# Determination of Thimerosal in Biological Products by Liquid Chromatography With Inductively Coupled Plasma Mass Spectrometric Detection*

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A liquid chromatography system has been interfaced to an inductively coupled plasma mass spectrometer in order to analyse injectable biological products for thimerosal. Thimerosal is a mercury containing antimicrobial agent used as a preservative in these materials. Sample types analysed ranged from vaccines and toxoids (influenza virus vaccine and tetanus toxoid) to diluents, containing only the preservative in a saline solution. Samples were analysed quantitatively for thimerosal content and qualitatively for the presence of decomposition products detectable by this method, such as methylmercury chloride, dimethylmercury and mercury(II) chloride. Flow injection was used to confirm that all mercury species in the samples were determined by liquid chromatography.

**Keywords:** *Inductively coupled plasma mass spectrometry; liquid chromatography; mercury determination; speciation; thimerosal*

Thimerosal (sodium ethyl mercurithiosalicylate) is a mercury containing antimicrobial agent used as a preservative in certain biological products. A previous study of these materials was based on total mercury content only, not on thimerosal content specifically.[1] It is known that the effective concentration of thimerosal may decrease with time; however, whether this decrease is through loss to the environment or by decomposition to other less useful mercury species needs to be determined.

Recent work by Bushee has demonstrated the applicability of liquid chromatography (LC) with inductively coupled plasma mass spectrometry (ICP-MS) to the determination of mercury species, including thimerosal,[2] by showing that thimerosal can be easily resolved from compounds, such as mercury(II) chloride, methylmercury chloride and dimethylmercury. It was discovered during this work that ethylmercury is not completely separated from thimerosal. Therefore, it was not possible to determine all decomposition products in the samples. However, useful information was obtained regarding the loss of thimerosal from solution and possible decomposition to other mercury compounds.

## Experimental

### Apparatus and Operating Conditions

The chromatographic system and the VG PlasmaQuad† ICP-MS instrument used for this work have been described previously.[2] The separation was performed on a Waters PicoTag $C_{18}$ column with a mobile phase containing of 0.06 mol $l^{-1}$ ammonium acetate, 3% *V/V* acetonitrile and 0.005% *V/V* 2-mercaptoethanol at a pH of 5.3.[3,4] A flow-rate of 1.0 ml $min^{-1}$ and sample volumes of 100 μl were used throughout.

The spray chamber of the ICP-MS instrument was cooled to 8 °C. A length of FEP tubing (*ca.* 60 cm) was used to connect the end of the chromatographic column to the nebuliser inlet. The ICP mass spectrometer was set to monitor the $^{202}Hg$ isotope as this is the most abundant.

### Reagents

All reagents were of analytical-reagent grade and were used without further purification. Thimerosal was obtained from Alfa Inorganics and used as received.

### Samples

The thimerosal containing products used in this study were prepared commercially by various manufacturers in the USA. Dates of manufacture are indicated in Table 1.

All sample preparation was carried out in a class 1 clean-air environment. Each sample was diluted prior to analysis. For LC - ICP-MS, samples were diluted to contain approximately 2.5 μg $ml^{-1}$ of Hg with sub-boiling distilled water.[5] Ten samples of each injectable product were analysed. Total mercury was then determined in five of these solutions by flow injection (FI) - ICP-MS. For FI, samples were diluted to approximately 0.5 μg $ml^{-1}$ of Hg.

The tetanus toxoid, adsorbed, required special handling because it contained an aluminium adjuvant. This solid material was removed from solution prior to dilution by filtering under vacuum through 0.22-μm membranes. Each aluminium filter cake was rinsed with sub-boiling distilled water and then air dried in a clean FEP beaker. One to three ml of concentrated HCl were used to dissolve the aluminium residue, followed by dilution to 10 ml with mobile phase and analysis by FI - ICP-MS. The solution was analysed by FI - and LC - ICP-MS as described above.

The stoppers were removed from the sample bottles, rinsed with sub-boiling distilled water and allowed to dry. The top was separated from the bottom of each stopper and the bottom quartered. The bottom of the stopper is classed as that part which came into physical contact with the sample. These five pieces of stopper constituted one analysis set. Stopper sets which were expected to contain little or no mercury (unused stoppers and those from Diluent Lot A) were pooled into

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† Certain commercial equipment, instruments or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best for the purpose.

