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Evaluation of the capability of different chromatographic systems for the monitoring of thimerosal and its degradation products by high-performance liquid chromatography with amperometric detection

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ABSTRACT

Several liquid chromatographic systems using electrochemical detection on carbon electrodes were compared for the analysis of thimerosal and its degradation products, thiosalicylic acid and dithiodibenzoic acid. The studied separation systems included reversed-phase ion-suppressed chromatography, reversed-phase ion-pair chromatography and ion chromatography. Amperometry and coulometry were evaluated as electrochemical detection techniques. The best method for thimerosal determination in ophthalmic solutions in terms of selectivity and sensitivity was ion-pair chromatography.

INTRODUCTION

Thimerosal (sodium ethylmercury thiosalicylate, TMS) is an organomercurial compound widely employed as a topical antiseptic and antimicrobial preservative for ophthalmic use and, in particular, is routinely employed in the formulation of hard and soft contact lens antiseptic solutions. Stability studies have revealed that this compound is unstable to light in aqueous solutions [1,2], mainly in glass bottles, and can be adsorbed by plastic [3,4], both of which factors influence the potential shelf-life of pharmaceutical products. It has also been reported that the presence of halides can have an adverse influence on the stability of thimerosal [5].

The decomposition of thimerosal in aqueous solution has been reported [2,4], and it has been shown that the major degradation products are thiosalicylic acid, (TSA) and 2,2′-dithiodibenzoic acid (DTDBA). In studies of degradation, and for routine analytical purposes, a number of analytical methods have been developed, including colorimetry [3,5,6], atomic absorption spectrometry [7–9] and polarography [10–11]. These techniques, based on total mercury or total organic mercury assay, do not indicate accurately the amount of intact thimerosal present in solution. Liquid chromatography has been suggested as a simple, specific method for analysis of intact thimerosal and its degradation compounds [3,4,7,12–17]. In all these methods, UV detection was used, except one [16] in which coulometric detection was applied for TMS determination, but no information regarding degradation products was given.

In our laboratory, we are developing different

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chromatographic methods for the determination of TMS and its degradation products in manufactured samples. In order to obtain more sensitive and selective detection of these samples, and considering the easy oxidation of these compounds on carbon electrodes, electrochemical detection was chosen as the most appropriate technique. Two electrochemical detection modes, amperometric and coulometric, were evaluated.

Related to these studies, in a previous paper [18] we described the use of amperometric detection on a glassy carbon electrode for determination of TMS, TSA and DTDBA by reversed-phase ion-suppressed chromatography. The method was applied to the determination of these compounds in ophthalmic formulations and, although detectability was adequate, high limits of detection were obtained, of the same order of magnitude as with UV detection. The present work improves previous results by increasing the sensitivity obtained using coulometric detection compared with amperometric ion-suppression chromatography (ISC).

Another problem associated with using ISC with real samples is the significant decrease in the retention time of TMS observed as a result of the presence of polymers in the samples. To resolve this problem, we propose the use of reversed-phase ion-pair chromatography (IPC) and ion-exchange chromatography (IEC) as separation techniques.

EXPERIMENTAL

Reagents and samples

Thimerosal (Alcon Iberhis, Madrid, Spain), thiosalicylic and 2,2′-dithiodibenzoic acids (Sigma, St. Louis, MO, USA) were used without further purification. Methanol, acetonitrile, 85% phosphoric acid, 99% acetic acid, 30% ammonium hydroxide, sodium perchlorate, potassium hydrogenphthalate, potassium nitrate (Carlo Erba, Milan, Italy), tetrabutylammonium perchlorate (TBAP) and tetraethylammonium perchlorate (TEAP; Sigma) were analytical reagent grade. Stock solution of thimerosal was made up in water, whereas stock solutions of TSA and DTDBA were made up in methanol, in a concentration of about 100 μg/ml. These solutions were stable for a week when stored at 4°C and protected from light.

Soft lens care products were obtained from Alcon Iberhis. These samples contain TMS in a concentration of 0.01 mg/ml. Other ingredients were disodium edetate, sodium chloride, phosphate buffer and a catalase.

