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FORMULATION FACTORS AFFECTING THIMEROSAL STABILITY

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ABSTRACT

A high-performance liquid chromatographic (HPLC) assay has been developed for thimerosal and some of its degradation products: thiosalicylic and 2,2'-dithiosalicylic acids. Using this method, the influence of formulation factors as: isotonic agent, initial concentration, addition of tromethamine, pH and container material, over thimerosal stability was studied.

INTRODUCTION

Thimerosal, the sodium salt of (2-carboxyphenylthio) ethylmercury, is an organomercurial preservative widely employed in pharmaceutical preparations, but it is mainly used in contact lens solutions. However, it has been reported to be unstable in aqueous solutions[1,2].

The literature reports various analytical procedures for quantifying thimerosal, including polarography[3,4], colorimetry[2], atomic absorption[5] and HPLC with UV[1,6-10] and electrochemical detection[11]. Only the HPLC techniques were specific for thimerosal in presence of its decomposition products[1,11].

The loss of thimerosal from contact lens solutions was previously studied[1,2,9,10,12]. However, these works offer only prompt remaining data of thimerosal and thiosalicylic acid, after a fixed lapse of time.

In the present work, an HPLC method[13] carried out by us was used for the quantification of thimerosal, thiosalicylic acid and 2,2'- dithiosalicylic acid.

Using this method, the effect of formulation factors on the evolution of thimerosal, thiosalicylic acid and 2,2'-dithiosalicylic acid concentrations was studied.

METHODOLOGY

1. Materials and Reagents

The following chemicals were used as received: thimerosal (Acofarma, Tarrasa, Spain), thiosalicylic acid (Aldrich-Chemie, Steinheim, Germany), 2,2'-di[thiosalicylic acid] (Sigma Chemical, St. Louis, USA), propylene glycol (Acofarma, Tarrasa, Spain), mannitol (Acofarma, Tarrasa, Spain), sodium chloride (Acofarma, Tarrasa, Spain), tromethamine base and hydrochloride (Sigma Chemical, St. Louis, USA), o-phosphoric acid (Acofarma, Tarrasa, Spain), nitric acid (Panreac, Barcelona, Spain), hydrochloric acid (Panreac, Barcelona, Spain) and sodium hydroxide (Acofarma, Tarrasa, Spain).

Methanol used was HPLC grade (Panreac, Barcelona, Spain) and the water was freshly distilled.

2. High-Performance Liquid Chromatograph

The HPLC system consisted of a constant-flow pump (Kontron Instruments, type 420), a Rheodyne type 7125 injector equipped with a 20 μL loop, a variable wavelength detector (Kontron Instruments, type 432) and an integrator (Konik Instruments, type DataJet 4600). The column used (Brownlee Labs., Spherisorb RP-18, 5 μm particle size, 21 cm X 4.6 mm ID) was packed with silica particles bonded with octadecylsilane.

3. Chromatographic conditions

A flow rate of 0.6 mL/min for the mobile phase (methanol : water : phosphoric acid (65/35/0.9 v/v) was employed and the variable wavelength detector was set at 222 nm.

Each peak area was computed automatically by the integrator. The elution was carried out in isocratic conditions at ambient temperature.

4. Preparation of Stock and Standard solutions

Stock solutions were prepared by dissolving amounts of each product, accurately weighed, in purified water. The standard solutions were obtained by diluting the stock solutions with water to the concentrations showed in table 1.

5. Preparation of test solutions

Using a crioscopic osmometer (Gonotec, type Osmomat 030), the thimerosal contribution to the osmotic pressure of formulations was calculated to be insignificant. Therefore we decided to add the isotonic agents and the other substances in the amounts showed in tables 2 and 3. These products were placed in their containers and the necessary volume of thimerosal standard solution was added.

pH adjustments were carried out employing 0.001 N NaOH solution for alkaline pH's, and using separately either, 0.001 N HCl or 0.001 N HNO_3 solutions for acidic pH's.

6. Sample assay

The solutions were sampled directly for assay. Determinations were carried out in duplicate.

TABLE 1
Concentration of Stock and Standard Solutions of Thimerosal, Thiosalicylic acid and 2,2'-dithiosalicylic acid

	T (ppm)	TSL (ppm)	DTS (ppm)
Stock sol.	200	9.30	5.00
Standard solutions	100	4.77	2.50
	50	4.51	1.00
	20	2.38	0.50
	10	1.85	0.25
	5	0.96	0.10
		0.88	
		0.44	
		0.22	
		0.14	

Where T: thimerosal, TSL: thiosalicylic acid and DTS: 2,2'-dithiosalicylic acid

RESULTS AND DISCUSSION

1. Analytical studies

To optimize the assay parameters, the effect of methanol concentration on the retention time (R_t) was studied. The retention time values for the three products were substantially affected by the variation of methanol composition in the mobile phase. At high methanol concentrations, thimerosal and thiosalicylic acid gave sharp peaks but very close to each other (almost overlap). This fact indicates that the resolution was inadequate. Lowering methanol below 65 %, the peaks become a little bit broad with longer retention time for 2,2'-dithiosalicylic acid. Therefore, 65 % methanol concentration was selected since it provided sharper peaks and reasonable elution times.

