), Ho (<0.002), In (<1), Ir (<0.002), Mo (<0.01), Nd (<0.002), Os (<0.002), Pd (<0.005), Pr (<0.0004), Pt (<0.003), Rb (<0.002), Re (<0.001), Rh (<0.001), Ru (<0.004), Sc (<0.01), Se (<0.4), Sm (<0.003), Tb (<0.0002), Tc (<0.03), Th (<0.0004), Tl (<2), Tm (<0.0003), Yb (<0.0005), Zn (<0.005).

Ni (0.008–0.816 ppm). The lowest concentrations of these metals were found in Alhydrogel® lots 4074 and 3877 and the highest were found in lot 4230. In addition, lot 4230 contained detectable amounts of Cu (0.064 ppm). The metal concentrations in the “improved” Alhydrogel® were not significantly different from those

in the commercially available, standard Alhydrogel® preparations. Thus, the additional washing steps performed for the improved product did not reduce the residual metal content, suggesting that, either the impurities were strongly bound to the surface of the aluminum hydroxide particles, or they had co-precipitated as

Table 3
Summary of quantitative analysis of metal ions in Alhydrogel® (2% solution) performed with ICP-MS. Note: A 2% stock solution contains 10 mg/mL Al.

Alhydrogel® (2% solution) Metal content (ppm)	Al	Fe	Ni	Cu	Co	Cr	Ag	Cd	W	Pb	V	Rb	Mo
Lot 4074	9130	0.266	0.015	<0.025	<0.005	0.020	<0.005	<0.005	<0.025	0.019	<0.005	<0.005	<0.005
Lot 4230	9570	5.640	0.816	0.064	0.007	1.139	<0.005	<0.005	<0.025	0.024	0.013	<0.005	0.011
Lot 4470	9560	1.179	0.018	<0.025	<0.005	1.637	<0.005	<0.005	<0.025	0.020	<0.005	<0.005	0.022
Lot 4414	10272	2.790	0.036	<0.025	<0.005	1.710	<0.005	<0.005	0.035	<0.025	<0.007	n.a.	n.a.
Lot 4539	n.a.	0.943	0.119	<0.025	<0.005	0.276	<0.005	<0.005	<0.025	0.027	<0.005	<0.005	<0.005
Lot 3877	10766	0.183	0.012	<0.025	<0.005	0.048	<0.005	<0.005	<0.025	0.010	<0.005	<0.005	<0.005
Lot 4187	10100	3.617	0.172	<0.025	<0.005	2.333	<0.005	<0.005	<0.025	0.018	<0.005	<0.005	<0.005
Lot 4287	9800	2.047	0.296	<0.025	<0.005	0.620	<0.005	<0.005	<0.025	0.030	0.010	<0.005	<0.005
Lot 4563	9360	2.485	0.009	<0.025	<0.005	1.874	<0.005	<0.005	<0.025	0.008	<0.005	<0.005	<0.005
Lot 4621	9760	1.183	0.008	<0.025	<0.005	1.333	<0.005	<0.005	<0.025	0.008	<0.005	<0.005	<0.005
Lot 4580 (“improved”)	10497	2.610	0.027	<0.025	<0.005	1.470	<0.005	<0.005	<0.025	<0.025	<0.005	n.a.	n.a.
Lot 4596 (“improved”)	10776	3.530	0.027	<0.025	<0.005	1.710	<0.005	<0.005	<0.025	<0.025	<0.005	n.a.	n.a.
Lot 4577 (“improved”)	10720	3.060	0.024	<0.025	<0.005	1.650	<0.005	<0.005	<0.025	<0.025	<0.005	n.a.	n.a.
Lot 4230 Supernatant	n.a.	0.082	0.012	<0.025	<0.005	0.007	<0.005	<0.005	<0.025	<0.025	<0.005	n.a.	n.a.
Lot 4230 Sediment	n.a.	6.200	0.920	<0.025	<0.005	1.200	<0.005	<0.005	<0.025	0.045	0.013	n.a.	n.a.

n.a.: not analyzed.

Table 4
Analysis of residual Cu, Fe, and Ni impurities in 2% aluminum hydroxide.

