Clinica Chimica Acta 412 (2011) 1563–1566

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Speciation of methyl- and ethyl-mercury in hair of breastfed infants acutely exposed to thimerosal-containing vaccines

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ARTICLE INFO

Article history:
Received 25 January 2011
Received in revised form 2 May 2011
Accepted 2 May 2011
Available online 7 May 2011

Keywords:
Methylmercury
Ethylmercury
Thimerosal
Mercury
Hair
Breastfeeding

ABSTRACT

Background: Different chemical forms of mercury occur naturally in human milk. The most controversial aspect of early post-natal exposure to organic mercury is ethylmercury (EtHg) in thimerosal-containing vaccines (TCV) still being used in many countries. Thus exclusively breastfed infants can be exposed to both, fish derived methylmercury (MeHg) in maternal diets and to EtHg from TCV. The aim of the study is to evaluate a new analytical method for ethyl and methyl mercury in hair samples of breastfed infants who had received the recommended schedule of TCV.
Methods: The hair of infants (<12 months) that had been exposed to TCV (Hepatitis B and DTaP) was analysed. A method coupling isothermal gas chromatography with cold-vapor atomic fluorescence spectrometry was used for MeHg which can also speciate EtHg in biological matrices.
Results: In 20 samples of infants' hair, all but two samples showed variable amounts of MeHg (10.3 to 668 ng/g), while precise and reliable concentrations of EtHg (3.7 to 65.0 ng/g) were found in 15 of the 20 samples. A statistically significant inverse association ($r = -05572$; $p = 0.0384$) was found between hair-EtHg concentrations and the time elapsed after the last TCV shot.
Conclusions: The analytical method proved sensitive enough to quantify EtHg in babies' hair after acute exposure to thimerosal in vaccine shots. Provided that the mass of hair was above 10 mg, organic-mercury exposure during early life can be speciated, and quantified in babies' first hair, thus opening opportunities for clinical and forensic studies.

1. Introduction

Mercury (in both its inorganic and organic chemical forms) is a neurotoxic agent capable of causing both lasting and transient damage to the central nervous system (CNS) of young and adult individuals. Depending on the stage of development of the CNS and the severity of insults a wide range of mental damage can result. During the perinatal period, infants have a highly diminished metabolism (sequestration and excretion) for mercury that improves with physiological maturation [1]. Coupled with this, the sensitivity of the developing CNS makes young children highly vulnerable to mercury insults. As both methylmercury (MeHg) and ethylmercury (EtHg) are capable of affecting the CNS at low doses, it is important to identify Hg species during the critical period of neuro-sensorial development.

Breastfeeding, which is capable of attenuating and reversing the detrimental effects of early exposure to neurotoxic substances [2] can also be a source of mercury (organic and inorganic). Environmental exposure to mercury during infancy is linked to maternal fish consumption (MeHg) in breastfed babies and to EtHg in infants immunized with thimerosal-containing vaccines (TCV). It has long been known that MeHg has an affinity for hair and can be an indicator of fish consumption [3]. Studies have indicated that thimerosal-EtHg can be detected in adult hair as a result of occupational and chronic exposure [4] and also in infant's hair after acute exposure to TCV [5].

The toxicity of acute exposure to low doses of EtHg has only recently received attention; experimental studies, both in vitro and in vivo, have shown that even in small doses (relevant to vaccine exposures) thimerosal-Hg shows toxicity [6]. Indeed, neurological risks from thimerosal-EtHg, other than autism, are plausible after exposure to TCV, at least in susceptible infants. Collectively, epidemiological studies suggest an increased risk of untoward neurodevelopment outcomes [7]. Epidemiological studies have also shown hypersensitivity reactions to thimerosal as a result of immunization with TCV [8,9].