At neutral or alkaline pH's, interferences have occurred for the three peaks because the three substances are ionized, having a high affinity for the eluent. 0.9 % phosphoric acid provides the necessary acidic pH (2.4 units) to avoid interferences.

To determine the linearity of the detector response, calibration standard solutions of thimerosal, thiosalicylic acid and 2,2'-dithiosalicylic acid were prepared as described previously. Since thiosalicylic acid quickly undergoes a dimerization phenomenon to 2,2'-dithiosalicylic acid in solution, the amount of 2,2'-dithiosalicylic acid found in the

TABLE 2
Composition and Container Material of solutions 1 to 16

Sol.	T	ClNa	Man.	P.G.	Tr(B)	Ct
1	20	0.9				G
2	200	0.9				G
3	20		5.07			G
4	200		5.07			G
5	20			2.0		G
6	200			2.0		G
7	20					G
8	200					G
9	20	0.9			0.067	G
10	200	0.9			0.067	G
11	20		4.97		0.067	G
12	200		4.97		0.067	G
13	20			1.96	0.067	G
14	200			1.96	0.067	G
15	20				0.067	G
16	200				0.067	G

Where T: thimerosal (ppm), Man.: mannitol (% w/v), P.G.: propylene glycol (% w/v), Tr(B): tromethamine base (% w/v), Ct: container material, G: Glass.

standard solutions of thiosalicylic acid has been calculated, and the concentration of thiosalicylic acid in these solutions has been corrected.

A plot of peaks areas versus concentrations was linear in the ranges of 5 - 200, 0.15 - 10 and 0.1 - 5 ppm of thimerosal, thiosalicylic acid and 2,2'-dithiosalicylic acid, respectively. Regression analysis of the calibration curves gave the statistical parameters showed in tables 4, 5 and 6.

The specificity of the method is illustrated in Fig. 1 where a complete separation was noticed for thimerosal, thiosalicylic acid, and 2,2'-dithiosalicylic acid in a test solution. The retention times for thiosalicylic acid, thimerosal and 2,2'-dithiosalicylic acid were 5.8, 7.6 and 9.8 min., respectively.

TABLE 3
Composition, Container Material and pH Adjustment of solutions 17 to 37

Sol.	T	ClNa	Tr(B)	Tr(H)	Ct	pH adj
17	20	0.9	0.007	0.07	G	
18	20		0.007	0.07	G	
19	20	0.9			PE	
20	20	0.9	0.007	0.07	PE	
21	20		0.007	0.07	PE	
22	20				PE	
23	20	0.9			PP	
24	20	0.9	0.007	0.07	PP	
25	20		0.007	0.07	PP	
26	20				PP	
27	20				G	4 H
28	20				G	4 N
29	20				G	5.5 H
30	20				G	7 S
31	20				G	8.5 S
32	20				G	10 S
33	20	0.9			G	4 H
34	20	0.9			G	5.5 H
35	20	0.9			G	7 S
36	20	0.9			G	8.5 S
37	20	0.9			G	10 S

Where T: thimerosal (ppm), Tr(B): tromethamine base (% w/v), Tr(H): tromethamine hydrochloride (% w/v), Ct: container material, G: glass, PE: polyethylene, PP: polypropylene, pH adj: pH adjustment (H: hydrochloric acid, N: nitric acid and S: sodium hydroxide).

TABLE 4
Calibration Curve data of Thimerosal

Coeff. of Determ : 0.9999			Estimated constant term : -2.4E-4		
Multiple corr. Coeff: 0.9999			Standard Err. of Estimate: 0.0088		
Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
Regress.	1	27.9115	27.9115	351757	0.0000
Residuals	10	7.934E-4	7.934E-5		
Total	11	27.9123			
Regression Coeff.		Stand. Coeff.	Stand. Err.	T	Prob.
221.985		0.9999	0.3742	593.091	0.0000

TABLE 5
Calibration Curve data of Thiosalicylic acid

Coeff. of Determ : 0.9990			Estimated constant term :-0.0085		
Multiple corr. Coeff: 0.9995			Standard Err. of Estimate: 0.0064		
Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
Regress.	1	0.80391	0.80391	19855.6	0.0000
Residuals	20	8.097E-4	4.048E-5		
Total	21	0.80472			
Regression Coeff.		Stand. Coeff.	Stand. Err.	T	Prob.
0.07096		0.9995	5.0362E-4	140.910	0.0000

TABLE 6
Calibration Curve data of 2,2'-dithiosalicylic acid

Coeff. of Determ : 0.9994			Estimated constant term :-6.61E-4		
Multiple corr. Coeff: 0.9997			Standard Err. of Estimate : 0.0021		
Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
Regress.	1	0.07401	0.07401	16879.1	0.0000
Residuals	10	4.384E-5	4.384E-6		
Total	11	0.07405			
Regression Coeff.		Stand. Coeff.	Stand. Err.	T	Prob.
0.04534		0.9997	3.4897E-4	129.920	0.0000