Lot number	Metal concentration (ppm)		
	Copper	Iron	Nickel
Brenntag (Alhydrogel®)			
4712	0.009	0.870	0.071
4746	0.015	0.780	0.071
Chemtrade (Rehydragel LV)			
2329401	0.083	1.600	0.180
2339401	0.081	1.700	0.220
3092402	0.020	1.600	0.160
3136401	0.020	1.500	0.150
3197402	0.063	1.600	0.200
3184401	0.076	1.700	0.210
3273401	0.072	1.500	0.180
2321401	0.120	2.000	0.240
3262401	0.057	1.500	0.180
3197402	0.072	1.700	0.230
3205401	0.056	1.600	0.200
SPI Pharma (VAC 20)			
201200198	0.490	3.500	0.085
201300059	0.370	2.800	0.095

hydroxides during the production process. Presence of metals in the solid particle sediment only was also confirmed by analyzing the solid and aqueous phase of lot 4230 obtained after centrifugation showing that hardly any of the metals could be detected in the supernatant. Preliminary evaluation of lot-to-lot consistency in aluminum hydroxide samples from other suppliers (Table 4) was done for three metals selected (Cu, Fe, Ni). We found that VAC 20 had much higher levels of Cu (0.370–0.490 ppm) than the samples from other suppliers. Rehydragel LV had Cu levels from 0.020 to 0.120 ppm. VAC 20 had Fe levels of 2.800–3.500 ppm, which was in the same broad range as the levels found in Brenntag's Alhydrogel® and Chemtrade's Rehydragel LV. Interestingly, all Rehydragel lots showed a tight Fe range, which indicated good consistency across multiple batches. VAC 20 had Ni levels of 0.085 and 0.095 ppm, similar to that found in Brenntag's Alhydrogel® (71 ppm). Rehydragel LV had Ni concentrations of 0.150–0.240 ppm. Due to the higher Cu levels observed in Rehydragel LV and VAC 20, they were excluded at that time from further testing using inactivated JEV as a model antigen.

3.3. Stability of inactivated JEV (NIV) in a solution spiked with metals

The stability of NIV in the presence of spiked Cu(II), Ni(II), and Cr(III), formulated at pH 7.0 and 8.0, was determined by monoclonal/polyclonal ELISAs. A much lower antigen stability could be observed in the presence of Cu(II) at 0.5 and 1.0 ppm after storage for 7 weeks at 22 °C (Fig. 1), with faster degradation at higher metal concentration and lower pH. Ni(II) and Cr(III) did not influence antigen stability.

3.4. Stability of inactivated JEV (NIV) adsorbed on aluminum hydroxide spiked with metals

In this study, we used adsorbed JEV FBV 11D87 (formulated with Alhydrogel® Lot 4539) to simulate conditions where an antigen was adsorbed on aluminum hydroxide. The solution was adjusted to pH 7 or pH 8 and spiked with Ni(II), Cu(II), or Cr(III) at 0.1, 0.5, or 1.0 ppm, to evaluate the effects of metal concentrations in combination with pH changes. A trend of decreasing antigen stability in the presence of Cu(II) at pH 7 after 7 weeks at 22 °C (Fig. 2) was observed. Interestingly, the differences were not as pronounced compared to the experiments that used NIV

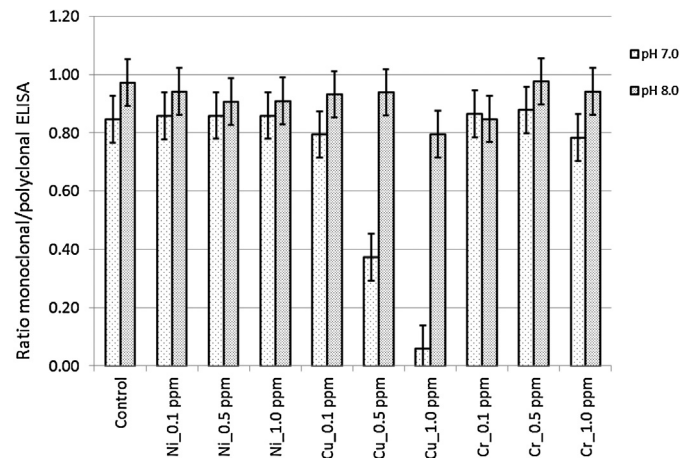


Fig. 1. Stability of NIV in the presence of contaminating metal ions Ni, Cu, Cr. NIVs were formulated at pH 7.0 or 8.0 and spiked with metal ions at 0.1, 0.500, and 1.0 ppm. Samples were incubated for 7 weeks at 22 °C, followed by ELISA analysis. Control samples were prepared without any spiked metals and stored under the same conditions.