The toxicokinetics (TK) and toxicodynamics (TD) of mercury depend on its chemical characteristics including solubility, bioavailability and mobility, which are governed by the chemistry of the various forms of Hg. Therefore species-specific analysis that distinguishes the various organic mercury forms is crucial for understanding their metabolism in young infants. There is a scarcity of information regarding EtHg levels in hair after exposure to TCV. Studies of chronic

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0009-8981
doi:10.1016/j.cca.2011.05.003

feeding experiments in adult animals related to EtHg derived from fungicides showed that pigs [10], cattle and sheep [11] could retain organic Hg species in hair. Experiments with rodents undergoing acute exposure to EtHg also showed that Hg could be detected in mice hair [12].

To support clinical studies related to the use of thimerosal, Gibičar et al. [4] developed a simple, sensitive and reliable analytical method to separate and quantify MeHg and EtHg in biological samples. The aim of the study is to evaluate this new analytical method for ethyl and methyl mercury in hair samples in the hair of Brazilian breastfed infants who had received the recommended schedule of TCV.

2. Materials and methods

This protocol was part of ongoing studies designed to monitor and evaluate the exposure of toxic metals to nursing infants (0 to 12 months age). The protocol was approved by the Ethics Committee for Studies in Humans (CAAE-0041.9.912.000-07) of the Universidade de Brasilia. Participating mothers were properly informed and gave written consent; participation was voluntary and confidentiality assured. A simple questionnaire captured basic information on the infant's age and type of feeding, immunization with TCV, and the frequency of fish or sea food in the maternal diet.

2.1. Sample collection

Hair samples were taken from healthy nursing babies during a regular visit to a pediatric clinic by volunteering mothers contacted by the principal investigator. Hair strands were cut with clean scissors from the occipital area near the scalp, placed in a paper bag, individually identified, stored and taken to the laboratory for analysis. Prior to processing, hair strands were cut to 2-mm lengths, and because of the small amount of sample available, the hair was analyzed without pre-washing and only speciation of organic mercury was carried out to meet the objective of the present work.

2.2. Analytical system

All chemical assays for organic mercury were conducted at the analytical laboratory in the Department of Environmental Sciences, Jožef Stefan Institute (Ljubljana, Slovenia), utilizing the routine method described by Gibičar et al. [4]; this method permits the separation of the two forms of Hg likely to be present in hair of breastfed infants that have been inoculated with TCV, namely: methylmercury and ethylmercury. Both forms of organic mercury were measured by atomic fluorescence spectrometry; this new, sensitive and reliable analytical method for determination of low concentrations of ethyl mercury was adapted for hair samples. Briefly, after leaching the samples in acid (H_2SO_4–KBr–$CuSO_4$) and extracting MeHg and EtHg as bromides into an organic solvent (CH_2Cl_2), they were backextracted into Milli-Q water and subsequently propylated with $NaBPr_4$ (room temperature precollection on Tenax), and then mercury was determined by isothermal gas chromatographic separation (GC), pyrolysis and cold vapor atomic fluorescence spectrometric detection (CV AFS). The method imprecision calculated as the coefficient of variation for duplicate measurements was 4.1% [4,13]. The lowest peak that could be reliably measured was estimated to be 5 pg/ml for MeHg and 10 pg/ml for EtHg of the final extracted sample (30 ml) if 10 mg of hair sample was taken for analysis. The limit of detection for MeHg was determined on the basis of three standard deviations of the blank. For EtHg the blank was not observed (not recorded), so we assessed the lowest readable peak signal, multiplied by 5 and expressed it as limit of quantification (LOQ). In order to express it on the real practical manner, we calculated the LOQ for 10 mg of the sample; the corresponding limit of quantification (LOQ) for the method was estimated to be 0.5 ng/g for MeHg and 1 ng/g for EtHg (taking 30 ml of the extracted sample for analysis); we assume that better LOQs can be achieved if larger quantities of hair sample can be taken for analysis.

2.3. Statistical analysis

Data was summarized with Microsoft Office EXCEL software (ver 2007; Microsoft Corp, Redmond, WA). Both the Pearson and Spearman (non-parametric) correlation tests between variables were performed with PRISM software (ver 4.0; San Diego, CA); the significance level was set at $P<0.05$.