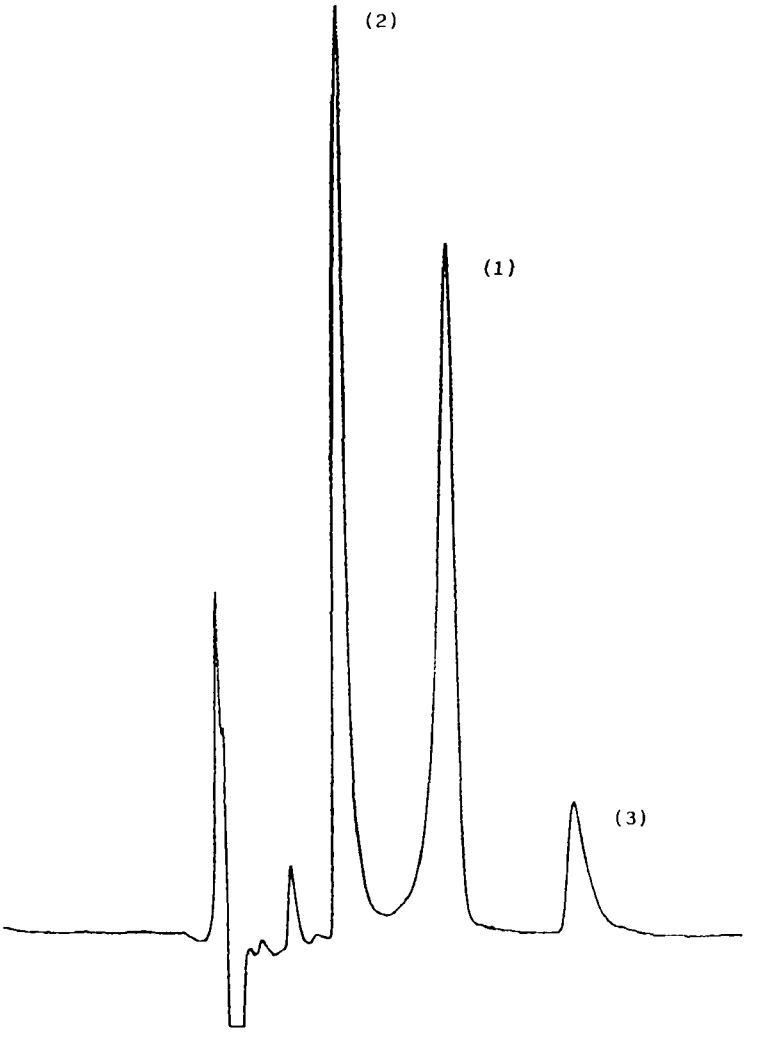

FIGURE 1

Chromatogram of a test solution, showing the peaks corresponding to thimerosal (1), thiosalicylic acid (2) and 2,2'-dithiosalicylic acid (3).

To asses the reproducibility of this method, four replicate samples from a solution containing thimerosal (20 ppm) were analyzed on each of five different days. Two of these samples were injected at the begin, and the other ones at the end of the workday. Table 7 shows the most important parameters of this statistical analysis.

In all cases, satisfactory recoveries with small coefficients of variation are given as indication of the high reproducibility and accuracy.

TABLE 7
Analytical Method Validation Data

Source	Variance	D. F.	C. V.
Apparatus	42.01	10	1.44
Within-day	25.74	5	1.13
Between days	60.29	4	1.72
Mean area: 450.72 (n = 20)			

2. Influence of formulation factors on the stability of thimerosal

As we have found some contradictions and several hypothesis about the influence of formulation factors over thimerosal stability, we have carried out a wide study, using 37 test solutions. The composition and the most important characteristics of these solutions are shown in tables 2 and 3.

All solutions were placed in darkness to prevent photolytic processes interferences; they were sampled and analyzed at previously fixed times: 4, 24, 44, 91, 189, 378, 766, 1531 and 3056 h. For several solutions, the first analysis was carried out as soon as possible to know the degradation process at first moments.

Also, we have analyzed the initial and the final pH's (3060 h.) for each solution (see table 8). pH values of these solutions had not important changes. However, solutions without tromethamine showed a little rise of its pH values. This fact can be attributed to hydrolytic reactions: thimerosal is the sodium salt of a weak acid.

For the solutions containing tromethamine, pH variation has not occurred because this compound has buffer characteristics.

2.1. Influence of isotonic agents on the stability of thimerosal

To asses this influence, we have carried out a three ways ANOVA based on the thimerosal concentration values of solutions 1 to 16. Qualitative variables were: initial concentration (low, high), tromethamine addition (+, -) and isotonic agent. This later included solutions isotonized with sodium chloride, mannitol, propylene glycol and solutions without isotonic agent. Principal parameters of this ANOVA are showed in table 9.

This analysis shows that there is influence, with statistical significance, considering type of isotonic agent over thimerosal stability.

Solutions containing sodium chloride, underwent the greatest degradation. As shows Fig. 2, this degradation occurred mainly at first. For this reason, we have made new test solutions with similar composition and we have analyzed these solutions at times 2, 4, 6 and 8 minutes. So, we have established that initial decrease in thimerosal concentration in presence of sodium chloride is almost instantaneous.