not adsorbed to aluminum hydroxide. This slower degradation kinetics might be explained by a reduction in the mobility of the adsorbed antigen on the aluminum hydroxide which could minimize the interactions with the spiked metals. Also, any differences between the local pH on the surface of the adjuvant and bulk pH were predicted to minimize degradation of an adsorbed antigen [7,8].

3.5. Accelerated testing of inactivated JEV stability adsorbed on different Alhydrogel® lots

A single NIV batch (JEV11A74) from a commercial production was formulated with various Alhydrogel® lots produced by Brenntag, which covered a broad range of residual metals (see Table 3). Alhydrogel® lot 4230 had the highest Fe, Cu, Ni, and V contents, whereas Alhydrogel® lot 4074 had a low content of residual metals. We prepared mixtures of lot 4230 (low purity) and 4074 (high purity) to get “intermediate” levels of Ni and Cu and any other metals present. The final formulated vaccine samples were stored in plastic tubes at 2–8 °C (control conditions) or

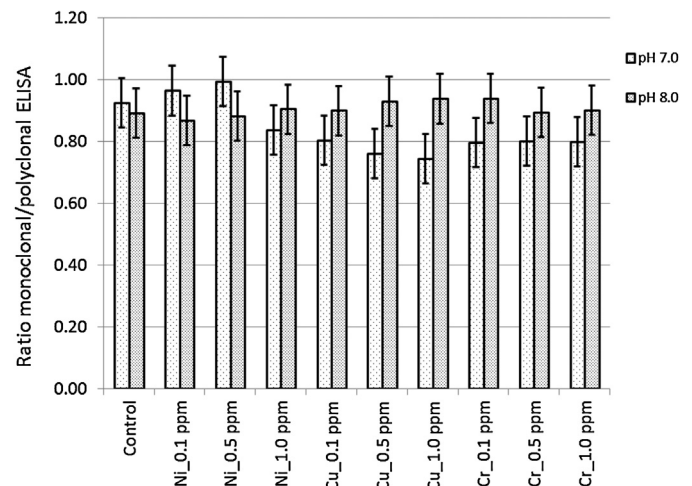


Fig. 2. Stability of NIV adsorbed on aluminum hydroxide in the presence of contaminating metal ions Ni, Cu, Cr. FBVs were formulated at pH 7.0 or 8.0 and spiked with metal ions at 0.1, 0.5, and 1.0 ppm. Samples were incubated for 7 weeks at 22 °C, followed by antigen desorption and ELISA analysis. Control samples were prepared without any spiked metals and stored under the same conditions.

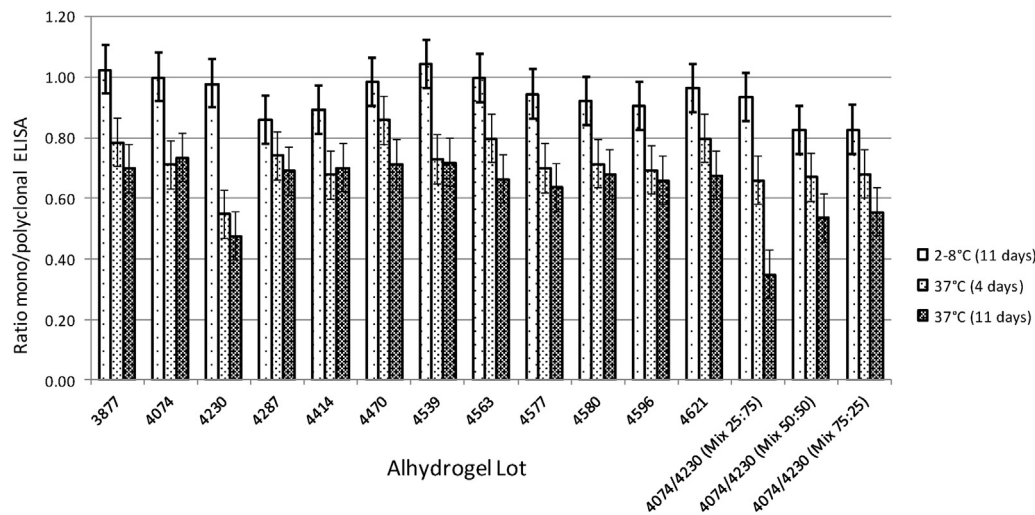


Fig. 3. Stability of inactive JEV formulated with different Alhydrogel® lots. Samples were stored for 4 or 11 days at 2–8 °C (control) or under accelerated conditions (37 °C) followed by desorption and ELISA analysis.