3. Results

The analytical results for infants' hair are summarized in Table 1. The principal constraint of the study was the small amount of hair

Table 1
Concentrations of methyl- and ethyl-mercury in hair samples of young children immunized with thimerosal-containing vaccines.

Sample #	Sex	FFC, w	Age, d	BM, d	TCVs	Total[a] EtHg, μg	Time after TCV, d	Sample weight, mg	MeHg, ng/g	EtHg, ng/g
02	M	<1	150	150	HpB(2);DTP(2)	100	ND	16.4	205	32.2
04	F	1	240	120	HpB(3);DTP(3)	150	39	20.1	142	32.2
05	M	0	85	60	HpB(2);DTP(1)	75	25	5	43.2	<LOD
06	M	2	285	90	HpB(2);DTP(2)	100	7	11.9	97.4	10.2
07	M	2	270	50	HpB(3);DTP(3)	150	90	24.1	42.6	30.0
08	M	<1	220	120	HpB(3);DTP(3)	150	14	11.7	388	65.0
09	M	1	185	180	HpB(2);DTP(2)	100	44	7.9	688	47.1
10	F	1	340	150	HpB(3);DTP(3)	150	123	16.5	114	47.9
11	F	0	540	90	HpB(4)	100	5	18.7	23.1	<LOD
590733	F	1	370	365	HpB(2);DTP(2)	100	208	9.7	34.3	9.0
592150	M	<1	365	365	HpB(3);DTP(3)	150	176	11.9	116	14.3
592351	M	0	363	270	HpB(3);DTP(3)	150	184	12.1	22.6	3.74
601308	F	<1	222	210	HpB(3);DTP(3)	150	42	13.3	86.7	30.1
603638	M	0	172	120	HpB(2);DTP(2)	100	138	9.3	10.3	25.7
603653	F	1	180	180	HpB(2);DTP(2)	100	146	9.8	58.7	<LOD
604823	F	<1	150	150	HpB(2);DTP(2)	100	152	10.5	193	14.8
606631	M	1	125	90	HpB(2);DTP(2)	100	62	10.7	71.2	51.4
S-01	F	1	51	51	HpB(2)	50	20	1.6	164	<LOD
S-02	F	ND	346	300	HpB(3);DTP(3)	150	129	13.1	456	<LOD
F-01	F	1	120	120	HpB(2);DTP(1)	75	60	12.2	186	19.1

[a] Estimated from values declared by manufacturer; FFC, frequency of fish consumption – fish meals/week; BM, breast milk: d, days; TCV, thimerosal containing vaccines – (number of vaccines); ND, not determined; HpB, hepatitis B(doses); DTP, difteria, tetanus and pertussis (doses); MeHg, methylmercury; EtHg, ethylmercury; <LOD, below limit of detection (<7 ng/g for MeHg and 6 ng/g for EtHg in 10 mg of hair sample); sex: M, male; F, female.

samples that could be collected from these young babies. Two samples did not have enough mass to perform the analysis (<10 mg) taking into consideration their age and actual level of exposure. Nevertheless, 15 of 20 hair samples could be measured with precision and reliability. The median EtHg concentration of 32.0 ng/g showed that, when compared to the median MeHg concentration (86.7 ng/g), a considerable proportion of the organic mercury exposure is derived from intramuscular TCV; there was no other apparent source of EtHg. The range of MeHg concentration (10.3 to 668 ng/g) mostly represents maternal transfer through placenta or breast milk and also the variation of the mother's fish intake. In this sample, media fish consumption was one meal a week (Table 1). The wide variation in the concentrations of both measured forms of organic mercury was due to the time of exposure, i.e, length of breastfeeding (and age), as well as the time elapsed from the last TCV shot (Fig. 1a to c).