TABLE 8
Initial (I) and Final (F) pH Values

Sol.	pH(I)	pH(F)	Sol.	pH(I)	pH(F)	Sol.	pH(I)	pH(F)
1	7.0	7.5	14	9.5	9.7	27	4.1	4.3
2	7.0	7.4	15	9.7	9.8	28	4.0	4.3
3	6.2	7.4	16	9.5	9.8	29	5.6	5.6
4	6.1	6.8	17	7.4	7.3	30	7.0	6.9
5	6.2	7.5	18	7.0	7.0	31	8.6	8.7
6	6.2	7.0	19	7.2	6.7	32	10.0	9.9
7	6.1	7.6	20	7.4	7.3	33	4.2	4.5
8	6.7	6.9	21	7.0	7.0	34	5.5	5.7
9	10.4	10.1	22	5.6	5.7	35	7.2	6.7
10	10.4	10.1	23	7.3	6.6	36	8.9	9.0
11	9.9	9.6	24	7.5	7.3	37	10.0	9.9
12	9.5	9.7	25	7.0	7.0			
13	9.7	9.7	26	5.6	5.6			

TABLE 9
Influence of Formulation Factors. Multifactorial Analysis

Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
A	1	7741.71	7741.71	99.2037	0.0000
B	3	20606.7	6868.91	88.0195	0.0000
C	1	1504.04	1504.04	19.2731	0.0000
AB	3	2903.47	967.82	12.4019	0.0000
AC	1	0.02958	0.02958	3.79E-4	0.9845
BC	3	3011.31	1003.77	12.8625	0.0000
ABC	3	26.0809	8.69362	0.11140	0.9533
GLOBAL	15	35793.3	2386.22	30.5775	
RESID.	128	9988.93	78.0385		
TOTAL	143	45782.3			

Where A: Concentration, B: Isotonic agent, C: Tromethamine addition.

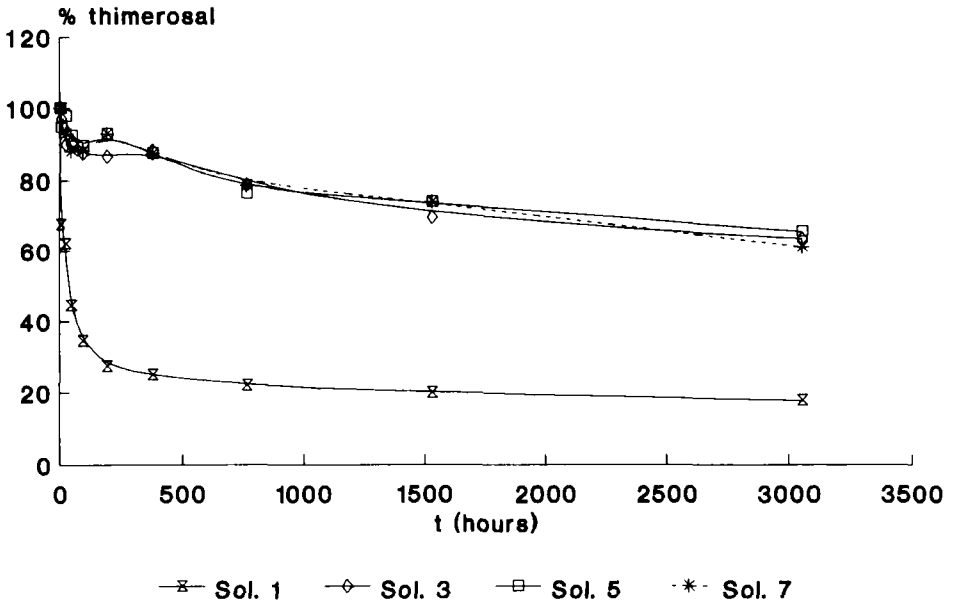

FIGURE 2

Influence of isotonic agent over stability of thimerosal. (Isotonic agents were NaCl (sol. 1), mannitol (sol. 3), propylene glycol (sol. 5) and sol. 7 was prepared without isotonic agent).

Since we have observed a great similarity between the different thimerosal concentration profiles of solutions without sodium chloride, we have decided to make another ANOVA, excluding solutions which contain sodium chloride (see table 10). Thus, we have established that there is not difference, with statistical significance in thimerosal stability, using as isotonic agent mannitol, propylene glycol or without isotonic agent.

In a previous work[12], READER has employed alternative isotonic agents and he has found that thimerosal undergoes smaller degradation using these substances. However, he has only reported prompt data without employed statistical analysis to asses the influence of isotonic agents on the stability of thimerosal.

The effect of sodium chloride can not be only attributed to ionic strength because a test solution (sol. 36) containing only thimerosal and hydrochloric acid (pH = 4), underwent a very greater degradation than a similar solution (sol. 37) containing nitric acid (pH = 4) but not hydrochloric acid. This fact shows the influence of chloride anions over thimerosal stability. So, we confirm experimentally the suspects of other authors[1,8,12]

2.2. Influence of initial concentration

Initial concentration was included in 3-ways ANOVA mentioned above (see tables 9 and 10) with two levels: high (200 ppm) and low (20 ppm).