at 37 °C (accelerated conditions), for 4 and 11 days followed by assessment of stability (Fig. 3). The higher temperature (37 °C) was chosen to accelerate degradation reactions and reduce incubation times.

Storage at 2–8 °C did not change the antigen stability in this time course. At the accelerated temperature, stability declined in all samples after 4 and 11 days. However, antigen stability was much lower in formulations of Alhydrogel® 4230 compared to all the other aluminum hydroxide lots. After 11 days at 37 °C, Alhydrogel® 4230 formulation showed a mono/polyclonal ELISA ratio of approximately 0.35, while all other formulations had maintained ratios of 0.7 ± 0.05 . Samples with mixed Alhydrogel® lots, which contained 50% or 25% of lot 4230, also showed reduced stability (ratio 0.56). Based on these results, we concluded that aluminum hydroxide lot 4230 caused antigen degradation under stressed conditions.

3.6. Real time testing of inactivated JEV stability in different Alhydrogel® lots

We tested whether accelerated stability testing was predictive of real-time stability under normal vaccine bulk storage conditions (2–8 °C) over 20 months. NIV obtained from a commercial production (NIV lot 09M39) was used to prepare exploratory formulations with different Alhydrogel® lots representing low (lot 4074), intermediate (lot 4414), and high (lot 4230) contents of residual elemental impurities (see Table 3). At the beginning and end of the storage period, samples were analyzed with ELISAs to determine antigen stability (Fig. 4) and with the JEV mouse potency assay to determine the functional antibody response (Fig. 5). The formulations prepared with Alhydrogel® lots 4074 and 4414 showed a minor decrease in ELISA ratios from approximately 0.9–0.8 after 20 months. The formulation prepared with Alhydrogel® lot 4230 had a much lower ratio of 0.58, which indicated much higher loss of the neutralizing epitope during the storage period. One month after preparation of formulations all potencies were within the specified range for the commercial vaccine ($ED_{50} \leq 460$ ng). After 20 months, the vaccine potency of formulations containing Alhydrogel lot 4074 ($ED_{50} = 150$ ng) and 4414 ($ED_{50} = 173$ ng) remained in the expected range. The formulation prepared with Alhydrogel® 4230 had a much higher ED_{50} (2960 ng), which indicated a large loss of potency due to antigen degradation.

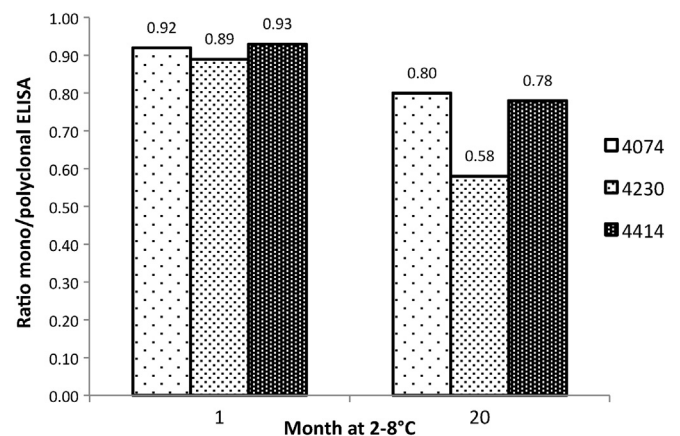


Fig. 4. Long time antigen stability of inactive JEV formulated with different Alhydrogel® lots representing low (lot 4074), intermediate (lot 4414), and high (lot 4230) contents of residual elemental impurities (see Table 3). Samples were desorbed and the antigen was analyzed by ELISAs after storage for 1 and 20 months at 2–8 °C.

