Analysis of Mercurial Preservatives in Bacterins, Vaccines, and Antisera by Atomic Absorption Spectrophotometry

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A flameless atomic absorption method was developed for the determination of mercurial preservatives in biologicals. The assay was based on a quantitative determination of the mercury content of these preservatives. This method was used to analyze a variety of samples and yielded reproducible results with satisfactory recoveries. The procedure is presented in a simplified block diagram and described in detail relating its suitability for routine assay of large numbers of samples.

The regulatory control of veterinary biologicals in the United States is under the direction of Veterinary Services Laboratories, APHIS, U.S. Department of Agriculture. They permit the use of mercurial compounds, such as thimerosal, phenylmercuric acetate, and phenylmercuric nitrate, as preservatives in veterinary biologicals. Regulatory control of these preservatives is important, as it provides assurance that the biological product has been adequately preserved and that excessive amounts of mercury are not being added to our meat supply (3, 8).

Mercurial preservatives are a difficult group of compounds to assay because they are normally used at low levels of concentration and their degradation compounds often react with the biologicals to form many intermediary products. Conventional chemical methods, such as the colorimetric procedure using dithizone, require tedious methods of digestion and extraction that are impractical as routine control procedures. Microbiological methods, similar to antibiotic assay methods, cannot easily be used to assay large numbers of samples. Microbiological methods also present problems as the results can be influenced by other ingredients (inactivating agents, bacterial by-products, etc.) in the commercially prepared biologicals.

Normally, mercurial preservatives are used at a 1:5,000 or 1:10,000 concentration in the bacterins, vaccines, and antisera. These mercurial compounds often react with components of the biologicals to form intermediary products which make specific identification of the original compound very difficult. A compromise to this problem would be to assay the bacterin or antiserum for its mercury content and mathematically estimate the amount of mercurial preservative in the product. The mercury content of these preservatives represent about 50% of their weight, which would mean that the theoretical amount of mercury in a preserved biological would be in the range of 50 to 100 µg/ml.

Atomic absorption spectrophotometry provides a sensitive and precise method for estimating mercury (1) which can be readily adapted to analyze numerous samples. A method is described which has been developed to assay the mercurial preservative content of bacterins, vaccines, and antisera used in veterinary medicine. The analytical procedure is a modification of the method developed by Hatch and Ott (4).

MATERIALS AND METHODS

Apparatus: (i) atomic absorption spectrophotometer. The present work was done with a Perkin-Elmer model 403 spectrophotometer. Other equipment having similar capabilities for mounting a long pathlength absorption cell and exciting the sample at the mercury line (253.7 nm) may also be used (5, 6).

(ii) Absorption cells. Cells were glass tubing (22-mm outer diameter) with quartz windows on each end and inlet-outlet ports placed near the ends. A cell with a 50-mm pathlength was used for analyses in the microgram range, and one with a 100-mm pathlength was used for analyses in the nanogram range.

(iii) Carrier gas system. Filtered compressed air was used as the carrier gas at a flow rate of 0.75 standard ft³ (ca. 0.021 m³) per h. The burner control
of the model 403 spectrophotometer was used for pressure and volume control of air. The bubbler referred to later in the procedure was constructed with a fritted glass tip for better aeration of the sample solutions and more efficient transfer of mercury vapor to the absorption cell. An efficient system for removing mercury vapor from the operating environment is necessary as a safety factor.

Reagents: (i) standard mercury solutions. Dissolve 10.8 mg of HgO in 5 ml of HCl (11.6 M) (1 + 1) and dilute to 100 ml with water to obtain a stock solution of 100 μg of Hg/ml. Make further dilutions to obtain standards of 80, 60, 40, and 20 μg of Hg/ml. (ii) Hydroxylamine hydrochloride (NH₂OH·HCl), 1.5% wt/vol. Make up with water. (iii) Stannous chloride (SnCl₂), 10% wt/vol. Dissolve 50 g in 75 ml of HCl (11.6 M) and dilute to 500 ml with water. This solution should be optically clear when freshly made. Discard when the solution becomes turbid.