TABLE 10
Influence of Formulation Factors. Multifactorial Analysis (excluding solutions containing sodium chloride)

Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
A	1	2428.66	2428.66	49.6560	0.0000
B	2	153.149	76.5746	1.56563	0.2142
C	1	40.5095	40.5095	0.82825	0.3651
AB	2	5.25669	2.62834	0.05374	0.9477
AC	1	3.24480	3.24480	0.06634	0.7973
BC	2	47.2708	23.6354	0.48325	0.6183
ABC	2	10.8664	5.43318	0.11109	0.8950
GLOBAL	11	2688.96	244.451	4.99799	
RESID.	96	4695.34	48.9097		
TOTAL	107	7384.30			

Where A: Concentration, B: Isotonic agent, C: Tromethamine addition.

Both analysis show that initial concentration has influence, with statistical significance, over degradation rate of thimerosal in solution. Therefore, we are in agreement with READER et al.[1]. Fig. 3 shows the influence of initial concentration over thimerosal stability in solution containing sodium chloride.

2.3. Influence of tromethamine addition

In a previous work[14], DOULAKAS affirms that tromethamine increases thimerosal stability in aqueous solution, and it can be used as protective in thimerosal formulations. So, we have included in our study solutions containing tromethamine base (sols 9 to 16, pH ≃ 10), and a tromethamine base : tromethamine hydrochloride mixture (sols. 17, 18, 20, 21, 24, 25; pH ≃ 7).

Four analysis of variance were carried out separately for the two formulations. Two of these including solutions containing sodium chloride, which principal parameters are shown in tables 9 and 11, and two excluding them, which most important parameters are shown in tables 10 and 12.

These ANOVA show that tromethamine (base or hydrochloride), increases thimerosal stability in solutions containing sodium chloride; this influence is shown in Fig. 4. Tromethamine effect was similar when we added tromethamine base or a tromethamine base : hydrochloride mixture. However, considering the solutions without sodium chloride, tromethamine addition has no protective effect with statistical significance.

Therefore, we have shown that tromethamine addition decreases the degradation rate of thimerosal in aqueous solution. In this aspect, we are in agreement with DOULAKAS[14].

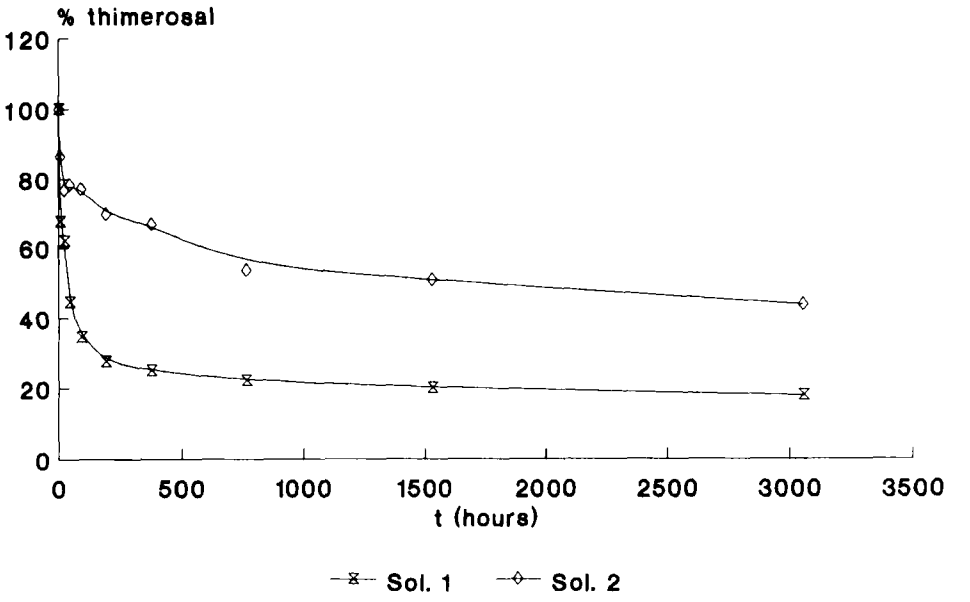

FIGURE 3

Influence of initial concentration over stability of thimerosal. (sol. 1: 20 ppm, sol. 2: 200 ppm).

TABLE 11
Influence of Formulation Factors. Multifactorial Analysis

Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
A	2	306.034	153.017	5.55401	0.0068
B	1	188.160	188.160	6.82959	0.0119
AB	2	2.12333	1.06167	0.03853	0.9622
GLOBAL	5	496.317	99.2635	3.60294	
RESID.	48	1322.43	27.5507		
TOTAL	53	1818.75			

Where A: Container, B: Tromethamine addition.

TABLE 12

Influence of Formulation Factors. Multifactorial Analysis (excluding solutions containing sodium chloride)

Source	D. F.	Sum. of Squares	Mean of Squares	F	Prob.
A	2	164.267	82.1335	0.50404	0.6072
B	1	3608.04	3608.04	22.1418	0.0000
AB	2	252.489	126.245	0.77473	0.4665
GLOBAL	5	4024.79	804.959	4.93985	
RESID.	48	7821.68	162.952		
TOTAL	53	11846.5			

Where A: Container, B: Tromethamine addition.

