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Applied methodology

In vitro study of thimerosal reactions in human whole blood and plasma surrogate samples

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ABSTRACT

Because of its bactericidal and fungicidal properties, thimerosal is used as a preservative in drugs and vaccines and is thus deliberately injected into the human body. In aqueous environment, it decomposes into thiosalicylic acid and the ethylmercury cation. This organomercury fragment is a potent neurotoxin and is suspected to have similar toxicity and bioavailability like the methylmercury cation. In this work, human whole blood and physiological simulation solutions were incubated with thimerosal to investigate its behaviour and binding partners in the blood stream. Inductively coupled plasma with optical emission spectrometry (ICP-OES) was used for total mercury determination in different blood fractions, while liquid chromatography (LC) coupled to electrospray ionisation time-of-flight (ESI-TOF) and inductively coupled plasma-mass spectrometry (ICP-MS) provided information on the individual mercury species in plasma surrogate samples. Analogous behaviour of methylmercury and ethylmercury species in human blood was shown and an ethylmercury-glutathione adduct was identified.

1. Introduction

In 1931, ethylmercury thiosalicylate (thimerosal, THI) was introduced into the market as a bactericidal and fungicidal additive to drugs that are distributed in multi-dose ampullae. Since its market introduction for many decades this mercury-containing agent has been used with little or no attention to possible dangerous effects which might occur upon its intramuscular or intravenous injection into the human body. Although the methylmercury cation ("methylmercury", MeHg) is a strong toxin that is to be avoided even at small levels when consumed in foods such as seafood and rice (in Asia), the World Health Organization considers small doses of thimerosal safe regardless of multiple/repetitive exposures to vaccines that are predominantly taken during pregnancy or infancy. Anyhow, an ongoing discussion about suspected neurotoxic effects [1–3] in patients treated with THI-preserved drugs lead to the recommendation of government organizations towards the pharmaceutical industry to phase out thimerosal as an adjuvant in vaccines in 2001. Temporarily withdrawn from routine childhood vaccination schedules in Europe and the US, thimerosal is still in use in the United States of America and in developing countries, and was present in most anti-flu vaccines against the H1N1 virus (swine influenza) in 2009. In aqueous media, THI undergoes a hydrolysis equilibrium reaction, dissolving into thiosalicylic acid (TSA) and the ethylmercury cation ("ethylmercury", EtHg) [4,5]. Recently, Dorea et al. reviewed the toxicity of thimerosal and ethylmercury in comparison to methylmercury [6] and concluded that the *in vitro* toxicity of ethylmercury and thimerosal is comparable with the toxicity of methylmercury, but the different pharmacokinetics leading to a shorter residence time of ethylmercury in the blood warrants special attention for studying the *in vivo* toxicity. However, since the target organ for the toxicity of organic mercury compounds is the brain, the shorter residence time in blood is not necessarily a risk reducing factor.

Due to the strong affinity of mercury towards sulphur and the almost universal presence of this chalcogen in the human body in the form of thiols and disulphides in peptides, proteins and DNA, sulphur is the major binding partner of mercury compounds under physiological conditions. Mercury unfolds its neurotoxic effects by binding to thiols or disulphides in the nervous system, thus inhibiting enzyme activities, distorting protein structure or

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blocking biologically active thiols [7]. Case-related medical studies, focusing on the toxicity of THI and other mercury compounds often regard total mercury (Hg_{tot}) in human tissue, blood, urine, faeces or hair samples [2]. Other investigations with *in vivo* experiments, using mice or monkeys, also often rely on atomic absorption spectrometry (AAS) for total element determination [1,8,9]. Further works often neglect physiological conditions, thus limiting the relevance for processes within the human body [5,10]. However, to understand the details of mercury toxicity, its distribution in the human body, its transport and pathways of metabolism and excretion, investigations on a molecular level are necessary [11] focusing on the chemical forms in which mercury is present. Such investigations, differentiating between mercury-containing compounds (speciation analysis) allow gaining much deeper insight than the use of methods for total element determination [12]. Basically, speciation analysis requires a separation technique like gas or liquid chromatography (GC, LC) or capillary electrophoresis (CE), coupled to an element-selective detection technique.

The classical approach for mercury speciation analysis, as applied for environmental analysis and food control [13], requires the extraction and derivatisation prior to analysis by gas chromatography (GC) coupled to element-selective detection by inductively coupled plasma-mass spectrometry (ICP-MS) or other techniques. Several studies employ this methodology for different analytical challenges [14,15]. However, with this approach, the mercury species are removed from the sample matrix and from their binding partners – in our study biothiols. In order to investigate mercury binding partners in biological samples some research groups have used the separation of mercury species in the liquid phase [16–23]. Li et al. used CE coupled to electrothermal atomic absorption spectrometric detection (ETAAS) for the investigation of the interaction of several mercury species with herring sperm DNA or human serum albumin (HSA) [16,17]. Bushee [18] had used high performance liquid chromatography (HPLC) coupled to ICP-MS already in 1988 for mercury speciation analysis avoiding such derivatization. ICP-MS is the most often applied detection technique for the coupling with HPLC, allowing for very sensitive element detection as well as isotope selectivity but does not provide any molecular information. For this reason, Krupp et al. [19] used both ICP-MS and electrospray ionisation-mass spectrometry (ESI-MS) for simultaneous detection, gaining structural information about adducts with biothiols like cysteine (Cys) and glutathione (GSH) with inorganic mercury (Hg(II)) and methylmercury (MeHg) while Kutscher et al. investigated the binding partner of methylmercury in tuna fish muscle [20]. Because of its capability to provide such molecular information, ESI-MS is steadily gaining significance. Trümpler at al. studied the interaction of mercury species with human serum albumin [4] and antidotes [21], Wilken et al. the interaction of mercurochrome with proteins [22] and Janzen et al. [23] compared the interaction of thimerosal with human and rat hemoglobin by using LC-ESI-MS.

