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Analytical methodology

# Investigations on the binding of ethylmercury from thiomersal to proteins in influenza vaccines

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ABSTRACT

This study investigates the binding of ethylmercury ($EtHg^+$) released from the preservative thiomersal by hydrolysis to proteins in influenza vaccines *via* ultrafiltration and subsequent total reflection x-ray fluorescence (TXRF) analysis as well as size exclusion chromatography (SEC) hyphenated to inductively coupled plasma-mass spectrometry (ICP-MS).

Binding of $EtHg^+$ to the protein fraction was shown by means of ultrafiltration and TXRF in a qualitative matter. SEC/ICP-MS was applied to gain more information about the molecular weight of the bound protein and quantitative information. First experiments showed the necessity of a rinsing step during elution with a thiol-containing compound to prevent unspecific binding or mercury species to the chromatographic system. Adduct formation of $EtHg^+$ and a high-molecular compound could be observed for different concentrations of $EtHg^+$ applied. The mercury-containing fraction was larger than 133 kDa, indicating binding to hemagglutinin, which is the active ingredient in influenza vaccines. The applied SEC/ICP-MS method allowed for external calibration with $EtHg^+$ and a binding of 141 µg $L^{-1}$ Hg was shown for a vaccine solution that was incubated with $EtHg^+$ (25 mg $L^{-1}$ Hg).

## 1. Introduction

Mercury is a ubiquitous pollutant and its toxicity is known to depend strongly on the occurring chemical species, leading to a necessity for speciation analysis [1,2]. Organic mercury species are potent neurotoxins and in this class of mercurials, humans are exposed to methylmercury ($MeHg^+$) and ethylmercury ($EtHg^+$) [3]. The consumption of seafood leads to the uptake of $MeHg^+$, which can be traced back to seafood contamination resulting from emission of mercury from diverse anthropogenic and geological sources [4–7], as well as deposition of inorganic mercury to the hydrosphere and subsequent biomethylation to $MeHg^+$ by microorganisms [8–11]. Accumulation along trophic webs can be observed, leaving predatory fish, like tuna, swordfish or sharks, with the highest concentration of $MeHg^+$ [12–14].

In contrast, exposure to $EtHg^+$ is strictly anthropogenic since ethylation processes are not known to occur naturally. In 1931, the mercury-containing compound thiomersal (THI) was introduced for its fungicidal and bactericidal properties in multidose vials of vaccines. The antimicrobial effect is based on the formation of $EtHg^+$ induced by the decomposition of THI in aqueous media, promoted by sodium chloride present in solution [15]. Banned in the EU since 2001, thiomersal-containing vaccines (TCV) are still in use in the USA and in developing countries. The use of TCV is discussed controversially in the scientific community [16–18].

Toxicity and toxicokinetics of $EtHg^+$ differ to $MeHg^+$ and are still poorly understood [19]. Mercury species often show a high affinity to sulphur and tend to form adducts with biogenic thiols, like glutathione (GSH) or cysteine-containing proteins. It is stated to be important to investigate those adducts to understand distribution routes within the human body [20]. So far, adduct formation with organic mercury species and GSH [21,22] or blood proteins like human serum albumin (HSA) [23], hemoglobin [24] and carbonic anhydrase [25] have been shown. Adducts of mercury and xenobiotic proteins can lead to a formation mercury-specific antibodies in mice [26,27]. This leads to the conclusion that adducts of $EtHg^+$ and antigens are not only important to investigate for understanding toxicokincetics, but may also induce a mercury-specific immune response. Albeit, such unwanted reaction by the use of TCV has not been reported to our knowledge.

