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Reversed-phase high-performance liquid chromatographic study of thimerosal stability in Cuban recombinant hepatitis B vaccine

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Abstract

Non-degraded thimerosal was determined in the presence of its decomposition products by directly assaying recombinant hepatitis B vaccine using a reversed-phase liquid chromatographic method. Methanol–water–orthophosphoric acid (65:35:0.9, v/v/v) was used as the eluent. Salicylic acid was employed as an internal standard. The calibration graph was linear ($r = 0.9995$) up to 2.5 μg of thimerosal. Interference from aluminium hydroxide was eliminated by centrifugation. Good stability of thimerosal in the hepatitis B vaccine was demonstrated. The results obtained were in agreement with the recently proposed mechanism of degradation.

Keywords: Vaccines; Thimerosal; Salicylic acid; Thiosalicylic acids

1. Introduction

Thimerosal has been used as a preservative in parenteral vaccines for decades. However, it displays a complex chemistry of degradation in aqueous solutions [1,2], which has not been studied in vaccines. The importance of the problem is related to the formation of unknown compounds at advanced stages of degradation [2], which could produce adverse reactions in humans exposed to a drug containing the degraded thimerosal. Another major application of thimerosal is in ophthalmic solutions, where it presents stability problems [1,3]. Many attempts to develop an effective method for investigating the loss of thimerosal from these preparations with storage have been conducted in recent years because common analytical procedures, such as spectrophotometry, polarimetry and atomic absorption spectrometry are not specific for this purpose.

Among the numerous HPLC procedures [4–17] developed for the determination of inorganic and organic mercury-containing compounds, none has proved to be useful in practice for studying thimerosal degradation. The techniques using inductively coupled plasma (ICP) mass spectrometry [4], atomic absorption spectrometry [5,6] and ICP atomic emission spectrometry [7] for detection have the drawback of expensive equipment. The electrochemical detection of mercury compounds after on-column derivatization with mercaptoethanol have been reported [8–10]. This detection mode is specific for study-

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ing thimerosal degradation [18]. Another approach is the use of UV–visible detection of mercury-containing compounds based on their off- or on-column derivatization with thiol-containing organic substances [11–17]. However, these derivatizing agents (e.g., dithiocarbamate) can easily displace the bound ethylmercury group from the undecomposed thimerosal, forming a more stable complex than the thiosalicylate group [16,17]. Hence, the measured ethylmercury content may not represent the true degree of degradation of thimerosal in the original sample.

HPLC techniques [2,19,20] allow the determination of thimerosal in presence of its non-mercury-containing degradation products, thiosalicylic and 2,2′-dithiosalicylic acids. Owing to the specificity for non-degraded thimerosal, the methods have been of great practical value for studying the kinetics of preservative degradation in ophthalmic solutions. Using this approach, the influence of container material [1], isotonic agents [21] and formulation factors [22,23] on thimerosal stability in these preparations has been clarified. Introduced originally by Reader and Lines [1] and improved by Rabasco and Caraballo [2], the method with modifications has been extended here to a more complex pharmaceutical formulation such as a vaccine. Its application to recombinant hepatitis B vaccine showed the good stability and safety of using thimerosal as a preservative. The results were in agreement with the proposed mechanism of degradation [23].

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Pharmacia Model 2248 pump, an LKB valve injector equipped with a 200-μl loop, a Shandon Hypersil C_{18} column (210 × 4.6 mm I.D., 5 μm), a Pharmacia Model 2141 variable-wavelength UV detector operated at 222 nm and a Pharmacia Model 2221 integrator. A 5 × 4 mm I.D. guard column packed with 5-μm Hypersil C_{18} particles was placed in front of the analytical column. A Jouan M 14-11 centrifuge was used for sample preparation.

2.2. Reagents

The chemicals used were thimerosal, 2,2′-dithiosalicylic acid (Sigma, St. Louis, MO, USA), thiosalicylic acid (Aldrich-Chemie, Steinheim, Germany), salicylic acid (BDH, Poole, UK), 85% orthophosphoric acid (Fluka, Buchs, Switzerland) and methanol (HPLC grade) (Merck, Darmstadt, Germany). The water used was purified with a Milli-Q system (Millipore, Milford, MA, USA).

