ELSEVIER

Journal of Chromatography A, 907 (2001) 173–179

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Reversed-phase high-performance liquid chromatography versus spectrophotometric assay for thimerosal in Cuban recombinant hepatitis B vaccine☆

Lourdes Costa*, Maribel Vega, Yenai Díaz, José Luis Marcelo, Juana M. Hernández, Tamara Martino

Center for Genetic Engineering and Biotechnology, Ave 31 e/158 y 190, P.O. Box 6162, CP 10600 Havana, Cuba

Received 31 May 1999; received in revised form 13 October 2000; accepted 20 October 2000

Abstract

A reversed-phase liquid chromatographic method was applied to study the stability of thimerosal in Cuban recombinant hepatitis B vaccine samples stored under different temperature conditions. Salicylic acid was used as internal standard, it allowed one to determine the thimerosal in the presence of its degradation products. Good stability of the preservative was demonstrated in vaccine samples for as long as 6 years. The same results were obtained when the vaccine samples were incubated at 37 and 45°C during 30 days. The results were in compliance with the microbiological test for determining the effectiveness of antimicrobial preservative in these samples.

Keywords: Pharmaceutical analysis; Stability studies; Thimerosal; Salicylic acid

1. Introduction

Pharmaceutical products are stored in the presence of antimicrobial preservatives in order to avoid microbiological contamination and spoilage that reduces the likelihood of microbial growth in aqueous products. The aim of pharmaceutical product preservation is to ensure that the product is microbiologically safe and stable [1]. The preservative action of a formulation is often considered to be due solely to the preservatives used. However, preservative chemicals do not act independently of the product [2], factors such as pH, water activity, presence of surfactant concentration and the nature of pharmaceutical product may influence the preservative action. On the other hand the use of wrong preservatives may affect the potency of vaccines [3,4]. Therefore, stability studies should include the study of the preservative [5]. Thimerosal has been used as a preservative in pharmaceutical systems for a long time. However the degradation of thimerosal in aqueous solutions leads to inconsistency in the results provided by the methods measuring intact thimerosal [6] and total mercury-containing compounds [7]. Methods for thimerosal analysis include atomic absorption spectroscopy [8,9], polarography [10,11] colorimetry [12], and variety of methods using high-performance liquid chromatography (HPLC) [13–15]. Most of these chromatographic

☆Presented at the 23rd International Symposium on High-Performance Liquid Phase Separations and Related Techniques, Granada, 30 May–4 June 1999.

*Corresponding author.

0021-9673/01/$ – see front matter
PII: S0021-9673(00)01073-6

methods measure intact thimerosal and hence give significantly different results when they are applied to degraded samples compared with methods which measure total mercury such as atomic absorption spectroscopy and colorimetry.

Drug manufacturers are responsible for demonstrating and routinely ensuring that their commercial products maintain their purported identity, potency, purity and security throughout their shelf life [16]. Generally techniques used to assess the stability of vaccines and their ingredients are not standardized. To evaluate the stability of pharmaceutical products it is necessary to establish analytical methods that permit one to detect and quantify any and every chemical degradation [17,18]. Stability-indicating and stability-specific methods are based on analytical techniques with good specificity. Techniques such as UV spectroscopy are usually considered non-specific methods and are not considered for stability assessment. Separation methods such as HPLC, thin-layer chromatography (TLC), gas chromatography (GC) and capillary electrophoresis (CE), are the methods choice for stability-indicating methods [19–21]. Although the reversed-phase (RP) HPLC method was shown to be feasible for the analysis of vaccine samples [22], the concentration of thimerosal is still commonly determined by a non-specific spectrophotometric assay. In this paper we were interested to compare both methods in order to establish the HPLC techniques for the routine thimerosal testing in Cuban recombinant hepatitis B vaccine samples and for monitoring the stability of this preservative in vaccine samples stored under different temperatures in comparison with a preservative efficacy test.

2. Experimental

2.1. Reagents

The chemicals used were thimerosal (Merck, Darmstadt, Germany), 2,2′-dithiosalicylic acid (Sigma, USA), thiosalicylic acid (Aldrich, Steinheim, Germany), salicylic acid (BDH, Poole, UK), 85% orthophosphoric acid and methanol (both Merck). The water used was purified with a Milli-Q system (ELGA, UK). All reagents were analytical or HPLC grade. The recombinant hepatitis B vaccine was produced at the Center for Genetic Engineering and Biotechnology (Havana, Cuba).