Apparatus

For reversed-phase chromatography, the HPLC system consisted of a Gilson Model 302C pumping system, equipped with a membrane damper, a Rheodyne Model 7125 injector equipped with a 20-μl loop, and a Spectra Physics SP 4290 integrator. When ion chromatography was used, the HPLC system consisted of a Shimadzu Model LC9A pumping system and a CTO-6A column oven and the injector was equipped with a 100-μl loop. A Shimadzu CR4A Cromatopac was used in this case. As a detector we used a Metrohm Model 461 amperometric detector equipped with a Metrohm Model 656 flow cell, of less than 1 μl volume, or an ESA Model 5010 coulometric cell of 2 μl volume. A glassy carbon electrode (Metrohm 6.0805.010) or a carbon paste electrode (Metrohm 6.0807.000) with an area of 7 mm^2 was used as the amperometric working electrode. The detector was operated in amperometric mode at a sensitivity of 10 nA full scale. When coulometric mode was used, the detector was operated at a sensitivity of 0.1 μA full scale.

Chromatographic conditions

For reversed-phase chromatography the column used was a 150 × 4 mm stainless-steel prepacked column containing 5-μm Spherisorb C_{18} particles (Tracer, Madrid, Spain). In IPC, the mobile phase was methanol–water (55:45, v/v) containing 2 mmol of TBAP or methanol–water (50:50, v/v) containing 2 mmol of TEAP, both adjusted to pH 4.8 with $HClO_4$. For ISC with coulometric detection, the mobile phase was methanol–water (60:40, v/v) containing 0.005 mol/l phosphoric acid. The flow-rate was 1.0 ml/min. For IEC the column was a 50 × 4.6 mm plastic column prepacked with 10-μm ammonium quaternary polymeric resin of low capacity

(30 μequiv./g) (TR-anion; Tracer). The mobile phase was 7.5 m*M* potassium perchlorate, and the flow-rate was adjusted in this case to 1.5 ml/min.

Mobile phase solutions were filtered through a Millipore Durapore filter (0.45 μm pore size) and deaerated by stirring under vacuum for at least 15 min. Standard solutions of all compounds were prepared in mobile phase and filtered through a similar filter before injection into the chromatograph.

The aqueous manufactured samples of TMS were injected without modification or after extraction with a C_{18} Sep-Pak cartridge (Millipore). The extraction procedure was as follows. A 0.1-ml aliquot of concentrated phosphoric acid was added to 10 ml of soft lens care solution. The mixture was pumped with a syringe through a Sep-Pak cartridge preconditioned with methanol and water. The cartridge was washed with 5 ml of water and 2 ml of 20% of methanol solution and then TMS, TSA and DTDBA were eluted with 2 ml of methanol. The extract was diluted to 10 ml with eluent and injected into the chromatograph.

Electrode pretreatment procedure

The pretreatment procedure for LC working electrodes was as follows. Before each set of measurements (every day), the amperometric glassy carbon electrode surface was polished with the polishing cloth for 60 s and rinsed with water before being attached to the detector. The coulometric cell was cleaned by flushing the cell with 5 ml each of water, 3 *M* nitric acid and water again before attaching the cell to the chromatograph. A suitable working potential was applied to the electrode while the mobile phase was passed through the system until a stable baseline was obtained.

RESULTS AND DISCUSSION

Chromatographic studies

Chromatographic conditions for TMS, TSA and DTDBA in reversed-phase ion-suppression chromatography have been reported elsewhere [18]. In IPC, the effect of the counter-ion concentration on separation and signal-to-noise ratio was studied for both TEAP and TBAP, varing the concentration between 0.01 and 0.001 *M*. Adequate resolutions and analytical signals were obtained at concentrations of 0.002 *M* for both types of counter-ions studied. Mobile phases containing 55 and 50% methanol for TBAP and TEAP, respectively, were chosen in order to obtain a similar retention of the products for both types of counter-ions. The pH value of the eluent does not affect the retention times of TMS and TSA, as long as it is higher than 4 (pK_a values are lower than 4); in this case the retention time of TSA is lower than that of TMS. For DTDBA, which contains two acidic groups, the pH value of mobile phase must be kept between 4 and 5 in order to obtain an adequate resolution. Under these conditions, DTDBA appears at retention times higher than TMS.