The recombinant hepatitis B vaccine and the placebos were obtained from the Center for Genetic Engineering and Biotechnology (Havana, Cuba). The mobile phase was methanol–water–orthophosphoric acid (65:35:0.9, v/v/v) of pH 2.5. This solution was filtered through a 0.45-μm membrane filter and degassed ultrasonically before use. The internal standard stock solution contained salicylic acid dissolved in the mobile phase–water (1:4) at a concentration of 2.0 mg ml^{-1}. This solution was stored in a glass bottle at 4°C. Thimerosal stock standard solution contained 100 μg ml^{-1} of thimerosal in water and was prepared daily. Working standard solutions of lower concentration were prepared by appropriate dilution with water at the time of injection. Each one contained the internal standard at a concentration of 40 μg ml^{-1}. Stock standard solutions of thiosalicylic and dithiosalicylic acids contained 40 μg ml^{-1} of the compound and were prepared in the same manner as for thimerosal. Their working standard solutions contained the internal standard at a concentration of 15 μg ml^{-1}.

2.3. Sample preparation

Working standard solutions were briefly vortexed, centrifuged for 3 min at 12 000 *g* and 25 μl of the supernatant were injected.

The vaccine containing the internal standard at a concentration of 40 μg ml^{-1} was centrifuged for 15 min at 3400 *g* and 25 μl of the supernatant were injected. Determinations were carried out

in duplicate. Samples were stored at 4°C. The conversion of peak areas to concentrations was carried out using programmable integration.

The percentage area method was used in the same LC system but without the internal standard. Peak areas were previously normalized using detector response factors for thimerosal, thiosalicylic and dithiosalicylic acids. Quantification was carried out using programmable integration.

2.4. Adsorption test

The placebos were placed in glass containers and the necessary volume of 100 mg ml^{-1} thimerosal solution was added to give concentrations of 50, 70, 1000 and 2500 $\mu g\ ml^{-1}$, respectively. Three replicate samples were prepared for each concentration. All manipulations were carried out under aseptic conditions. The samples were sealed and stored in the dark at 4°C for 1, 2 and 3 months. After centrifugation for 15 min at 3400 *g*, the thimerosal concentration in supernatant was determined spectrophotometrically [24,25].

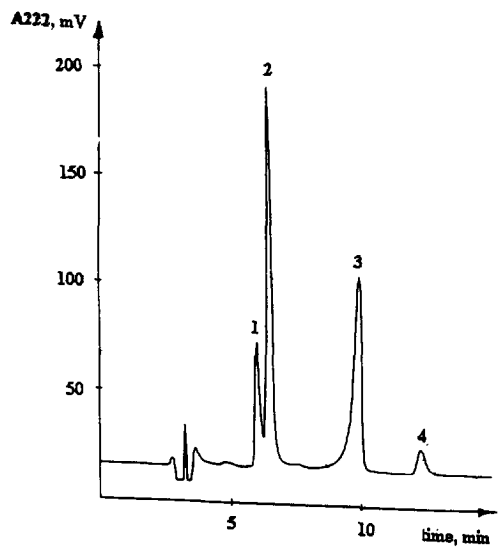

Fig. 1. Chromatogram of the vaccine supernatant containing the internal standard. Conditions: Shandon Hypersil C_{18} column (210 × 4.6 mm I.D., 5 μm); eluent, methanol–water–orthophosphoric acid (65:35:0.9, v/v/v) (pH 2.5); flow-rate, 0.6 ml min^{-1}; detection, UV at 222 nm; injection volume, 25 μl. Initial thimerosal concentration, 63 $\mu g\ ml^{-1}$; concentration of non-degraded thimerosal, 41.9 $\mu g\ ml^{-1}$. Peaks: 1 = thiosalicylic acid; 2 = salicylic acid; 3 = thimerosal; 4 = dithiosalicylic acid.

3. Results and discussion

The method described by Rabasco and Caraballo [2] has been crucial for studying thimerosal degradation and it was modified here to be applied to the vaccine. The use of a Hypersil C_{18} column instead of Spherisorb RP-18 [2] led to a considerable increase in the resolution of the separation of thimerosal from thiosalicylic acid. This allowed us to include salicylic acid as an internal standard (Fig. 1). The retention times were 6.0, 6.5, 10.1 and 12.4 min for thiosalicylic acid, salicylic acid, thimerosal and dithiosalicylic acid, respectively.