**Table 1.** Dates of production of samples

| Sample type | Date of production |
|---|---|
| Diluent, Lot A | January 1988 |
| Diluent, Lot B | November 1984 |
| Tetanus toxoid, adsorbed | January 1987 |
| Influenza virus vaccine | June 1974 |

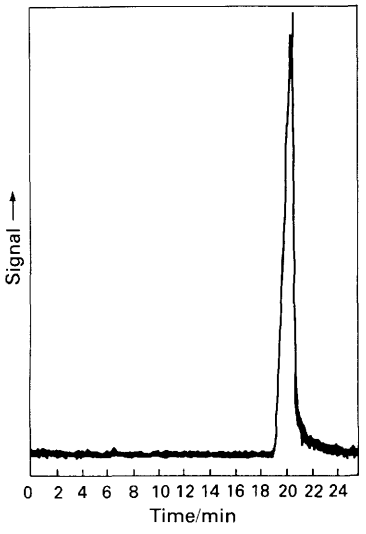


**Fig. 1.** LC - ICP-MS chromatogram of a typical sample, influenza virus vaccine at 2.5 μg ml$^{-1}$. Conditions as given in text

**Table 2.** Determination of thimerosal in Diluent Lot A

| | Hg in thimerosal/μg ml$^{-1}$ | |
|---|---|---|
| Sample | LC - ICP-MS* | FI - ICP-MS† |
| A1 | 48.1 | 49 (1) |
| A2 | 47.5 | 47 (3) |
| A3 | 49.1 | 48 (1) |
| A4 | 47.7 | 48 (1) |
| A5 | 47.3 | 46 (1) |
| A6 | 47.4 | — |
| A7 | 47.4 | — |
| A8 | 48.7 | — |
| A9 | 48.3 | — |
| A10 | 47.7 | — |
| Mean | 47.9 | 48 |
| Standard deviation | 0.6 | 1 |

* Values represent the average of duplicate injections.
† Values represent the average and standard deviation (in parentheses) of three injections.

groups of five sets. Stoppers which were suspected of absorbing mercury (Diluent Lot B, tetanus toxoid, adsorbed and influenza virus vaccine) were pooled into groups of two sets. The stoppers were placed in clean FEP beakers to which 5 ml of mobile phase were added. The 2-mercaptoethanol in the mobile phase acts to extract the absosrbed mercury. The extract was then analysed by FI - ICP-MS for total mercury content.

Thimerosal standard solutions were prepared fresh daily and injected alternately with samples. Injections of mobile phase and/or distilled water were used as blanks. One to three ml of concentrated HCl diluted to 10 ml with mobile phase, were then used as a blank for the analysis of the aluminium residue from the tetanus toxoid samples.

**Table 3.** Determination of thimerosal in Diluent Lot B

| | Hg in thimerosal/μg ml$^{-1}$ | |
|---|---|---|
| Sample | LC - ICP-MS* | FI - ICP-MS† |
| B1 | 42.7 | 42.8 (0.1) |
| B2 | 42.5 | 45.0 (1) |
| B3 | 41.5 | 43.6 (0.6) |
| B4 | 41.5 | 42.5 (0.8) |
| B5 | 41.9 | 45.9 (0.1) |
| B6 | 44.2 | — |
| B7 | 43.6 | — |
| B8 | 45.3 | — |
| B9 | 41.3 | — |
| B10 | 41.4 | — |
| Mean | 43 | 44 |
| Standard deviation | 1 | 1 |

* Values represent the average of duplicate injections.
† Values represent the average and standard deviation (in parentheses) of three injections.

**Table 4.** Determination of thimerosal in tetanus toxoid, adsorbed

| | Hg in thimerosal/μg ml$^{-1}$ | |
|---|---|---|
| Sample | LC - ICP-MS | FI - ICP-MS† |
| T1 | 49.7 | 47 (1) |
| T2 | 48.7 | 46 (1) |
| T3 | 50.9 | 44 (1) |
| T4 | 49.3 | 46 (1) |
| T5 | 50.6 | 46 (2) |
| T6 | 49.4 | — |
| T7 | 47.9 | — |
| T8 | 45.3 | — |
| T9 | 45.5 | — |
| T10 | 47.4 | — |
| Mean | 48 | 46 |
| Standard deviation | 2 | 2 |

* Values represent the average of duplicate injections.
† Values represent the average and standard deviation (in parentheses) of three injections.