The scope of the present work was to investigate the compounds binding EtHg from THI in human plasma and their respective adducts. Therefore, we avoided the extraction and subsequent analysis by GC/ICP-MS, but focused on the separation in the liquid phase. Several other techniques were also applied in this study: ICP-OES was used for Hg_{tot} determination in human whole blood fractions to achieve comparability with present literature data. In the following experiments, LC/ICP-MS and LC/ESI-TOF-MS were applied to gain complementary element-selective and molecular information from plasma surrogate samples.

This work presents the distribution of different mercury species among fractions of human whole blood. Furthermore, human blood plasma was simulated and spiked with THI. The complementary use of total element determination and element-selective and molecular speciation techniques allows the identification of EtHg-biothiol adducts under physiological conditions. Mimicking key conditions of physiological processes, this study is intended to help understanding toxicological effects which result from the application of THI-preserved vaccines.

2. Materials and methods

2.1. Reagents and chemicals

THI, L-glutathione (GSH, reduced) and TSA were obtained from Sigma–Aldrich (Steinheim, Germany). Hexamethyl disilazane (HMDS), methylmercury chloride and mercury(II) chloride were purchased from ABCR (Karlsruhe, Germany). HPLC-grade Methanol and acetonitrile (ACN), ammonia 25%, sodium chloride and ammonium formate were obtained from Fluka (Buchs, Switzerland). Cysteine (Cys) was purchased from Acros Organics (Geel, Belgium). All chemicals were of the highest purity available. Water was purified using a Milli-Q Gradient A10 system and filtered through a 0.22 μm Millipak 40 filter (Millipore, Billerica, MA, USA).

A physiological solution with pH 7.4 was prepared by adjusting the pH of a 20 mM ammonium formate solution containing 154 mM sodium chloride with 25% aqueous ammonia. Stock solutions of THI, mercury chloride, TSA, GSH and Cys were prepared daily by dissolving the solids in the physiological buffer. Methyl mercury chloride was dissolved in a mixture of physiological solution/methanol 25/75 (v/v). Volumetric flasks were silanised to prevent adsorption of analytes to container walls. For silanisation, 100 vials were heated in a 1 L flask together with 300 μL of HMDS for 3 h at 120 °C, rinsed once with water and dried before use.

2.2. Instrumentation and settings

2.2.1. ICP-OES

A Spectro CIROS^{CCD} ICP-OES (Spectro Analytical Instruments, Kleve, Germany) instrument with axial plasma viewing was used for total mercury determination. The free-running generator was operated at 27.12 MHz with a maximum output of 2 kW. A standard Fassel-type torch (No. 75160526, Spectro Analytical Instruments) was employed. For sample introduction, the system's peristaltic pump with a cross flow nebulizer and a double-pass spray chamber (Scott type) was used. Mercury emission was detected at 194.227 nm and at 253.653 nm under the following conditions: 1,400 W (radio frequency power), outer plasma gas flow: 12.0 L/min, auxiliary plasma gas flow: 1.0 L/min, sample carrier gas flow: 1.05 L/min.

2.2.2. Liquid chromatography

Separations were performed on a Shim-Pack XR-ODS (2.0 × 30 mm, 2.2 μm) reversed-phase C_{18} column (Shimadzu Europa, Duisburg, Germany) at 40 °C. Injection volume was 10 μL. A binary gradient with 0.1% formic acid (eluent A) and methanol (eluent B) was used with a total flow rate of 300 μL/min. Separation was started at 5% B which was held for 0.5 min. 60% B were reached at 2 min and held until 3.5 min, before re-equilibrating at 5% B which were reached at 4.5 min. Rinsing and re-equilibration proceeded until 9 min. This LC system was coupled either to an ICP-MS or an ESI-TOF-MS, which are described in the following.

2.2.3. LC/ICP-MS

An Agilent 1200 series LC system was used, consisting of a G1379B micro vacuum degasser, a G1376A capillary pump, a G1329A autosampler, a G1316A column oven and a G1315B diode array detector (Agilent Technologies, Waldbronn, Germany). An ELAN 6000 ICP-MS system from PerkinElmer (Rodgau, Germany) was used as detector. Platinum sampler and skimmer cones were used. Sample introduction was performed by means of a cooled