Injection of thimerosal into the described LC system resulted in a thimerosal peak with slight forward tailing (Figs. 1 and 2). The same phenomenon was reported previously [2,23]. The forward tailing could be explained by some dissociation of thimerosal into thiosalicylic acid and ethylmercury(II) chloride during the elution

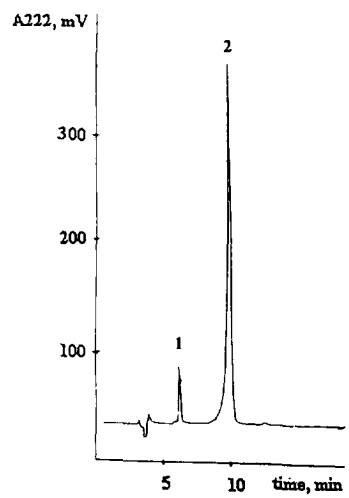

Fig. 2. Chromatogram of the fresh stock standard solution containing 100 $\mu g\ ml^{-1}$ of thimerosal. Conditions as in Fig. 1. Peaks: 1 = thiosalicylic acid; 2 = thimerosal.

at an acidic pH of the mobile phase, because the retention of thimerosal is higher than that of its degraded form. The addition of an excess of thiosalicylic acid to the sample solution should prevent the dissociation by displacing the equilibrium in Eq. 1 to the left [26]. However, this did not affect the peak shape (data not shown).

$$Na^+ {}^-OOC\text{–}C_6H_4\text{–}S{:}HgCH_2CH_3 \rightleftharpoons Na^+ {}^-OOC\text{–}C_6H_4\text{–}S^- + CH_3CH_2Hg^+ \quad (1)$$

The forward-tailing was increased in samples with high levels of degradation (Fig. 3). We did not use the method for the accurate determination of thimerosal in such samples. We believe that this phenomenon did not influence the analytical results owing to the use of calibration, which was linear and reproducible in the analysed range.

3.1. Calibration

The chromatogram of the freshly prepared stock solution of thimerosal already showed the peak corresponding to thiosalicylic acid (Fig. 2). This is due to establishing the equilibrium in Eq. 1. Since the dissociation depends on the initial thimerosal concentration, each working standard solution was previously analysed without internal standard and the percentage of undecomposed thimerosal was determined by the percentage area method. The concentration was corrected and plotted on the calibration graph.

The linearity between peak areas and amounts was corroborated in the ranges 0.01–2.5 μg for thimerosal ($r = 0.9995$), 0.005–1.0 μg for thiosalicylic acid ($r = 0.9990$) and dithiosalicylic acid ($r = 0.9993$).

The reproducibility of the method was tested with three replicate injections of stock solutions of thimerosal, thiosalicylic and dithiosalicylic acids on three different days. The relative standard deviation (R.S.D.) was 1.13–1.35% (within-day) and 1.44–1.87% (between days). The

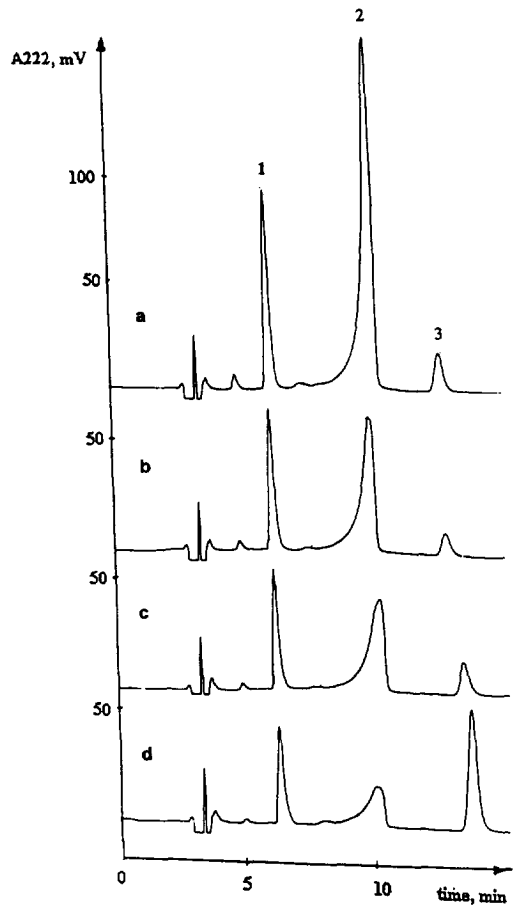

Fig. 3. Thimerosal degradation in the placebos stored (a, b) for 6 months at 4°C in the dark, (c) for 6 months at 28°C in the dark and (d) for 1 week at 28°C in the light. Conditions as in Fig. 1. Initial thimerosal concentration, (a) 70 and (b–d) 30 μg ml^{-1}. Peaks: 1 = thiosalicylic acid; 2 = thimerosal; 3 = dithiosalicylic acid.