## Results

The LC and FI results on the sample solutions are presented in Tables 2–5. Fig. 1 illustrates a typical sample chromatogram with an injection of influenza virus vaccine. The nominal thimerosal concentration for all of the samples is 49.6 μg ml$^{-1}$ as mercury (100 μg ml$^{-1}$ as thimerosal). Samples from the manufacturer are considered to be within the specifications if the Hg concentration is within ± 10 μg ml$^{-1}$ of this level. Diluent Lot A (Table 2) showed no apparent loss of thimerosal. As the total mercury concentration obtained by FI - ICP-MS agrees with the value for the concentration of mercury as thimerosal obtained by LC - ICP-MS, this indicates that all mercury in the sample is present as thimerosal. The LC and FI results for Diluent Lot B in Table 3 also agree, however, while the result is about 13% lower than the nominal thimerosal level, it is well within the manufacturers stated uncertainty range (40–60 μg ml$^{-1}$). A second peak, which eluted at about 30 min, was noted in the chromatograms for this sample. The peak was very small with an area of less than 2% of the area of the thimerosal peak. This was suspected to be a phenylmercury compound, however subsequent injection of a concentrated solution of phenylmercury(II) nitrate did not support this hypothesis. Phenylmercury ion did not elute from the column within 60 min, hence the identity of this small peak remains unknown.

The results presented in Table 4 for the tetanus toxoid, adsorbed, are similar to those for Diluent, Lot A. There is no loss of thimerosal from the solution and all of the mercury is accounted for, as can be seen by comparing the LC and FI data. The fourth sample, influenza virus vaccine, showed a

**Table 5.** Determination of thimerosal in influenza virus vaccine

| Sample | Hg in thimerosal/μg ml$^{-1}$ LC - ICP-MS* | FI - ICP-MS† |
|---|---|---|
| F1 | 23.9 | 25.1 (0.2) |
| F2 | 21.0 | 23.4 (0.2) |
| F3 | 27.6 | 27.5 (0.4) |
| F4 | 26.1 | 26.6 (0.2) |
| F5 | 14.9 | 18.3 (0.7) |
| F6 | 28.5 | — |
| F7 | 33.4 | — |
| F8 | 36.0 | — |
| F9 | 25.6 | — |
| F10 | 23.7 | — |
| Mean | 26 | 23 |
| Standard deviation | 6 | 3 |

* Values represent the average of duplicate injections.

† Values represent the average and standard deviation (in parentheses) of three or four injections.

**Table 6.** Analysis of stoppers for mercury content

| Stopper | Amount of Hg/μg per stopper* | Sample volume/ml | Hg found in stopper, % of total |
|---|---|---|---|
| Unused, grey | 0.0017 (0.0004) | | |
| Diluent Lot A | 0.0143 (0.0008) | ≈6.5 | 0.005 |
| Diluent Lot B | 0.55 (0.01) | ≈6.5 | 0.17 |
| Tetanus toxoid, adsorbed, Lot A | 0.40 (0.04) | ≈6.2 | 0.13 |
| Influenza virus vaccine | 52.7 (0.8) | ≈5.8 | 18.4 |

* Values represent the average and standard deviation (in parentheses) for a minimum of three injections of each solution.

significant loss of thimerosal from solution, Table 5. Approximately 50% of the thimerosal that would be expected to be in the solution is missing. The FI data confirm that all of the mercury in solution is accounted for by LC - ICP-MS. This is the oldest sample, well over 14 years past its expiry date. The degree of loss over this time period was slightly different for each sample and this is reflected in the poor between-sample precision indicated in Table 5. Qualitative analysis by LC - ICP-MS of both the tetanus toxoid and the influenza virus vaccine revealed none of the degradation products currently detectable by this method (*i.e.*, methylmercury, dimethylmercury and inorganic mercury).

Results of the analysis of the rubber closures are summarised in Table 6. It is of interest to note that the first three samples, which exhibited minimal loss of mercury, were closed with grey coloured stoppers, while the influenza virus vaccine sample was closed with a red stopper. This sample is also the oldest. There is a direct relationship between the loss of thimerosal from the sample solution (Table 2–5) and the presence of mercury in the stopper extract. The results of this experiment are semi-quantitative and represent the lower limits for mercury absorption by the stoppers. The actual percentage recovery of mercury from the stoppers was not determined. Mercury absorbed from solution could also pass through the stopper and be lost to the environment.

The analysis of the tetanus toxoid, adsorbed, aluminium residue yielded an average of 60.5 ng of mercury per filter residue. This corresponds to a loss of 0.098 μg ml$^{-1}$ of mercury from the sample solution through adsorption on to the aluminium adjuvant. This is insignificant compared with the concentration of the solution which is 48 ± 2 μg ml$^{-1}$.

## Conclusion

The usefulness of LC - ICP-MS in the study of long-term stability of thimerosal in a variety of sample matrices has been demonstrated. Evidence of long-term loss is suggested in some products, such as the influenza virus vaccine. In each instance, however, the material was beyond the normal expiry date and would not represent a safety hazard. The stopper as a route for loss of thimerosal from solution has been confirmed. While a minor, unidentified mercury compound was found in one of the samples, no other significant decomposition products were observed using the analytical system described. Further study of the separation problem between thimerosal and ethylmercury is currently underway.

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