HPLC Analysis of Thimerosal in Ophthalmic Solution. Comparing Electrochemical with Spectrophotometric Detectors

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Abstract: Electrochemical and spectrophotometric detectors for the reversed phase HPLC analysis of thimerosal in an ophthalmic formulation were evaluated with respect to response linearity, recovery, and lower limit of detection. The electrochemical oxidation of thimerosal at a glassy carbon electrode was characterized by both cyclic voltammetry and thin layer amperometry. The hydrodynamic half-wave potential for thimerosal (+0.6 V) was determined using a coulometric detector. The relatively low oxidation potential for thimerosal forms the basis for this highly sensitive and selective analytical technique. The amperometric lower limit of detection for thimerosal (at signal/noise = 2) was less than 400 pg. The detection limit with spectrophotometric detection at 254 nm was approximately 20 ng, a 50 fold increase.

Thimerosal (sodium ethylmercurithiosalicylate) is a widely used antimicrobial preservative. Stability studies with thimerosal reveal that it is unstable in aqueous solution (1, 2). Analytical methods previously developed for thimerosal have included: polarography (3), atomic absorption spectroscopy (4), colorimetry (5) and HPLC (6, 7, 8). With respect to stability specificity, only the HPLC procedures are acceptable for analyzing thimerosal in the presence of its degradation products. The sensitivity of HPLC assays depends upon the signal/noise ratio obtained under the defined operating parameters. Because thimerosal is often present in microquantities (usually less than 0.001 to 0.005 % of the formulation), spectrophotometric detection may not be the optimal choice for quantitative analysis.

Electrochemical detectors have been successfully used for the sensitive HPLC analyses of organosulfur compounds, including: chlorpromazine, levomepromazine, glutathione and parathion (9–11). The advantages of electrochemical HPLC detection include: a wide linear dynamic range, excellent reproducibility, and detection limits in the low nanogram to picogram range. Additionally, response selectivity with electrochemical detection is controllable by appropriate choice of potential and electrode material.

The objectives of this study were to develop an HPLC assay for thimerosal in an ophthalmic solution, and to directly compare electrochemical with spectrophotometric detection.

Materials and Methods

Chemicals and Reagents

Ethylmercurithiosalicylic acid sodium salt was obtained commercially (Aldrich; Milwaukee, WI) and used without further purification. All other chemicals and reagents were analytical grade Mallinkrodt; Paris, KY). Distilled water was further purified by passing it through a nanopure system (Barnstead U.S.A.).

HPLC System – A Spectra Physics-8100 ternary solvent delivery system (Spectra Physics; San Jose, CA) was used to deliver the eluant to a 10-cm RACII (C-8) column (Whatman Chemical Separation Inc.; Clifton, NJ) at 1.0 ml/min. Fifteen microliter injections were made with a 710B WISP autosampler (Waters Associates; Milford, MA). A fixed-wavelength UV detector SP-8300 (254 nm) (Spectra Physics; San Jose, CA) was used for all spectrophotometric detection studies, and it was connected in series with the electrochemical detector. An SP-4100 integrator was used to record and integrate all chromatograms (Spectra Physics; San Jose, CA). The coulometric detector used was an ESA Model 5100A (ESA Inc.; Bedford, MA) with dual porous graphite working electrodes and a solid state hydrogen reference electrode. Amperometric detection studies were performed with a BAS model LC-4B/17 (Bioanalytical Systems; West Lafayette, Indiana) controller, glassy carbon working electrode and a Ag /AgCl reference electrode.

The mobile phase was methanol and sodium acetate buffer (0.1 M, pH 4.5) in a 30 : 70 ratio with 0.2 mM EDTA added. Backpressure at 1.0 ml/min was approximately 3000 PSI.

Cyclic Voltammetry – CV scans of thimerosal were performed with an EC-225 voltammetric analyzer (IBM Instruments Inc.; Danbury, CT) using glassy carbon working electrode, Ag/AgCl reference, and platinum wire auxiliary electrodes.

Standards – Thimerosal stock solutions were prepared by dissolving 20, 25 and 30 mg in 100 ml methanol. Calibration standards prepared by diluting the stock standard solutions 1:20 with mobile phase.

Analytical Validation – Linearity and recovery data were generated by spiking ophthalmic placebo (a proprietary formulation) in duplicate with thimerosal at levels approximating 0, 70, 80, 90, 100, 110, and 120 % of thimerosal label strength (25 μg/ml). Sample preparation required diluting the spiked samples 1:2 with mobile phase before injection onto the chromatograph.

Analytical Precision – Ten spiked placebo samples ($\simeq$ 24 μg/ml) were analyzed for thimerosal content by serial UV-EC detection. The amperometric detector was used for this study, and on-column injections were limited to 60 ng of the analyte.