R.S.D.s for retention time were 0.40% for thiosalicylic acid, 0.35% for the internal standard, 1.03% for thimerosal and 0.41% for dithiosalicylic acid.

To assess the reproducibility of the percentage area method, five replicate samples from a solution containing 50 μg ml^{-1} of thimerosal, 20 μg ml^{-1} of thiosalicylic acid and 5 μg ml^{-1} of dithiosalicylic acid were analysed on different days. The R.S.D. was 1.55–1.81% (within-day) and 1.64–1.90% (between days).

3.2. Interferences

Since the vaccine is a heterogeneous system consisting of an antigen adsorbed on aluminium hydroxide gel in aqueous solution, thimerosal added as a preservative would be distributed into this two-phase system. However, the adsorption test showed that this does not occur. Placebos containing known amounts of thimerosal were centrifuged after storing in dark at 4°C and the thimerosal concentration in the supernatant was determined by spectrophotometry [24,25]. No adsorption was detected even for the placebos with the highest content of the reagent. The vaccine samples and their respective supernatants obtained by centrifugation were separately assayed by spectrophotometry. Both had the same value of thimerosal concentration. All this led us to conclude that thimerosal and its mercury-containing degradation products were completely found in the aqueous phase. Metallic mercury formed in the second degradation stage should be in the pellet after centrifugation. Its amount was either too small to be detected by spectrophotometry or, more probably, it was not formed at all in the vaccine.

No adsorption of thiosalicylic and dithiosalicylic acids on aluminium hydroxide was demonstrated in the same manner but assaying the centrifuged samples by the described HPLC method (data not shown). This fact was determinant for the stability of thimerosal in the vaccine. If thiosalicylic acid were to be adsorbed on the gel, its removal from the aqueous phase due to adsorption would displace the equilibrium in Eq. 1 to the right, causing rapid degradation of the preservative. The use of the internal standard corrected for the loss of thimerosal and its decomposition products due to their non-specific adsorption on aluminium hydroxide after sample centrifugation.

The lack of adsorption of thimerosal is understandable in view of the structure of aluminium hydroxide. In aqueous solutions, aluminium hydroxide exists primarily as a ring unit joining six aluminium ions by double hydroxide bridges, where each aluminium is in octahedral coordination with water molecules, $Al_6(OH)_{12}(OH_2)_{12}^{6+}$ [27]. It can undergo ionic, hydrogen, hydrophobic, van der Waals or ligand-exchange interactions with substances present in the solution. The nature of the substance and the solvent conditions determine that interaction. In the vaccine, where aluminium hydroxide is in a solution containing 7.9 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, 137 m*M* NaCl and 2.7 m*M* KCl, phosphate anions replace hydroxyl groups or water molecules from the gel surface owing to their having the highest affinity with aluminium hydroxide [28]. Since thimerosal is composed of an anion of a weak organic acid, there is a low possibility of its adsorption on the gel by ionic or ligand-exchange interactions, particularly in the presence of competitive phosphate and chloride anions. On the other hand, it is less probable that thimerosal could undergo hydrophobic interactions with the highly hydrated gel surface. The lack of adsorption of thimerosal and its degradation products on aluminium hydroxide obviously indicates the absence of their interaction with the antigen.

To exclude the possible interference from the unbound protein, all analysed samples were previously checked to determine the adsorbed percentage of the antigen by enzyme-linked immunosorbent assay (ELISA) [24]. Its concentration in the vaccine supernatant was found to be less than 0.1 μg ml^{-1}. The purified antigen injected onto the column did not elute under the described conditions.