Procedure. The complete procedure, including digestion and aeration, was done in 120-ml Erlenmeyer flasks with 24/40 standard taper ground-glass necks. Fifty microliters of standard or sample was pipetted, using a micropipette with disposable tip, into flasks containing 5 ml of H₂SO₄ (17.8 M) and 5 ml of HNO₃ (15.4 M). The flasks were then covered with loose-fitting plastic stoppers and placed in an 80-C water bath for 10 min. The stoppers were removed, and approximately 40 mg or a sufficient amount of KMnO₄ was added until a dark purple color persisted. Heating was continued for an additional 10 min. The flasks were removed from the heating bath, covered, and allowed to cool to room temperature. All standards and samples were analyzed in duplicate.

Digestion and oxidation of the samples to completely break down the organic matter was necessary to release the bound mercury, oxidize it to mercuric ion, and eliminate foaming (7) during subsequent steps of the procedure. The digested samples were diluted to approximately 100 ml with water and again cooled to room temperature.

A reagent blank was aerated while final instrument and recorder adjustments were made. NH₂OH·HCl (5 ml) was added to each sample to react with the excess KMnO₄. The solution changed from the dark permanganate color to colorless, indicating that all the KMnO₄ had reacted.

At this point, each sample must be treated individually as this step is probably the most critical step in the procedure. Addition of 5 ml of SnCl₂ and insertion of the bubbler must be completed as rapidly as possible to avoid loss of mercury vapor which is immediately converted to the metallic form by SnCl₂. After each absorption peak was recorded, the bubbler was returned to the blank solution to purge the mercury vapor from the system. This procedure was repeated with each additional standard and sample. The temperature of the solutions and the air flow were held relatively constant because variations produced a slight effect on absorption values.

Concentration of standards was plotted versus recorder response, and mercury concentration of the samples was determined from the resultant curve. The concentration of mercury determined from the curve multiplied by the reciprocal of the mercury fraction in the preservative times the dilution factor provides the concentration of the preservative in the original sample.

The complete procedure is presented in a simplified block diagram in Fig. 1.

RESULTS AND DISCUSSION

Figure 2 is an actual recording of absorption peaks for five duplicate standards. The peak height may be adjusted by changing instrument settings or sample volumes. The peaks are always sharp and always return to base line quickly, allowing samples to be analyzed in rapid sequence. Time lapse for analyzing the 10 predigested standards was slightly over 20 min. A short digestion time and fast instrument response makes this method suitable for automation (2). Standard curves derived from plot-
Three samples of bovine serum were spiked with known amounts of mercury in the form of thimerosal. These spiked samples were subjected to the same analysis sequence as the aqueous solutions, and the recoveries were determined. Results from these analyses are presented in Table 2 and indicate satisfactory recovery in the presence of biological material. These data also reflect the reproducibility of the method. Fresh standards and samples were prepared during the time period represented. Precision of the method was checked by analyzing 12 samples of phenylmercuric acetate solution during 1 day. The calculated precision for these results was 3.0 ± 0.07 μg of mercury for a theoretical 3-μg mercury sample.

The assay system has been used in this laboratory for analysis of mercurial preservatives in over 200 samples of bacterins, vaccines, and antisera. The mercury content was determined quantitatively, and the preservative content was calculated. Some results are listed in Table 3, along with the maximal amount of preservative and related mercury levels permitted according to the outline of production for each product. The mercury level in the products varied, but all results were below the maximum permitted. Some products tested, such as those indicated by the zero levels in the table, were not preserved with mercurial preservatives and therefore should be free of mercury.

As an additional test of the sensitivity and versatility of this method, standards and samples were prepared in a lower (nanogram) mercury concentration range. The 50-mm absorption cell was replaced with a 100-mm cell, and instrument controls were adjusted for optimal peak height. Mercury solution was added to bovine serum to obtain samples containing 25 ng of Hg/50-μlter sample. The average recovery from a series of six of these serum samples was 24.6 ± 1.1 ng of Hg.

The recoveries reported in this work were based on freshly preserved samples. Additional experiments need to be designed to study the

![Graph](image)

**Fig. 2.** Actual recorder tracing for mercury standards. The peaks correspond to 5, 4, 3, 2, 1 μg of Hg/50-μlter sample of HgO standard solutions.