*Abbreviations:* DMSA, *meso*-2,3-Dimercaptosuccinic acid; $EtHg^+$, ethylmercury; GSH, glutathione; HA, hemagglutinin; HSA, human serum albumin; ICP-MS, inductively coupled plasma-mass spectrometry; IV, influenza vaccine; $MeHg^+$, methylmercury; SEC, size exclusion chromatography; TCV, thiomersal-containing vaccine; THI, thiomersal; TXRF, total reflection x-ray fluorescence

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Size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) has great potential for the identification of Hg-binding proteins [28]. Kutscher *et al.* found binding of $MeHg^+$ to large proteins in tuna fish muscle tissue *via* SEC/ICP-MS [29]. Skeletal muscle myosin heavy chain was identified as possible binding partner for $MeHg^+$. Another study uses SEC/ICP-MS for the analysis of the water soluble fraction of dolphin liver extract and finds most of $MeHg^+$ bound to high-molecular fractions [30]. Investigations on brain cytosol of maternal and infant rats [31] and on liver and kidney extracts of red deer and wild boar [32] showed adduct formation of mercury to proteins also in terrestrial mammals by means of SEC/ICP-MS. A recent study facilitates SEC coupled to an inductively coupled plasma atomic emission spectrometer to investigate the fate of $MeHg^+$ and $EtHg^+$ from THI in the red blood cell fraction [33]. It was shown that adducts with hemoglobin and GSH were formed, with $EtHg^+$ showing a higher affinity to hemoglobin than $MeHg^+$. The majority of mercury was bound to GSH in both cases.

## 2. Materials and method

### 2.1. Reagents

Thiomersal (THI), sodium chloride and 25% aqueous ammonia solution were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium formate was purchased from Acros Organics (Geel, Belgium) and *meso*-2,3-dimercaptosuccinic acid (DMSA) was supplied by TCI (Eschborn, Germany). Selenium standard solution was purchased from Merck (Darmstadt, Germany). All chemicals were obtained in the highest purity available.

Water was freshly purified by an Aquatron A4000D system (Barloworld Scientific, Nemoers Cedex, France).

### 2.2. Incubation of vaccine solution with THI

For the preparation of stock solutions, THI was daily dissolved in purified water and diluted using a 0.9% sodium chloride solution. The seasonal tetravalent influenza vaccine (IV) Vaxigrip tetra (Sanofi, Paris, France), containing inactivated split virions of two influenza A and two influenza B strains (each 30 µg $mL^{-1}$ hemagglutinin (HA), propagated in chicken eggs), was diluted 1:20 and incubated with THI in concentrations of 0.35, 0.7, 3.5, 7 and 14 µg $L^{-1}$ Hg. Mixtures were stored for 20 d in a refrigerator to allow incubation under storage conditions of vaccines.

In addition, a triplicate of IV solutions was incubated with THI (final concentration: 25 mg $L^{-1}$ Hg) in a refrigerator for seven days.

### 2.3. Ultrafiltration and TXRF

IV solution containing THI (10 mg $L^{-1}$ Hg) and control sample (THI in 0.9% sodium chloride, 10 mg $L^{-1}$ Hg) were desalted in triplicate using Amicon Ultra-0.5 centrifuging filter units with a molecular weight cut-off of 10 kDa (Merck, Darmstadt, Germany). A BlueSpin Cryo centrifuge (Serva Electrophoresis GmbH, Heidelberg, Germany) was operated at 14,000 rpm 20 °C for 30 min and temperature was set to 20 °C. The filtrate was subsequently diluted with purified water and the centrifugation protocol was repeated four times to remove all non-bound mercury. The filtrate was obtained *via* reverse spin at 1000 rpm after the last centrifuging step. According to Holtkamp et al., 20 µL filtrate, 20 µL Se standard in 20% nitric acid (10 mg $L^{-1}$ Se) and 20 µL DMSA (2.5 mmol $L^{-1}$) were mixed for analysis by TXRF [34]. Analyses were carried out using a S2 PICOFOX system (Bruker AXS, Karlsruhe, Germany). Sample solutions were placed on siliconized quartz glass discs and dried at 80 °C for 10 min.