2.2. Equipment

The HPLC system consisted of a Pharmacia Model 2248 pump, an Rheodyne 7125 valve with a 200-μl loop, a LKB 2158 Uvicord SD BioChrom software (Center for Genetic Engineering and Biotechnology) was used for data acquisition and processing.

2.3. Chromatographic conditions

A Hypersil C_{18} column (210×4.6 mm I.D., 5 μm) was used. A guard column packed with 5-μm Hypersil C_{18} particles was placed in front of the analytical column. UV detection at 226 nm was performed. The mobile phase was methanol–water–orthophosphoric acid (66:35:0.9, v/v) of pH 2.5. The flow-rate was fixed at 0.6 ml/min.

2.4. Standard solutions

Thimerosal stock standard solution at 100 μg/ml in water was prepared daily. Working standard solutions of lower thimerosal concentrations were prepared from stock standard solution by appropriate dilution with water at the moment of injection. The internal standard stock solution contained salicylic acid dissolved in mobile phase–water (1:4) at a concentration of 1 mg/ml. This solution was stored at 4°C. Stock standard solutions of thiosalicylic and dithiosalicylic acids contained 40 μg/ml of the compound. Mercury standard solution (Merck) was appropriately diluted before injection.

2.5. Calibration curve

Working solutions containing 20, 40, 50, 60 and 80 μg/ml of thimerosal and 40 μg/ml internal standard were used to construct the calibration curve for determining thimerosal concentration in the vaccine samples. The logarithm of the internal standard area (A_i)/thimerosal area (A_t) ratio was determined for each point. The thimerosal concentration in the samples was obtained when the log A_i/A_t value was interpolated in the calibration curve.

Working standard solutions and the vaccine samples spiked (just before the analysis) with internal standard at 40 μg/ml, were centrifuged for 3 min at 12 000 rpm and 50 μl of the supernatant was injected.

2.6. Spectrophotometric determination of thimerosal

The total thimerosal concentration was spectrophotometrically tested after acid digestion and complexation of Hg^{2+} by dithizone [23].

2.7. Preservative efficacy testing

The inocula from given species of test microorganisms should be individually introduced into samples of the vaccine preserved. This challenge should include representatives of the Gram-positive and Gram-negative bacterial species, moulds and yeast and they should be presented in sufficient size to enable kinetic information to be obtained [24].

The inoculated samples were stored at temperatures between 20 and 25°C over 28 days. They were examined visually and by plate count procedures to determine the number of viable microorganism remaining each 7 days. The preservative is effective in the product examined if the concentrations of viable bacteria are reduced to ≤0.1% of the initial concentrations up to the fourteenth day and remains at or below these levels during the remainder of the test period. For yeast and moulds the concentration must remain be the same or lower than the initial concentration during the test period.

3. Results and discussion

The method described by Rabasco and Caraballo [6] was modified by Tleugabulova and González [22] for determining the thimerosal concentration in the vaccines containing aluminum hydroxide. The method showed a good selectivity to separate non-degraded thimerosal from its degradation products, thiosalicylic and dithiosalicylic acid, Fig. 1. The retention times were 6.1, 6.4, 9.3 and 11.6 min for thiosalicylic acid, salicylic acid, thimerosal and dithiosalicylic acid, respectively.

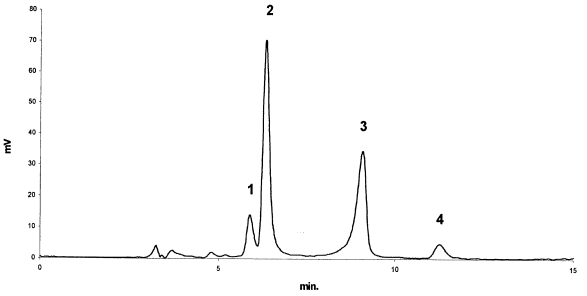

Fig. 1. Chromatographic profile of the vaccine supernatant. Conditions: Shandon Hypersil C_{18} column (210×4.6 mm), mobile phase: methanol–water–orthophosphoric acid (65:35:0.9, v/v), UV detection at 226 nm, flow-rate: 0.6 ml/min, injection volume: 50 μl. Peaks: 1=thiosalicylic acid, 2=salicylic acid (internal standard), 3=thimerosal, 4=dithiosalicylic acid.