3.3. Determination of thimerosal in hepatitis B vaccine

The thimerosal concentration in hepatitis B vaccine is commonly determined by the spectrophotometric determination of mercury ions as dithizone complexes after sample digestion. The method gives the concentration of all mercury-containing compounds present in the sample in both the undegraded and degraded forms. This value was defined here as the initial thimerosal concentration. The described internal standard HPLC method seems to be appropriate for accurately accessing only the non-degraded thimerosal concentration in the vaccine. We

assayed samples from different batches of the vaccine by this method, by the percentage area method and spectrophotometrically. The percentage of non-degraded thimerosal was calculated from the results obtained by the internal standard and spectrophotometric methods. The results are given in Table 1. The percentage area method gave the same values for non-degraded thimerosal as those calculated using the other two methods. We believe that this could be considered as a test of the reliability of both modes for thimerosal determination. The percentage area method was particularly useful for the rapid determination of thimerosal and its degradation products.

3.4. Thimerosal stability in recombinant hepatitis B vaccine

Samples from different batches of the hepatitis B vaccine were analysed to obtain information about the state of thimerosal degradation in the vaccine (Table 1). The samples assayed had different storage times and different initial thimerosal concentrations. Because of this, the concentration of non-degraded thimerosal also showed a batch-to-batch variation. However, it did not depend on storage time. It was surprising that the non-degraded thimerosal content was nearly the same in the freshly prepared vaccine and in that stored for 3 years. The concentration of thiosalicylic acid in the samples assayed ranged from 18 to 27%. The concentration of dithiosalicylic acid mostly varied from 2 to 7%; in rare case it was higher but never more than 10%.

Table 1
Thimerosal concentration in the different batches of hepatitis B vaccine determined by (A) the spectrophotometric method (μg/ml), (B) the HPLC internal standard method (μg/ml), (C) the HPLC percentage area method (%) and (D) calculated as $(C_2/C_1)\cdot 100$ (%), where C_1 is data from (A) and C_2 is data from (B)

Storage time (days)[a]	Thimerosal concentration			
	A	B	C	D
1	41	28.6	70.6	69.8
30	53	35.1	66.5	66.2
60	50	32.1	65.0	64.3
120	51	29.2	58.1	57.3
180	47	28.2	59.2	60.0
240	49	32.1	66.2	65.5
540	63	41.9	66.3	66.8
570	44	27.2	61.6	61.8
600	40	22.0	56.1	55.0
660	57	37.7	67.0	66.1
690	53	35.9	68.9	67.7
720	50	34.1	67.7	68.3
1080	50	33.8	67.6	68.7

[a] Storage time is from the date of vaccine production.

Since the range established for thimerosal concentration in the recombinant hepatitis B vaccine is 30–70 μg ml^{-1} [24], the placebos containing the reagent at concentrations of 30, 50 and 70 μg ml^{-1} were prepared and stored at 4°C. The non-degraded thimerosal and thiosalicylic and dithiosalicylic acid contents were checked periodically by the percentage area method. The initial concentrations were assumed to be 100% for thimerosal and 0% for thiosalicylic and dithiosalicylic acids. The results are given in Table 2. Thimerosal was already found to be partially decomposed in the freshly prepared placebo. The preparations contained thiosalicylic and dithiosalicylic acids, the latter in a small amount. The degradation was higher in the placebo containing 30 μg ml^{-1} of the reagent. The concentrations of thimerosal and its decomposition products varied insignificantly with storage.

The structure of more advanced degradation products is unknown. They have been reported as hydrophilic compounds eluting close to the solvent front in the same LC system [23]. No presence of unknown compounds in the vaccine was detected.

Analysing the results obtained, we conclude that the degradation of thimerosal in the vaccine occurs at the time of addition of the preservative to bulk. A similar initial decrease in thimerosal concentration in solutions containing sodium chloride has been reported [1,2,23]. This fact is in agreement with the proposed degradation mechanism [23]. The degradation consists of two basic stages, the first being the dissociation of thimerosal [1] and the second a redox reaction

Table 2
Simultaneous determination of thimerosal, thiosalicylic acid and dithiosalicylic acid in the placebos by the percentage area method