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Results and Discussion

The electrochemical behavior of thimerosal was characterized by both cyclic and hydrodynamic voltammetry. These experiments helped to define optimal operating parameters for the electrochemical analysis of thimerosal.

Figure 1 shows the cyclic voltammogram of 0.5 mM thimerosal in 0.1 M sodium acetate (pH 4.5). The voltammogram reveals a single anodic wave with a 1.1 V peak potential. Absence of a cathodic wave during the reverse scan of the CV cycle indicates an irreversible oxidation. The above electrochemical behavior is typical of sulfhydryl compounds at carbon electrodes and suggests slow electron transfer between thimerosal and the electrode surface (12).

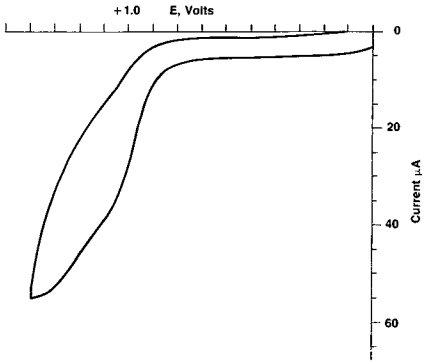

Fig. 1 Cyclic voltammogram of 0.5 mM thimerosal in 0.1 M sodium acetate buffer: pH 4.5; sweep rate 100 mV/S.

A hydrodynamic voltammogram of thimerosal (Fig. 2) obtained under established chromatographic conditions showed a characteristic sigmoidal current-voltage relationship. The half-wave potential for thimerosal determined from Figure 2 was + 0.6 V. Thus, a detector potential of + 0.75 V, provides diffusion-limited oxidation of thimerosal under the defined chromatographic conditions.

The electrochemical oxidation mechanism of thimerosal at glassy carbon probably involves one electron transfer from sulfur or the thimerosal aromatic nucleus to the electrode surface, yielding a transient radical cation. The redox behavior observed for thimerosal is similar to that found with another alkyl-aryl sulfide, chlorpromazine (13, 14), which undergoes one-electron oxidation to a radical cation.

The primary advantage of electrochemical detection for HPLC analysis of

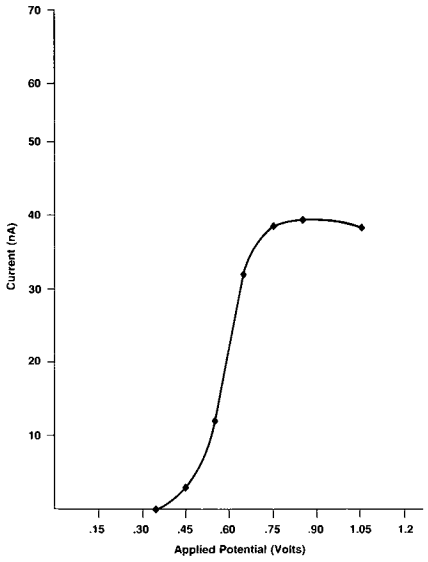

Fig. 2 Hydrodynamic voltammogram of repetitive injections of thimerosal, obtained by liquid chromatography/electrochemistry in mobile phase of 0.1 M sodium acetate pH 4.5, 30 % methanol (v/v).

Signal-to-noise ratio = 2
Amplitude of baseline noise = 15 mm
Slope = 104.9 ± 12 mm/ng
Intercept = O.7556 ± 10 mm

Lower limit of detection = 0.356 ng

thimerosal is high sensitivity. The detection limit of the electrochemical system depends on the working electrode applied potential, the analyte half-wave potential and diffusion coefficient, and the mobile phase background or capacitance current.

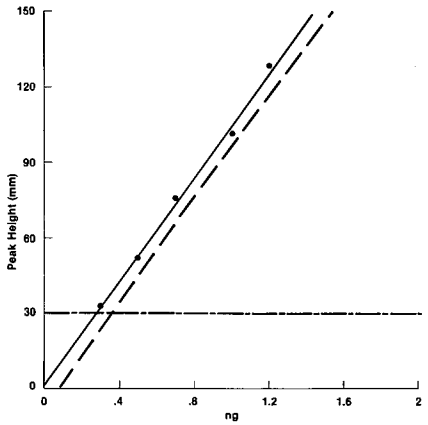

Fig. 3 Lower limits of detection of thimerosal with amperometric detection at + 0.75 Volts vs. Ag/AgCl.
— — Represents the lower 95 % confidence interval.
— — Represents lower limit of detection, S/N = 2.

The detection limit for thimerosal was determined using both amperometric and spectrophotometric detectors. Figure 3 plots amperometric detector response, (peak height) versus amount of thimerosal injected and illustrates the limit of detectability (at the 95 % confidence level) to be less than 400 pg of thimerosal. This detection limit is approximately 50 times lower than that achievable with UV (20 ng).