When IEC was employed, potassium hydrogenphthalate, potassium nitrate and potassium perchlorate were evaluated as eluents. The most efficient compound was potassium perchlorate as determined by both adequate separation and low noise in the detector. Appropriate resolutions for TMS and TSA were obtained using eluent concentrations of 0.0075 *M* at a flow-rate of 1.5 ml/min. Under these conditions, DTDBA presented retention times higher than 30 min, and no peak was observed at concentrations lower than 20 μg/ml. Under these conditions, peaks were broad and not baseline resolved (R_s = 0.95). No improvement in the resolution was obtained by including small amounts of methanol or acetonitrile in the eluent. Therefore IEC was not further considered.

Electrochemical studies

Fig. 1 shows the hydrodynamic curves obtained for the three compounds studied using ion-pair chromatography in amperometric mode. It was observed that TMS and TSA reached a maximum and almost constant signal at potentials higher than 0.8 V. For DTDBA, however, the higher signal was obtained at a potential of 1.2 V. In addition to these results, lower backgrounds and noises were obtained at potentials lower than 1.0 V. At higher potentials a significant increase in background and noise was ob-

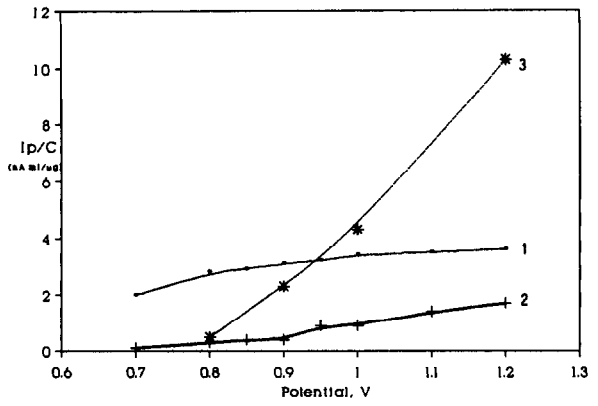

Fig. 1. Hydrodynamic voltammograms of compounds studied with amperometric detection. Eluent: 50% (v/v) methanol in water, containing 0.0020 *M* TEAP adjusted to pH 4.8 with perchloric acid. Concentrations: (2) TMS 11.1 μg/ml, (1) TSA 9.1 μg/ml and (3) DTDBA 9.6 μg/ml. Background (···).

served when the potential increased. Because of these results, a potential of 0.9 V for the simultaneous quantitation of TMS and TSA and a potential of 1.2 V for the quantitation of DTDBA were chosen in order to obtain the highest sensitivity for each compound.

The hydrodynamic curves obtained in the coulometric detection mode for IPC (Fig. 2) showed a higher signal at a potential value of 0.8 V for all studied compounds. Therefore, a potential of 0.8 V was chosen for simultaneous detection of TMS, TSA and DTDBA.

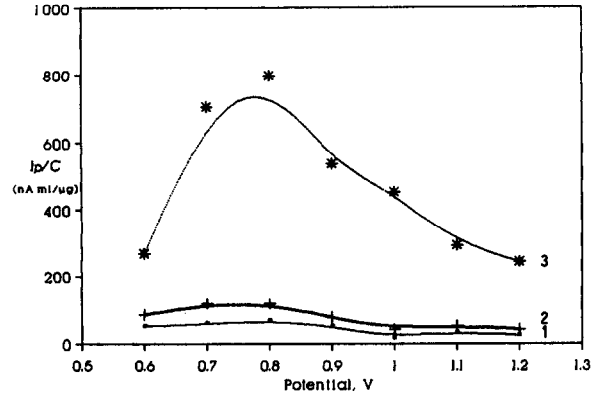

Fig. 2. Hydrodynamic voltammograms of compounds studied with coulometric detection. Concentrations: (1) TSA 1.00 μg/ml, (2) TMS 1.22 μg/ml and (3) DTDBA 1.06 μg/ml. Eluent as Fig. 1.

As in IPC, in ISC the efficiency of the coulometric detector observed allowed the detection of DTDBA, together with TMS and TSA, at potentials lower than 1.2 V with a better sensitivity. From coulometric hydrodynamic voltammograms, a potential of 0.9 V was chosen for simultaneous detection and quantitation of all compounds studied. The variation of signal-to-noise ratio with phosphoric acid concentration in the eluent, ranging from 0.01 to 0.001 *M*, was evaluated for this detector type. An eluent with a lower concentration of phosphoric acid, 5 m*M*, than in amperometric detection was chosen in order to obtain an optimum signal-to-noise ratio without a significant increase in the retention time.