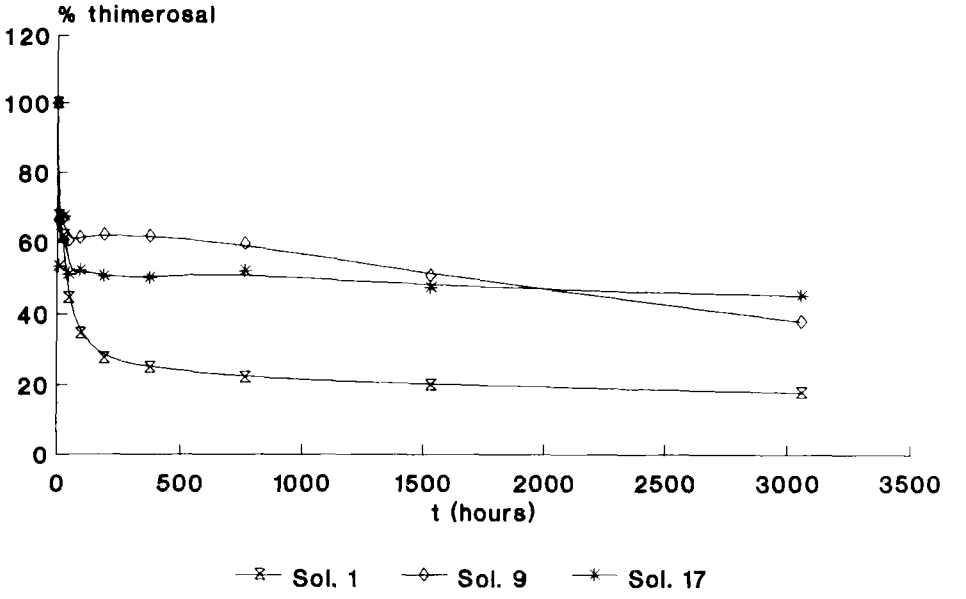

FIGURE 4

Influence of tromethamine addition over stability of thimerosal. (Sol. 1: without tromethamine, sol. 9: tromethamine base, sol. 17: tromethamine base and tromethamine hydrochloride).

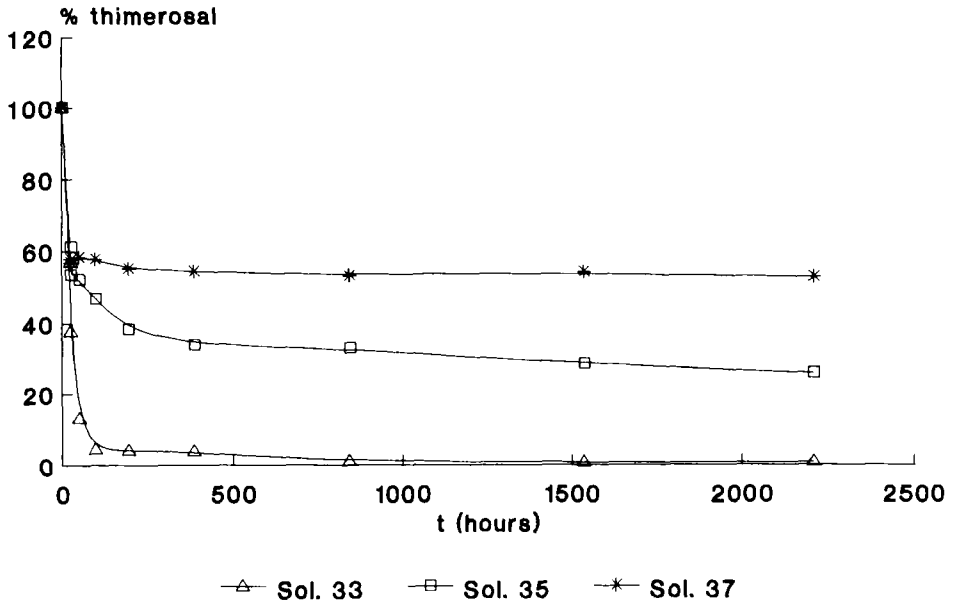

FIGURE 5

Influence of pH over stability of thimerosal. (Sol. 33: pH 4, sol. 35: pH 7, sol. 37: pH 10).

However, we have also found that tromethamine has two great handicaps to be used as thimerosal protective: thimerosal degradation is very high in solutions containing sodium chloride (as shows Fig. 3), even using tromethamine. The second problem is that in solutions without sodium chloride, tromethamine has no protective effect over thimerosal.

2.4. Influence of pH

To asses the influence of pH over thimerosal stability, we have carried out a study at five different pH values. We have employed solutions with and without sodium chloride (sols. 27 to 37). Their composition was shown in table 3.

For acidic solutions, pH adjustment was made separately using either, hydrochloric or nitric acid, to examine the anion chloride effect (this aspect has been discussed in section 2.1).

pH has shown a marked influence over stability of thimerosal in solutions with and without sodium chloride[15]. We have found that thimerosal undergoes greater degradation in acidic medium, than in neutral or alkaline pH's, as shows Fig. 5.

2.5. Influence of plastic container

As we have indicated previously, several investigators[1,2,9,16] affirm that the type of material used to contain thimerosal solutions, affects its stability.