### 2.4. Size exclusion chromatography

Separations were performed on a BioBasic SEC (150 × 7.8 mm, 5 µm) column and a MAbPac SEC-1 150 × 4 mm, 5 µm) column (both Thermo Scientific, Germering, Germany) at 30 °C. For both columns, a binary solvent mixture with ammonium formate (10 mmol $L^{-1}$, adjusted to pH 7.4 with 25% aqueous ammonia, eluent A) and ammonium formate (10 mmol $L^{-1}$, containing 1 mmol $L^{-1}$ DMSA), eluent B), was applied.

Conditions for BioBasic SEC column: Injection volume was 20 µL. With a flow rate of 600 µL $min^{-1}$, starting conditions were 0% B, which was held for one minute. 100% B was reached by linear increase within 0.5 min and held until 19 min, before column equilibration was performed at initial mobile phase composition for 10 min.

Conditions for MAbPac SEC-1 column: Injection volume was 10 µL. Flow rate was 230 µL $min^{-1}$ and starting conditions were 0% B. After one minute, the solvent was changed to 100% B by linear increase within 0.5 min and was held for 8 min. The initial solvent composition was equilibrated for 21.5 min. For external calibration, isocratic conditions with 100% B were applied and THI solutions (0.5, 1, 2 and 5 mg $L^{-1}$ Hg) were injected every two minutes for flow injection.

### 2.5. LC/ICP-MS

SEC was carried out using a Dionex UltiMate 3000 system (Thermo Scientific, Germering, Germany), consisting of a SRD-3400 vacuum degasser, a HPG-3200RS binary pump, a WPS-3000TRS autosampler, a TCC-300RS column oven, equipped with a PD715-000 six-port valve (Rheodyne, Cotati, USA), and a VWD-3400RS UV–vis detector, monitoring at 280 nm. An iCAP TQ (Thermo Scientific, Bremen, Germany), operating in SQ mode, was used as elemental detector. Since no organic eluent was used, nickel sampler and skimmer cones were applied. A PFA nebulizer (Elemental Scientific, Mainz, Germany) and a cooled (2.7 °C) cyclonic spray chamber were used for sample introduction. The ICP-MS was operating with a plasma power of 1550 W, a cool gas flow of 14 L $min^{-1}$, an auxiliary gas flow of 0.8 L $min^{-1}$ and a nebulizer gas flow of 0.79 L $min^{-1}$. Mercury isotopes $^{200}Hg$ and $^{202}Hg$ were monitored in dwell times of 100 ms each. Transient ICP-MS data was smoothened using a Savitzki-Golay filter prior to further evaluation.

## 3. Results and discussion

In this study, desalting *via* ultrafiltration and subsequent elemental analysis of the filtrate *via* total reflection x-ray fluorescence (TXRF) was used as a preliminary experiment to check on the binding of $EtHg^+$ to the protein fraction of a TCV. To exclude any false positive results based on secondary interactions of $EtHg^+$ with the membrane of the centrifuging filter, a solution of THI (10 mg $L^{-1}$ Hg, same as in TCV) in 0.9% NaCl was used as control sample. The quantification resulted in a concentration of 1.79 mg $L^{-1}$ Hg (SD: 0.34 mg $L^{-1}$ Hg, n = 3) in the concentrated filtrate, while the mercury concentrations of the control samples were below the limit of detection. These results indicate that $EtHg^+$ from THI is able to bind on the protein fraction of influenza vaccines. The comparably large standard deviation is likely to be due to the large number of filtration steps necessary for the complete removal of non-bound $EtHg^+$.

SEC/ICP-MS was used to further investigate these first results. Solutions of THI in 0.9% NaCl were injected on a Thermo Scientific BioBasic SEC column during method development, but no mercury species could be detected in the eluate. A slight increase of the baselines of both mercury isotopes monitored ($^{200}Hg$ and $^{202}Hg$) was observed instead. This indicates adhering of non-bound $EtHg^+$ to the column surface due to secondary interactions. Subsequent injections of the thiol-containing compounds HSA and GSH, which contained no mercury, lead to false positive peaks at their corresponding retention times in SEC/ICP-MS analysis. These findings show that secondary