Storage time (days)	Thimerosal			Thiosalicylic acid			Dithiosalicylic acid		
	P1	P2	P3	P1	P2	P3	P1	P2	P3
0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
1	60.9	72.0	73.4	36.1	25.5	24.6	2.0	2.0	2.0
50	61.4	73.7	74.1	30.2	20.1	20.1	6.1	4.0	4.1
104	66.4	74.7	74.4	24.2	19.5	18.6	8.1	5.3	5.9
226	65.8	71.0	73.6	25.5	19.2	18.8	8.1	7.1	7.1
550	64.9	70.5	73.6	24.6	19.5	18.5	8.8	7.1	6.9
720	64.4	70.5	73.4	24.0	18.5	19.0	9.0	8.0	7.0
1080	64.8	70.0	72.9	22.5	18.0	17.8	9.2	8.0	7.5

Conditions as in Fig. 1. The placebos had initial thimerosal concentrations of 30 μg ml^{-1} (P1), 50 μg ml^{-1} (P2) and 70 μg ml^{-1} (P3) and were stored at 4°C. The zero-time values were assumed to be 100% for thimerosal and 0% for thiosalicylic and dithiosalicylic acids.

involving the oxidation of thiosalicylic acid to produce dithiosalicylic acid with the corresponding reduction of ethylmercury(II) chloride to metallic mercury (Fig. 4). Since the second reaction hinders the reversible process of the first reaction reaching the equilibrium state, this is a step which really determines the degree of degradation [23].

During production, thimerosal is added to the vaccine bulk as a 100 mg ml^{-1} aqueous solution containing 96–99% of undecomposed thimerosal (data not shown). When this solution is added the vaccine medium, the equilibrium in Eq. 1 is immediately displaced to right as a result of a 2000-fold decrease in thimerosal concentration. The high content of sodium chloride in the vaccine medium steadies the dissociated ions (Eq. 1) that hinders the reverse process. Hence, this can be another cause of the displacement of the equilibrium. The equilibrium state reached after addition of thimerosal still leads to a prevalence of its undissociated form. Further reagent degradation depends on conditions which could induce the second stage. This reaction induces rapid thimerosal degradation to yield products of more advanced degradation [23]. Since the concentrations of thimerosal, thiosalicylic and dithiosalicylic acids in the samples assayed varied insignificantly with storage and products of more advanced degradation were not found, we believe that the degradation in the vaccine is stopped at the first stage. Our supposition could be questioned by the presence of dithiosalicylic acid in a small amount in the vaccine. This fact cannot evidence the reduction of ethylmercury(II) chloride to metallic mercury owing to the high tendency of thiosalicylic acid to oxidize to disulphide. If metallic mercury were to be formed, it should be in the pellet after centrifugation. We centrifuged 15 ml of the vaccine, digested the obtained pellet after successive washings with distilled water and assayed the final sample spectrophotometrically. No presence of mercury ions was found.

The concentration of non-degraded thimerosal in the batches assayed was 56–71%, while its

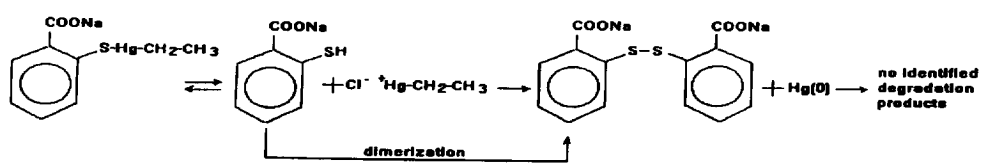

Fig. 4. Reaction scheme of thimerosal degradation.

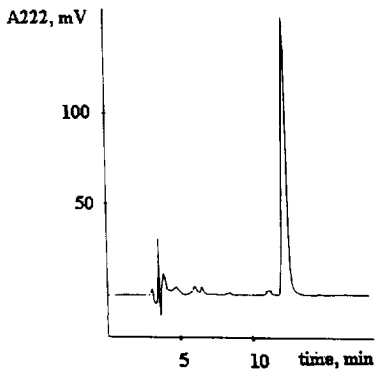

Fig. 5. Complete thimerosal degradation in the microbial contaminated placebo after 1 month of storage at 4°C. Conditions as in Fig. 1. Initial thimerosal concentration, 44 μg ml^{-1}. The peak corresponds to dithiosalicylic acid.

concentration in the contact lens solutions was about 50% in the best case when tromethamine had been added to protect thimerosal from degradation [23]. The pH of the vaccine and the storage conditions probably play an important role in thimerosal stabilization. Ethylmercury(II) chloride, the main degradation product, shows anti-microbial activity, as does thimerosal [2]. Hence, the degradation does not affect the preservative action.