EtHg concentrations varied not only with the age of the child but also with the number of vaccines and the time after the last TCV. Such variations are illustrated in Fig. 1a to c. The Spearman correlation between the number of vaccinations (total exposure to EtHg) and hair-EtHg concentrations (Fig. 1a) was not statistically significant (Spearman $r = 0.1579$; $p = 0.5742$). In view of the fact that most infants followed the recommended immunization schedule, the variation in age opened the opportunity to test the sensitivity of the method to capture temporal variations in hair-EtHg. Indeed, it is feasible to conduct studies with multiple hair samples from the same child. When the hair-EtHg concentration was plotted as a function of time elapsed after the last exposure to TCV (Fig. 1b), the correlation was statistically significant ($r = -0.5572$; $p = 0.0384$). However, there was no statistically significant correlation between hair-MeHg concentrations and days of breastfeeding ($r = 0.1189$; $p = 0.6175$; Fig. 1c) or weekly fish consumption (Spearman $r = 0.2590$; $p = 0.2842$).

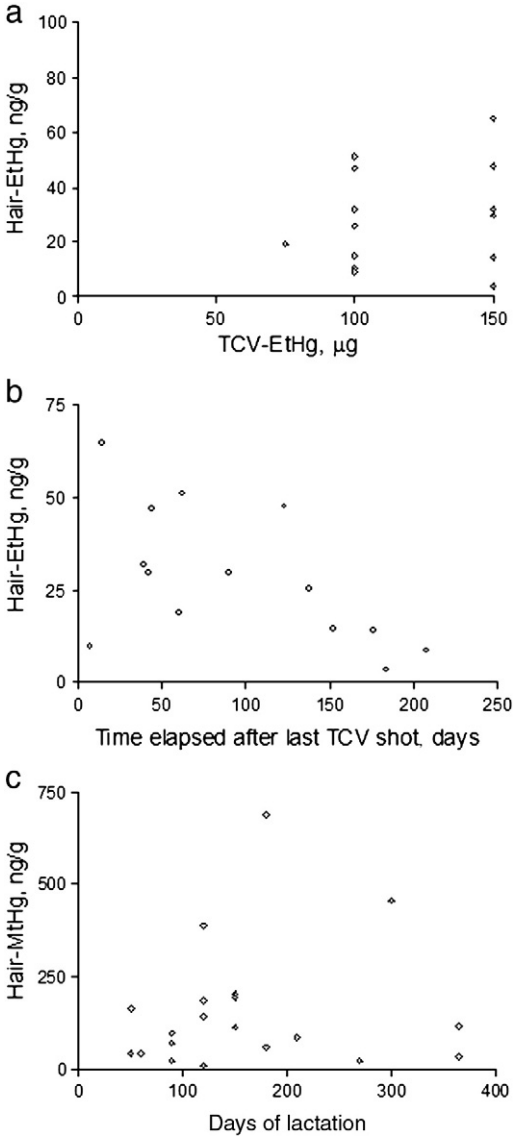

Fig. 1. Ethyl- and methyl-mercury (EtHg and MeHg) concentrations in hair of infants as a function of total thimerosal-containing vaccines — TCV (a), time elapsed after last shot of TCV (b), and days of lactation (c).

4. Discussion

This work demonstrates the possibility of using infant's hair to speciate organic Hg from maternal sources (MeHg) and from TCV (EtHg), allowing insight into a) the minimum hair mass that can produce useful results, and b) some aspects of the kinetics of retained organic Hg, especially the detection windows for capturing EtHg after vaccine exposure. As this was an exploratory study on very small amounts of babies' hair, no interpretations of the toxicity of the measured organic mercury could be made. Although there is much to be learned about physiological processes in the immature organism, it was seen that the intramuscular route of exposure allows a substantial amount of EtHg to reach mercury-storage/indicator tissue such as hair.

Sample pretreatment is an important step in the analytical procedure to speciate and quantify mercury in biological samples. However, given the small amount of hair that it was possible to collect and the fact that only organic mercury species were analyzed, we assumed that sample contamination was negligible. Maternal sources of MeHg in baby's hair could be from both pregnancy and breastfeeding. For EtHg, only exposure originating from the child's vaccine schedule could be ascertained though other sources seem improbable. Indeed, the only information regarding organic mercury (EtHg and MeHg) concentrations in vaccines indicates variability in the concentration of EtHg and also the residual presence of MeHg (1 to 1.5% of total Hg); furthermore, after one week of storage in the dark at low temperatures (4 °C) there was a 50% drop in EtHg concentration [4].