RICHARDSON et al.[2,16], attribute the loss of thimerosal from solutions to permeation phenomena through the plastic containers. Since thimerosal, thiosalicylic acid and 2,2'-dithiosalicylic acid have high melting points, they think that ethylmercury hydroxide will go through the plastic container walls, because it is the only compound sufficiently volatile to be lost from the container.

READER et al.[1], assert that in glass containers, there is reasonably good mass balance between thimerosal decomposed and thiosalicylic acid formed. They do not quantify in their experiments the 2,2'-dithiosalicylic acid formed.

However, in plastic containers, they did not find a good correlation between thimerosal and thiosalicylic acid. They think that this fact is due to some sorption on the surface of plastic containers. Because of these theories, we have decided to study the influence of container material over the stability of thimerosal solutions.

The types of plastic generally used for thimerosal solutions are polyethylene and polypropylene, which have the best characteristics of flexibility, permeability, etc.. So, we have elaborated four different thimerosal solutions and they were stored in polyethylene, polypropylene and glass containers in darkness (solutions 1, 7 and 17 to 26; see table 2). Solutions were assayed using the procedures outlined above. Replicate assays were determined for each solution and the results are shown in Figure 6.

In this Figure, we can observe how solutions stored in plastic containers have a similar behavior, but different to those stored in glass containers.

Formulations containing sodium chloride without tromethamine undergo a bigger degradation when they are stored in plastic containers than in glass containers. Therefore, in relation to these solutions, we are in agreement with the authors mentioned above[1,2,9,10,16].

Solutions containing sodium chloride and tromethamine undergo a very similar behavior when they were stored in the three different types of containers.

However, the solutions without sodium chloride (Figure 6) have shown a different behavior: the solutions stored in glass containers underwent a bit greater degradation than those stored in plastic containers. With the obtained results, we are out of keeping with other previous papers[1,2,9,16].

Considering the papers previously published, we want to notice some considerations: the analytic method used by RICHARDSON et al.[2,16] is not suitable to analyze thimerosal in presence of its decomposition products due to this method is sensitive to all mercury-containing species present in the solution[1]. In relation with READER et al. study[1], they used two test solutions, offering only prompt data. Moreover, they can not quantify 2,2'-dithiosalicylic acid concentration in solution and, therefore, they can not exactly calculate the mass balance of thimerosal, thiosalicylic acid and 2,2'-dithiosalicylic acid.

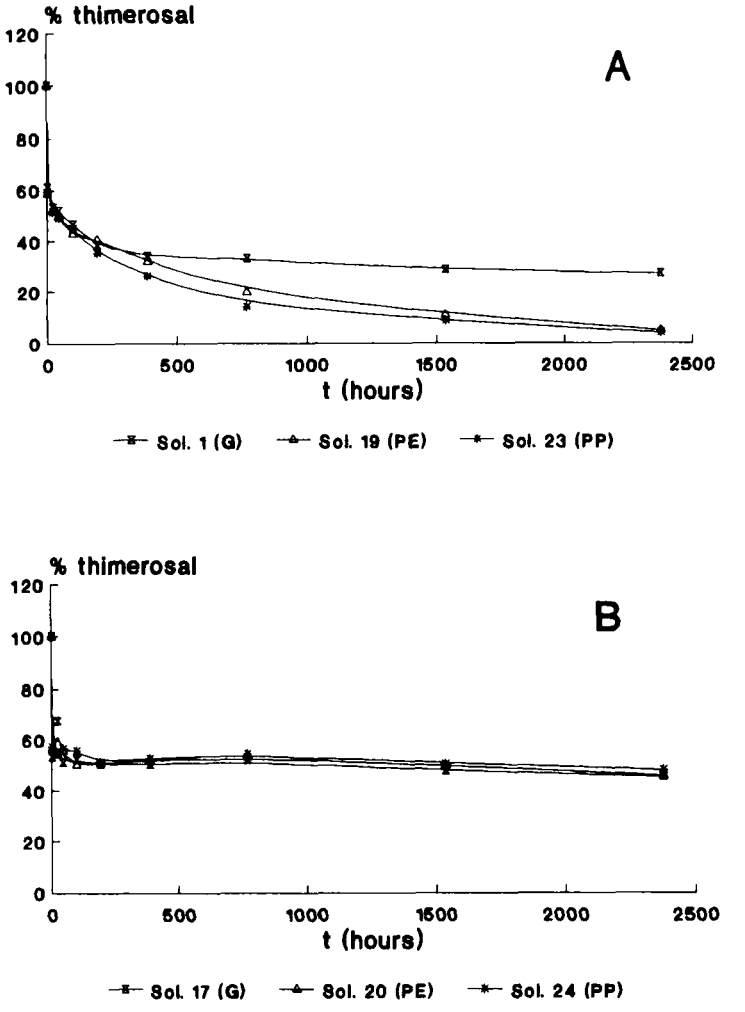

FIGURE 6

Influence of container material over stability of thimerosal. (G: glass container, PE: polyethylene container, PP: polypropylene container, A: solutions containing NaCl, B: solutions containing NaCl and tromethamine (base and hydrochloride), C: solutions containing tromethamine (base and hydrochloride) but not NaCl, D: solutions containing neither NaCl nor tromethamine).