Calibration graphs, sensitivity and precision

The linearity of the calibration graphs was checked up to 100 μg/ml in the amperometric detection mode and up to 5 μg/ml in the coulometric mode. For amperometric detection, linearity was observed at all concentration ranges assayed, whereas in coulometric detection no linearity was observed at concentrations higher than 2 μg/ml. The statistical treatment of the calibration graphs and limits of detection obtained for all compounds are reported in Table I for the ion-pair chromatographic method. As can be observed, the detection limits found in amperometric mode when TBAP was used as counter-ion were two orders of magnitude higher than when TEAP was used. In coulometric detection the results obtained were of the same order of magnitude for both counter-ions when the working electrode was treated daily with 3.0 *M* nitric acid. When no pretreatment was applied, the analytical signal decreased with increasing number of injections, especially when TBAP was used as counter-ion, obtaining under these conditions limits of detection higher than 0.1 μg/ml and poorer linearity. This could be due to adsorption of counter-ion on the electrode surface. This effect is less important for TEAP. Periodic cleaning of the glassy carbon electrode surface with 3.0 *M* nitric acid removes impurities and regenerates the analytical signal. This electrode pretreatment must be carried out very frequently when TBAP is used, whereas

TABLE I

STATISTICAL TREATMENT OF CALIBRATION GRAPHS AND DETECTION LIMITS (LD) ON CARBON ELECTRODES IN ION-PAIR CHROMATOGRAPHY

Working potential: amperometry, TSA and TMS 0.9 V and DTDBA 1.2 V; coulometry, 0.8 V (signal-to-noise ratio = 3:1). GCE = Glassy carbon electrode; CPE = carbon paste electrode.

Counter-ion	Detection technique	Compound	Sensitivity (μA ml/μg)	LD (μg/ml)	r
TBAP	Amperometry GCE	TSA	225	0.2	0.9967
		TMS	11	2.0	0.9990
		DTDBA	11	2.0	0.9996
	Coulometry	TSA	29.33	0.002	0.9998
		TMS	3.86	0.02	0.9997
		DTDBA	30.76	0.002	0.9993
	Amperometry CPE	TSA	0.049	0.04	0.9987
		TMS	0.022	0.09	0.9994
		DTDBA	0.011	0.2	0.9997
TEAP	Amperometry GCE	TSA	0.017	0.07	0.9994
		TMS	0.002	0.7	0.9950
		DTDBA	0.012	0.4	0.9997
	Coulometry	TSA	29.5	0.004	0.9995
		TMS	29.8	0.006	0.9997
		DTDBA	55.2	0.003	0.9995
	Amperometry CPE	TSA	0.089	0.03	0.9990
		TMS	0.045	0.09	0.9994
		DTDBA	0.023	0.1	0.9993

when TEAP was used the pretreatment was carried out weekly. These problems can be easily solved in amperometric detection using a carbon paste electrode. This electrode presents lower passivation effects, and lower detection limits were obtained without changing the electrode surface (see Table I). It is more advantageous to use TEAP as counter-ion because of its superior electrochemical behaviour. Fig. 3 shows typical chromatograms of mixtures of TSA, TMS and DTDBA obtained with eluents and potential values optimum for simultaneous detection.

In ion-suppressed chromatography, the limits of detection found for coulometric detection mode ranged between 1 and 12 μg/ml, and were only one order of magnitude lower than those obtained in amperometric mode [18], but three orders of magnitude higher than IPC (Table I). From these results it can be deduced that IPC is a more suitable separation method than ISC when electrochemical detection is used.

Replicate samples of the three compounds were injected at approximately 1.0 μg/ml concentration in amperometric detection and 0.1 μg/ml in coulometric detection for IPC, in order to obtain a measure of method reproducibility. For amperometric detection using carbon paste electrodes, the methods exhibited a relative standard deviation ranging between 1.0 and 3.0% when TEAP was used as counter ion. When TBAP was used, higher relative standard deviation values (up to 7.0%) were obtained. In coulometric detection, relative standard deviation values ranging from 0.0 to 4.0% for both counter ions were obtained.