It should be borne in mind that neonatal hair starts growing in utero during late pregnancy (approximately 28 weeks) and remains until 3–5 months post-partum [14]. Therefore, as it was not possible to differentiate hair grown before and after birth, the origin of MeHg (pregnancy or lactation) cannot be ascertained. Nevertheless, the relatively high EtHg concentration (median, 32.0 ng/g) in relation to MeHg concentration (median, 86.7 ng/g) indicates a considerable proportion of the organic mercury exposure is derived from TCV. Considering that chronic MeHg exposure (from maternal sources) starts *in utero* and persists throughout lactation, this work indicates that current acute doses of EtHg in vaccines are capable of being retained in the baby´s body.

Direct determination of the main chemical forms of organic mercury in baby's hair has been done using a thiourea-based chromatographic method [5]. Compared with that exploratory study, the range of both MeHg and EtHg found was higher here. Because EtHg is less stable than MeHg, it is possible that the lower HCl used by Dórea et al. [5] as an extraction solution could have affected EtHg stability during extraction and analysis [5]. As discussed elsewhere [5], the lower stability of EtHg may also cause breakdown to Hg^{II} more rapidly than MeHg, thus partly explaining the consistently lower concentrations of EtHg in babies' hair compared to MeHg in the present study. In breast-fed infants of mothers with a high level of fish consumption, hair MeHg concentration [5] was much higher than in the present study. The total organic Hg concentration in hair in this study and reported by others [5] is much below that predicted in the model used by Redwood et al. [15].

To date, most analytical methods for determining inorganic and organic mercury (MeHg and EtHg) in hair samples were carried out using the difference between total mercury and inorganic mercury. We know from experimental work that EtHg can be accumulated/retained in animal hair. Early feeding experiments with EtHg-based fungicides such as Tillatin-6 (methoxyethyl mercury) and Ceresan M (ethylmercury *p*-toluene sulfonanilide) showed EtHg in the hair of pigs [10], sheep and cattle [11]. In these animal experiments, EtHg was the only source of Hg exposure and, in contrast to human studies, there was no need for direct speciation. In mice, one injection of EtHg (1.4 mgHg/kg) could be assimilated in growing hair, similar to MeHg [12]. Zareba et al. [12] differentiated between MeHg and EtHg in exposed animal by decreasing the amount of the reducing agent (cadmium chloride/stannous chloride reagent) during Hg determination. These animal experiments confirm earlier observations that accidental poisoning with EtHg-based fungicides can be retained in human hair [16].

Analytical techniques to separate and quantify the chemical forms of mercury are fundamental to understand their metabolic differences as well as their toxicodynamics in animal and man. To date, only models based on methylmercury exist to predict the kinetics of EtHg [15]. Studies that emerged after discontinuation of the use of thimerosal by industrialized countries have shown that small doses are toxic to nervous cells (*in vitro*) and experimentally to mice, rats, and non-human primates [6].

WHO convened a group of experts that examined the complexities surrounding production and use of TCV [17] and recommends its use in pregnant women, infants, and young children. Thimerosal is still used in pharmaceutical and healthcare industries; as a result, it is likely to be of environmental concern at impact points of the drug industry and hospitals [18]. Therefore, as well as the clinical and forensic aspects, there is a need for specific methods to identify and detect this organic form of mercury in both health and environmental studies.

5. Conclusion

Both forms of organic Hg can be monitored in hair from breastfed infants: chronic exposure to MeHg from human milk plus acute EtHg exposure as a result of inoculation with TCV. It is possible to estimate the kinetics EtHg in hair after TCV exposure.

Acknowledgements

The analyses of MeHg and EtHg were carried out in the framework of the IAEA CRP project “Stable isotope techniques to assess human milk intake in infants living in areas contaminated with mercury, lead and cadmium” (E4.10.15./RC-1137.1) and ARRS programme P1-0143. We thank Dr. T. Byrne and Prof. Connie McManus for reading and correcting the manuscript.

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