Table 1. Recovery of mercury from various mercurial preservatives

| Theoretical concentration (μg of Hg) | Thimerosal | Phenylmercuric acetate | Phenylmercuric nitrate |
|-------------------------------------|------------|------------------------|------------------------|
| 2.00                                | 2.07 ± 0.12 (103.5)* | 2.00 ± 0.11 (100.0) | 1.98 ± 0.09 (99.0) |
| 3.00                                | 2.92 ± 0.12 (97.3)  | 3.03 ± 0.09 (101.0) | 2.93 ± 0.10 (97.7) |
| 5.00                                | 4.62 ± 0.19 (92.4)  | 4.78 ± 0.18 (95.6)  | 4.76 ± 0.14 (95.2) |

* One mercury equivalent of preservative equals 1 μg of Hg per 50-μliter sample.
* Average of duplicate analyses on 6 different days. SD, standard deviation.
* Number in parentheses indicates percentage of recovery from solution.
Table 2. Recovery of Hg (thimerosal) added to bovine serum

| Theoretical (μg/50-μliter sample) | Average* % recovery ± SD (%/50-μliter sample) | Average* % recovery ± SD |
|-----------------------------------|----------------------------------------------|--------------------------|
| 1.25                              | 1.27 ± 0.11 (3)*                              | 101.2 ± 8.3              |
| 2.50                              | 2.45 ± 0.10 (3)                               | 98.0 ± 4.0               |
| 5.00                              | 4.98 ± 0.10 (10)                              | 99.5 ± 2.0               |

* Average of data taken over a period of 18 days. SD, standard deviation.
* Number in parentheses indicates number of tests.

Table 3. Analysis of Hg in bacterins, vaccines, and antiserum

| Sample | Maximal permitted thimerosal level (μg/ml) | Maximal permitted mercury level (μg/ml) | Amt of Hg found (μg/ml) |
|--------|-------------------------------------------|----------------------------------------|------------------------|
| Bacterin 1 | 0                                        | 0                                       | 0                      |
|          | 2                                        | 0                                       | 0                      |
|          | 3                                        | 1:10,000                               | 49.6                   | 38.4       |
|          | 4                                        | 1:10,000                               | 49.6                   | 29.9       |
|          | 5                                        | 1:10,000                               | 49.6                   | 31.9       |
|          | 6                                        | 1:5,000                                | 99.2                   | 94.2       |
|          | 7                                        | 1:5,000                                | 99.2                   | 86.6       |
|          | 8                                        | 1:5,000                                | 99.2                   | 92.4       |
| Vaccine 1 | 0                                        | 0                                       | 0                      |
|          | 2                                        | 0                                       | 0                      |
|          | 3                                        | 0                                       | 0                      |
|          | 4                                        | 1:10,000                               | 49.6                   | 40.4       |
|          | 5                                        | 1:5,000                                | 99.2                   | 40.3       |
|          | 6                                        | 1:5,000                                | 99.2                   | 38.4       |
|          | 7                                        | 1:5,000                                | 99.2                   | 46.6       |
|          | 8                                        | 1:5,000                                | 99.2                   | 37.4       |
|          | 9                                        | 1:2,500                                | 198.0                  | 87.5       |
| Antiserum 1 | 0                                        | 0                                       | 0                      |
|          | 2                                        | 0                                       | 0                      |
|          | 3                                        | 1:10,000                               | 49.6                   | 37.6       |
|          | 4                                        | 1:10,000                               | 49.6                   | 36.9       |
|          | 5                                        | 1:10,000                               | 49.6                   | 36.4       |
|          | 6                                        | 1:5,000                                | 99.2                   | 79.4       |
|          | 7                                        | 1:5,000                                | 99.2                   | 73.7       |
|          | 8                                        | 1:5,000                                | 99.2                   | 73.1       |

Factors which may influence the recovery of mercurial preservative from biologicals. A versatile method has been presented which is suitable for the determination of the mercury content of several mercurial preservatives normally used in bacterins, vaccines, and antiserum. This procedure was developed for the assay of thimerosal, phenylmercuric acetate, and phenylmercuric nitrate at concentrations of near zero to 1:2,500, although it could be readily adapted for other mercurial preservatives and concentrations. Although this procedure lacks selectivity to differentiate between specific mercurial preservatives, it does have good precision and sensitivity for mercury. It is a rapid test and readily adapted to handling large numbers of samples, which makes it ideal for a regulatory control program.

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