Determination of TMS in ophthalmic solutions

Several soft lens products containing non-degraded 0.001% TMS were assayed using the proposed methods. Contaminants present in the sample did not interfere with the IPC method, but a small decrease in the retention time for TMS was observed when successive sample solutions were injected owing to the presence of

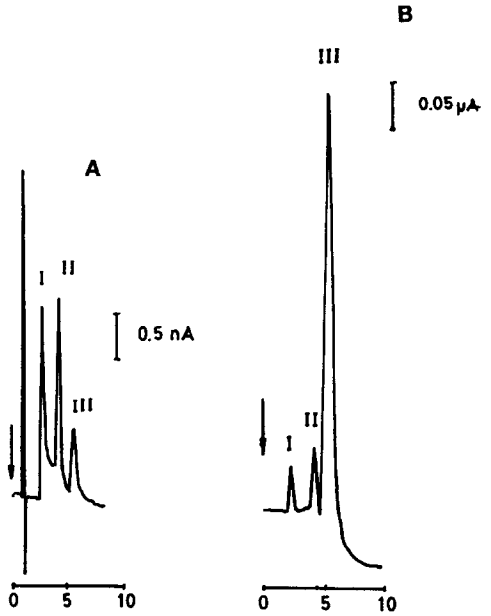

Fig. 3. Chromatogram obtained in ion-pair chromatography. (A) Carbon paste amperometric detection at 1.2 V. Concentrations: TSA (I) 5.05 μg/ml, TMS (II) 6.50 μg/ml and DTDBA (III) 5.00 μg/ml. (B) Coulometric detection at 0.8 V. Concentrations: TSA (I) 1.00 μg/ml, TMS (II) 1.22 μg/ml and DTDBA (III) 1.06 μg/ml. Eluent as Fig. 1.

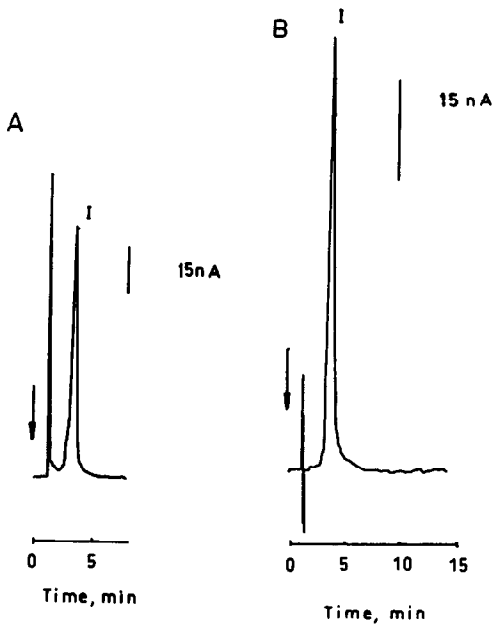

Fig. 4. Chromatogram of a manufactured sample containing 10 μg/ml thimerosal (I) in ion-pair chromatography using carbon paste electrodes and amperometric detection. (A) Mobile phase containing 20 n*M* TEAP as counter-ion concentration without extraction. (B) Mobile phase containing 2 m*M* TEAP, after Sep-Pak extraction.

polymers that can coat the column packing. This small decrease in the retention time of TMS did not affect the peak area evaluation. In ISC this effect was more important [18]. The changes in retention times can be almost removed by increasing the concentration of counter-ion in eluent up to 20 m*M* (Fig. 4A). In order to overcome this problem, a C_{18} Sep-Pak cartridge was used for quantitative extraction of TMS and degradation products. Using this method of separation, retention time and peak shape of TMS do not change and the external standard method can be used for quantitation (Fig. 4B). Recoveries higher than 95% were obtained in the extraction step for TMS, in concentrations ranging from 1.0 to 10 μg/ml.

The results obtained for TMS concentration by IPC methods are compared with those obtained by standard cold-vapour atomic absorption spectrometry (CVAAS) (Table II). The good agreement between these data and the absence of changes in the retention times when the IPC method was used indicate that this method is more useful for the determination of TMS and its degradation products in ophthalmic solutions than other methods described previously. The sensitivity of the coulometric detection mode is sufficient to detect small amounts of TSA and DTDBA in low degraded samples.

TABLE II

THIMEROSAL CONCENTRATION (μg/ml) IN SOFT CONTACT LENS PRODUCTS BY IPC ($n = 3$)

Sample	I	II	III
1	10.7 ± 0.7	10.3 ± 0.3	10.6 ± 0.4
2	10.5 ± 0.4	10.1 ± 0.7	10.7 ± 0.7
3	9.3 ± 0.6	10.1 ± 0.6	10.7 ± 0.3

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