The two electrochemical detectors and the spectrophotometric detector were compared with respect to observed percent recovery (= 100 x thimerosal found ÷ thimerosal added) and response linearity (linear least-squares regression slope of thimerosal found versus thimerosal added data). Using + 0.75 V applied potential, 500 ng thimerosal injected, and peak height quantitation, the coulometric detector gave 98.4 (±4.3 S.D.) per cent recovery, and response linearity according to equation (1):

$$\text{mg found} = (-5.6 \pm 4.1) + (1.06 \pm 0.046)\ \text{mg added} \quad (1)$$

where the error limits are expressed as 95 % confidence intervals and the correlation coefficient was 0.997. The spectophotometric detector gave essentially identical results.

The amperometric detector suffered performance degradation at high levels of injected thimerosal. Thus, for 500 ng injected, recovery was 95.1 ± 9.3 % and response linearity obeyed equations (2):

$$\text{mg found} = (12.1 \pm 8.6) + (0.79 \pm 0.09)\ \text{mg added} \quad (2)$$

with correlation coefficient = 0.976. However, decreasing the injected amount 10 fold gave satisfactory recovery (101 ± 2.6 %) and linearity, equation (3):

$$\text{mg found} = (0.38 \pm 3.1) + (1.00 \pm 0.031)\ \text{mg added} \quad (3)$$

For all three detectors, peak height quantitation proved more reliable than peak area quantitation, because peak tailing significantly influenced peak integration at the low analyte concentrations and high gain settings employed. Peak tailing commonly occurs in HPLC analysis of organosulfur compounds (15, 16), and thimerosal adsorption at electrochemical detector working electrode surfaces exacerbates peak asymmetry problems. Thimerosal adsorption at the

amperometric detector electrode probably accounts for the poor performance of this detector when the injected thimerosal amount exceeded approximately 100 ng.

We also examined the influence of electrode potential on analytical performance using the coulometric detector. Decreasing the applied potential to + 0.55 or + 0.45 V contributed to significant deviations from observed recovery and response linearity relative to the case of the coulometric detector at + 0.75 V. In principle, a linear current-concentration relationship holds for every point along the hydrodynamic current-potential curve in HPLC analysis with electrochemical detection. In practice however, small variations in potential yield relatively large current deviations when the applied potential resides on the upslope of the hydrodynamic curve. Thus possible advantages in the improved selectivity inherent with lower operating potentials (17) must be evaluated versus attendant losses in sensitivity and linearity before selecting an applied potential below the plateau region of a hydrodynamic voltammogram.

Finally, the spectrophotometric and amperometric detectors were tested for precision of response with 10 spiked placebo samples, and both detectors gave statistically identical results (paired t-test at the 95 % confidence level: UV = 24.1 ± 0.84 μg/ml, EC = 23.9 ± 0.53 μg/ml).

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Studies on the Dissolution of Drugs from Tablets using Perturbed Angular Correlation

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Abstract: The perturbed angular correlation (P.A.C.) technique was used to assess the dissolution of drugs coprecipitated with [^{111}In]indium chloride in tablets *in vitro*. The amount of drug in solution was monitored simultaneously with the amount of radioactivity in solution and these were correlated to the anisotropy values obtained by P.A.C. measurements. The results indicated that the rate of drug dissolution paralleled the change in measured anisotropy of the system. Thus, the measurement of anisotropic changes in drug-indium complexes by P.A.C. is a reliable indicator of drug dissolution and can provide meaningful dissolution data for noninvasive *in vivo* studies.

Imaging techniques, in particular gamma scintigraphy, are now well established as tools in pharmaceutical research (1). When studying solid dosage forms, one drawback of this technique is that it is impossible to distinguish between the radionuclide in solid form or in solution. This problem can, in part, be overcome by the combined use of gamma scintigraphy with perturbed angular correlation measurements (P.A.C.) (2). Indium-111 decays by emitting two gamma rays in cascade that exhibit a certain angular correlation between their direction of propagation. This correlation can be perturbed if the intermediate state of the nucleus, during decay, is perturbed, for example, by dissolution of material containing the radiolabel. Hence, Beihn and Digenis were able to monitor the dissolution of ^{111}indium chloride from tablets by P.A.C. and showed that changes in the angular correlation paralleled the rate of dissolution of ^{111}indium chloride as measured by sampling and counting (2). The technique could be used as a non-invasive assessment of dissolution *in vivo* and has since been successfully applied to the *in vivo* release from suppositories (3). As pointed out by Beihn and Digenis, the P.A.C. technique measures the dissolution of the radionuclide, and not the drug, and this may be considered as a limitation of its usefulness.

One approach to overcoming this problem would be to radiolabel the drug itself. In certain specific cases, this may be possible. This paper examines a more

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