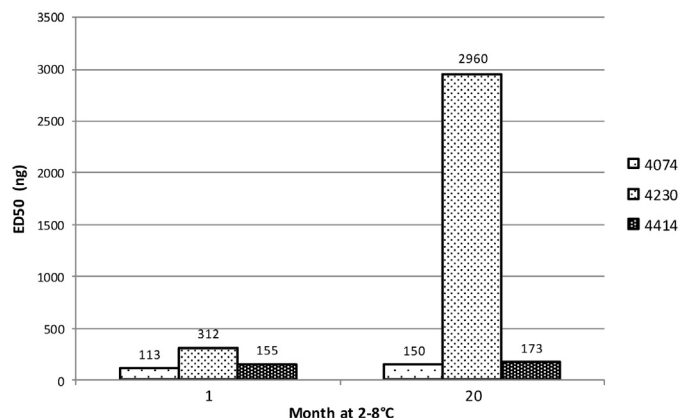


Fig. 5. Long time antigen stability of NIV prepared with different Alhydrogel® lots representing low (lot 4074), intermediate (lot 4414), and high (lot 4230) contents of residual elemental impurities (see Table 3). Samples were analyzed by mouse potency assay after storage for 1 and 20 months at 2–8 °C.

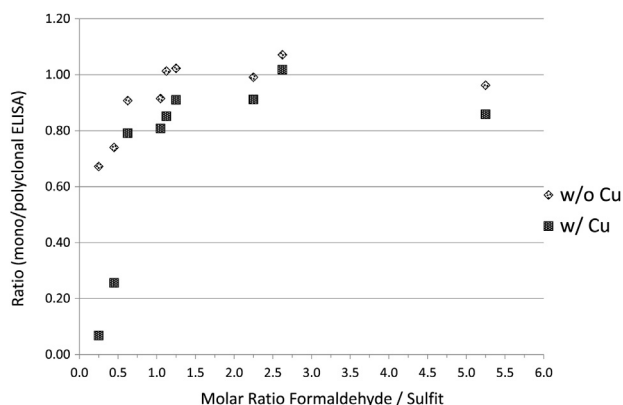


Fig. 6. Inactivated JEV stability adsorbed on aluminum hydroxide in presence of different molar ratios of formaldehyde and sulfite. In one formulation Cu(II) was added to a final concentration of 0.5 ppm. All formulations were incubated at 22 °C for 6 weeks. In the current IXIARO[®] manufacturing process, both substances (formaldehyde and sulfite) remain in the FBV at a slight molar excess of formaldehyde over sulfite with a residual average molar ratio of 1.17.

3.7. Exploratory FBV formulations spiked with Cu, formaldehyde, and sulfite

A final study was conducted to determine whether the degradation reaction might be caused by the single metal impurity, Cu, detected so far only in aluminum hydroxide lot 4230. NIV batch 11A74 was diluted two-fold with PBS (final pH 7.4) and formulated with Alhydrogel[®] batch 4287 (containing low elemental impurities). Exploratory formulations were prepared by adding different amounts of formaldehyde and sulfite to vary the molar ratio from 0.25 to 5.25. All formulations were split into two aliquots. One part was spiked with an additional 0.5 ppm Cu(II) to study the interaction of metal impurity with formalin and sulfite in more detail. Formulations were incubated at 22 °C for 6 weeks, followed by desorption and analysis with ELISA (mono/polyclonal, see Fig. 6). At lower molar formaldehyde/sulfite ratios of <1, higher antigen degradation was observed in presence of spiked Cu(II) as shown by low ELISA ratio of 0.07 at molar ratio 0.25. In the same formulations without Cu(II), the observed degradation rate was much lower (mono/polyclonal ELISA = 0.67 at a molar formaldehyde/sulfite ratio of 0.25). In all formulations, no significant antigen degradation was detected when formaldehyde was in molar excess over sulfite (molar ratio formaldehyde/sulfite > 1). These data suggested that the underlying degradation pathway observed in the presence of even a slight molar excess of sulfite was indeed catalyzed by Cu(II). In the current IXIARO[®] manufacturing process, both substances (formaldehyde and sulfite) remain in the FBV at residual concentrations of approximately 1.4 mM and 1.2 mM, respectively. The slight molar excess of formaldehyde over sulfite, with an average molar ratio of 1.17, appears to be insufficient to inhibit completely the formation of reactive substances in the presence of catalytic metals, such as Cu.