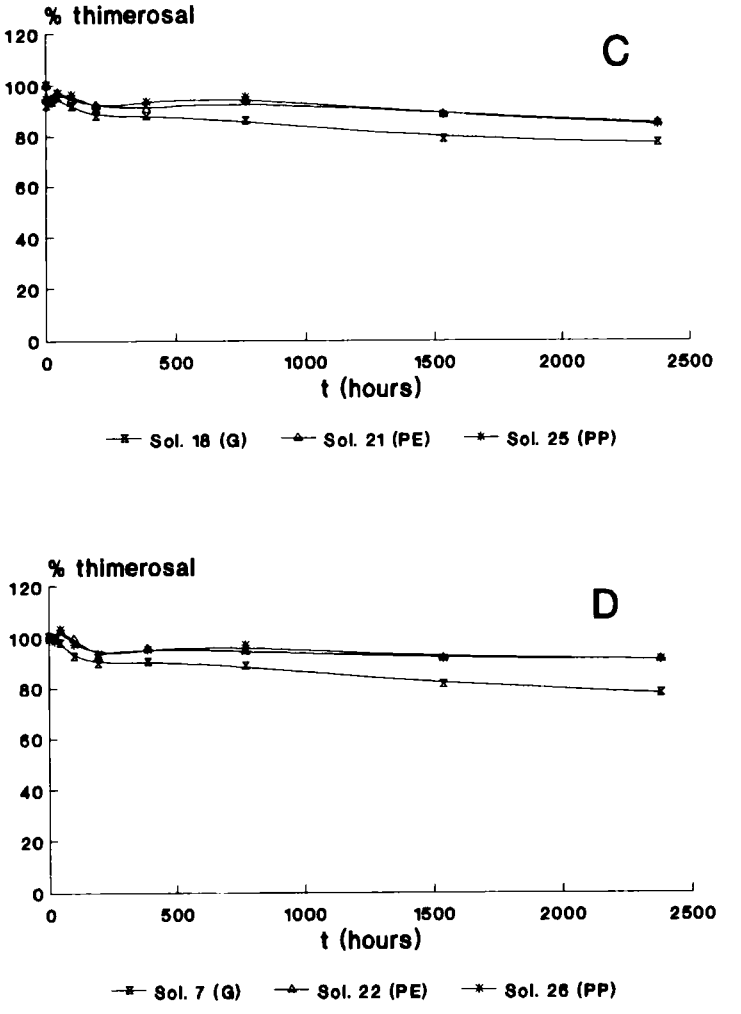

FIGURE 6 Continued

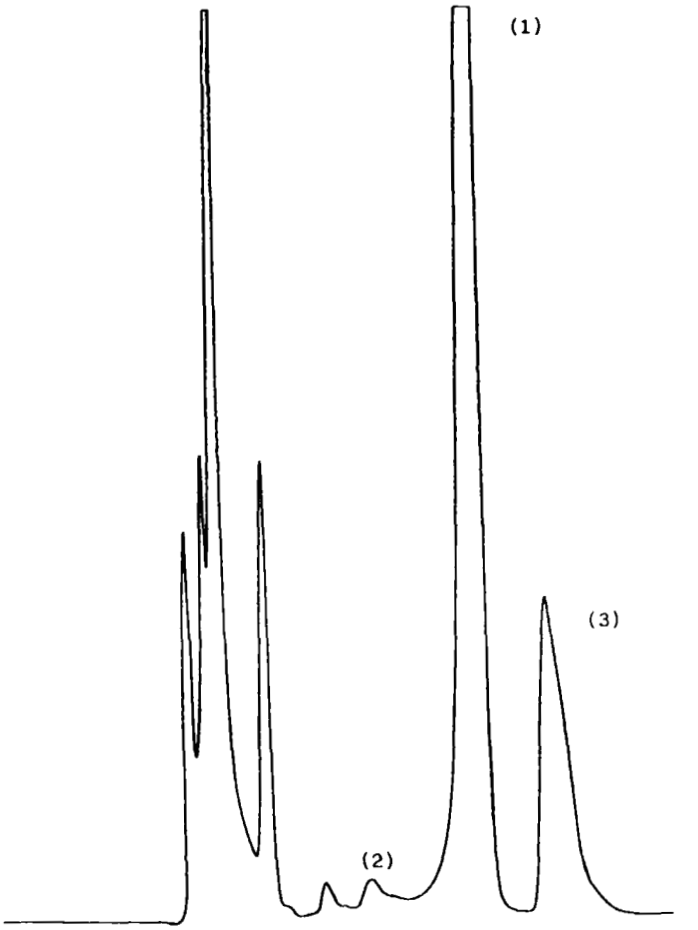

FIGURE 7

Chromatogram of a degraded thimerosal solution. The peaks corresponding to the new decomposition products appear near the solvent front. (1: thimerosal, 2: thiosalicylic acid, 3: 2,2'-dithiosalicylic acid).

We want to note that in the last stages of our study (when the thimerosal degradation has been high), we have not obtained a good mass balance between the substances mentioned above. We think that this fact is due to the presence of new decomposition products which, at the moment, we have not identify. These new products appear near the solvent front of the obtained chromatograms corresponding to the most degraded solutions (Figure 7).

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