We observed the complete degradation of thimerosal in the microbial contaminated placebo stored for 1 month at 4°C (Fig. 5). The contamination was shown by sterility testing [23]. We therefore suggest that the possible influence of contaminants on thimerosal degradation is taken into account.

4. Conclusions

A simple HPLC procedure for the determination of non-degraded thimerosal in a vaccine was developed. Interference from aluminium hydroxide was avoided by eliminating it by centrifugation. Both the internal standard method and the percentage area method can be used for accurately accessing non-degraded thimerosal using the described LC system. Their application to recombinant hepatitis B vaccine and placebos showed that the thimerosal concentration varied insignificantly after at least 3 years of storage, which demonstrated a good preservative stability in the drug. The non-degraded thimerosal concentration in the batches assayed varied from 56 to 71%. Thimerosal was found to be in equilibrium with thiosalicylic acid and ethylmercury(II) chloride. More advanced degradation of the preservative was not detected. The results obtained corroborated the proposed mechanism of degradation.

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References

[1] M.J. Reader and C.B. Lines, J. Pharm. Sci., 72 (1983) 1406.
[2] A.M. Rabasco and I. Caraballo, presented at the VIII Jornadas Farmaceuticas Españolas, Seville, 3–6 December, 1991.
[3] N.E. Richardson, D.J. Davies, B.J. Meakin and D.A. Norton, J. Pharm. Pharmacol., 29 (1977) 717.
[4] D.S. Bushee, Analyst, 113 (1988) 1167.
[5] W. Holak, J. Liq. Chromatogr., 8 (1985) 563.
[6] W. Holak, Analyst, 107 (1982) 1457.
[7] Y.S. Krull, D.S. Bushee, R.S. Schleicher and S.B. Smith, Analyst, 111 (1986) 345.
[8] W.A. MacCrehan, R.A. Durst and J.M. Bellama, Anal. Lett., 10 (1977) 1175.
[9] O. Evans and G.D. McKee, Analyst, 112 (1987) 983.
[10] O. Evans and G.D. McKee, Analyst, 113 (1988) 243.
[11] W. Langseth, Anal. Chim. Acta, 185 (1986) 249.
[12] S. Inoue, S. Hoshi and M. Mathubara, Talanta, 32 (1985) 44.
[13] J.E. Parkin, J. Chromatogr., 407 (1987) 389.
[14] J.E. Parkin, J. Chromatogr., 472 (1989) 401.
[15] W. Langseth, J. Chromatogr., 438 (1988) 414.
[16] J.E. Parkin, J. Chromatogr., 542 (1991) 137.
[17] Y.-C. Wang and C.-W. Wang, J. Chromatogr., 628 (1993) 133.
[18] C.G. Visor, R.A. Kenley, J.S. Fleitman, D.A. Neu and Y.W. Partridge, Pharm. Res., 2 (1985) 73.
[19] K. Thoma and O.E. Shubert, Dtsch. Apoth.-Ztg., 127 (1987) 1867.
[20] J.S. Fleitman and Y.W. Partridge, Drug Dev. Ind. Pharm., 17 (1991) 519.
[21] M.J. Reader, J. Pharm. Sci., 73 (1984) 840.

[22] A.M. Rabasco, I. Caraballo and M. Fernández-Arévalo, Drug Dev. Ind. Pharm., 19 (1993) 1673.
[23] I. Caraballo, A.M. Rabasco and M. Fernández-Arévalo, Int. J. Pharm., 89 (1993) 213.
[24] Norma Cubana 26-206:1991. Medicamentos. Vacuna Anti-Hepatitis B de Antígeno de Superficie Viral ADN Recombinante. Especificaciones.
[25] N.E. Richardson, D.J. Davies, B.J. Meakin and D.A. Norton, J. Pharm. Pharmacol., 30 (1978) 469.
[26] Cs. Horváth, W. Melander and A. Nahum, J. Chromatogr., 186 (1979) 371.
[27] S.L. Hem and J.L. White, J. Parent. Sci. Technol., 38 (1984) 2.
[28] R.J. Sepelyak, J.R. Feldkamp, T.E. Moody, J.L. White and S.L. Hem, J. Pharm. Sci., 73 (1984) 1514.