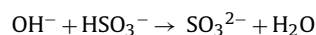
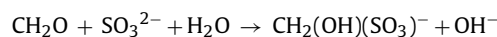
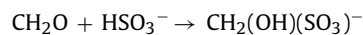
4. Discussion

4.1. Theory of metal-catalyzed sulfite auto-oxidation

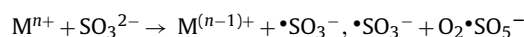
Our results suggested that the antigen degradation pathway involved the auto-oxidation of sulfite, through the formation of reactive free-radicals, which was catalyzed by residual metal impurity Cu. Previous studies have shown that metal-catalyzed oxidation is a common degradation pathway, resulting in covalent modifications of proteins and loss of biological activity [2,9]. In the current JEV inactivation process, formaldehyde is

neutralized with sodium-metabisulfite (Na₂S₂O₅). After dissolution of Na₂S₂O₅ in water, it is hydrolyzed into NaHSO₃ as follows: Na₂S₂O₅ ⇌ 2NaHSO₃. At neutral pH, we can assume the following equilibrium: HSO₃[−] ⇌ H⁺ + SO₃^{2−}, pK_a = 7.2.

During neutralization, formaldehyde forms a sulfite adduct according to the following equations [10]:



According to previous studies [11], transition metal ions (like those present in trace amounts in aluminum hydroxide) catalyze the auto-oxidation of (bi)sulfite via the formation of a sulfur trioxide anion radical (•SO₃[−]) and the highly reactive peroxy radical (•SO₅[−]) in presence of oxygen.



where M may be a copper (Cu²⁺), iron (Fe³⁺), oxovanadium (VO²⁺), manganese (Mn²⁺), Nickel (Ni²⁺), or chromate (CrO₄^{2−}) anion [12–16]. It was shown in the literature that sulfite radicals are highly reactive; they can oxidize various substances, including ascorbate, hydroquinone, and histidine [17,18]. All of these reactive compounds can oxidize biomolecules in a one-electron oxidation reaction [19,20].

We have shown that under certain conditions a particular lot of aluminum hydroxide can significantly reduce antigen stability. We found that for IXIARO[®] residual Cu even at in the low ppm concentration range may act as a catalyst and led to the proposal that degradation occurred with the formation of free sulfite radicals. However, adding additional formaldehyde and increasing the pH could stabilize the antigen to a certain extent.

5. Conclusion

To the best of our knowledge, this was the first study to characterize the specific content of elemental impurities in commercially available aluminum hydroxide adjuvant product with specific, sensitive, state-of-the art methods. Trace amounts of residual metal impurities were observed in commercially available aluminum hydroxide lots obtained from various suppliers. Residual metal impurities can be introduced at multiple steps in the processing of those products. In particular, Fe, Cu, Ni, and Cr were present at higher ppm levels. The variance in residual metal concentrations among different lots from the same supplier suggested that a larger number of lots should be tested before and during vaccine formulation development to define specifications for the internal raw material that might minimize any associated stability issues in the final drug product. Since the individual residual concentrations are not adequately addressed by current compendia of tests, we recommend that residual metal content in mineral adjuvants should be monitored in commercial vaccine manufacturing; we encourage the application of sensitive, quantitative, state-of-the art methods, such as ICP-MS, which may lead to the derivation of internal specifications, when appropriate. In the meanwhile the Ph. Eur. Commission work program has included a revision of these tests [21]. The new guidelines provide general descriptions of specific and sensitive analytical methods which are applicable to determine metals as impurities in pharmaceutical substances and give guidance on concentration limits [22,23].

Financial and competing interests' disclosure

Robert Schlegl, Jürgen Wruss, Michael Weber, Donald Low, Kirsten Queen, Shaun Stilwell and Michael Möhlen are employees of Vanleva SE, a European biotech company focusing on vaccine research and manufacturing. Erik B. Lindblad is an employee of the Brenntag Biosector, DK-3600 Frederikssund, Denmark. Brenntag Biosector is the manufacturer of Alhydrogel® that is one of the materials investigated in this article. All authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript, apart from those disclosed.

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