A standard solution of mercury was injected onto the column. There was no retention of this metal in the column. The chromatographic signal was added to in the solvent front (data no shown). Therefore, if metallic mercury were formed, it should elute close to the solvent front and never with the thimerosal peak or with the other degradation product peaks.

3.1. Calibration

The calibration curve for thimerosal determination was linear in the range from 20 to 80 μg/ml. The regression coefficient was $r=0.9996$. The detection limit (signal-to-noise ratio=3) was 5 μg/ml for thimerosal.

The reproducibility of the method was tested with three replicate injections of different working standard on 3 different days. The relative standard deviations (RSDs) were 1–2.5% (within day) and 1–4% (between day) for the thimerosal concentration. The different points of the calibration curve were stable at 4–8°C during a week. No significant differences between thimerosal concentrations were found during this time.

3.2. Determination of thimerosal in recombinant hepatitis B vaccine

The concentration of thimerosal in different batches of the Cuban recombinant hepatitis B vaccine stored up to 6 years was determined by the spectro-

Table 1
Thimerosal concentration in different batches of hepatitis B vaccine at different storage times at 4°C[a]

Batch	Storage time (months)	Spectrophotometric method	HPLC method
1	0	38	41
2	0	44	40
3	0	48	46
4	0	44	42
5	0	37	42
6	0	42	42
7	1	43	36
8	2	42	41
9	12	46	40
10	24	54	36
11	24	51	42
12	24	55	48
13	48	45	38
14	60	41	42
15	72	44	30
16	72	46	36
17	72	49	32
18	72	48	37
19	72	45	37

[a] In both methods the concentration is reported as μg/ml. Significant differences between methods for samples stored during 72 months at 4°C were found ($P>0.05$).

photometric and HPLC methods and the results are shown in Table 1. The values provided by both methods were similar for batches stored at 4°C for 24 months. For vaccine samples stored during 6 years at 4°C there were significant differences in thimerosal concentration using both methods. The values obtained with the HPLC method were lower than the values from the colorimetric method in these later samples. The determination of thimerosal concentration in hepatitis B vaccine using spectrophotometric determination of mercury ions gives the concentration of all mercury-containing compounds in the sample including non-degraded and degraded forms [23]. The HPLC method permits access to only the non-degraded thimerosal. Thus was obtained a decreasing thimerosal concentration in older vaccine samples. In spite of the thimerosal concentration in samples, vaccine stored for 6 years decreased, the concentration of preservative in this samples was in compliance with the quality vaccine specification for the concentration of this thimerosal, 30–70 μg/dose [25]. The HPLC method is appropriate for accurate determination of only the non-degraded thimerosal concentrations in the vaccine, therefore it can be used during the stability studies.

3.3. Stability of thimerosal in recombinant hepatitis B vaccine

Vaccine samples were tested to determine the state of thimerosal degradation in the vaccine after different storage times at 4°C. The concentration of non-degraded thimerosal depends on storage time. A decrease in the thimerosal peak area was obtained during time with a simultaneous increase of the degradation products peaks area as shown in Table 2.

Three good defined sample groups were observed for degradation products area peaks percent. As thimerosal degradation is increased, the conversion of the thiosalicylic acid peak to dithiosalycilic acid occurs. These data support the proposed degradation mechanism [26].

There are some reports on thimerosal degradation in the hepatitis B vaccines stored for a week at 28°C in the light [22]. However the chromatograms in Fig. 2 do not show degradation of thimerosal in vaccine samples stored for a month at 45°C. The same results were obtained for 37°C.

Poor stability of thimerosal in different solutions

Table 2
Area percent average of thimerosal, thiosalicylic and dithiosalicylic acid in vaccine samples stored at 4°C[a]

Storage time (years)	Area (%)		
	Thimerosal	Thiosalicylic acid	2,2-Dithiosalicylic acid
<1	84.1±3.4	12.9±1.7	3±1.6
2–4	76.2±1.4	11.6±2.3	12.2±2.0
5–6	74.7± 3.3	8±2.3	17.3±2.8

[a] The values reported are the mean±SD ($n=7$).

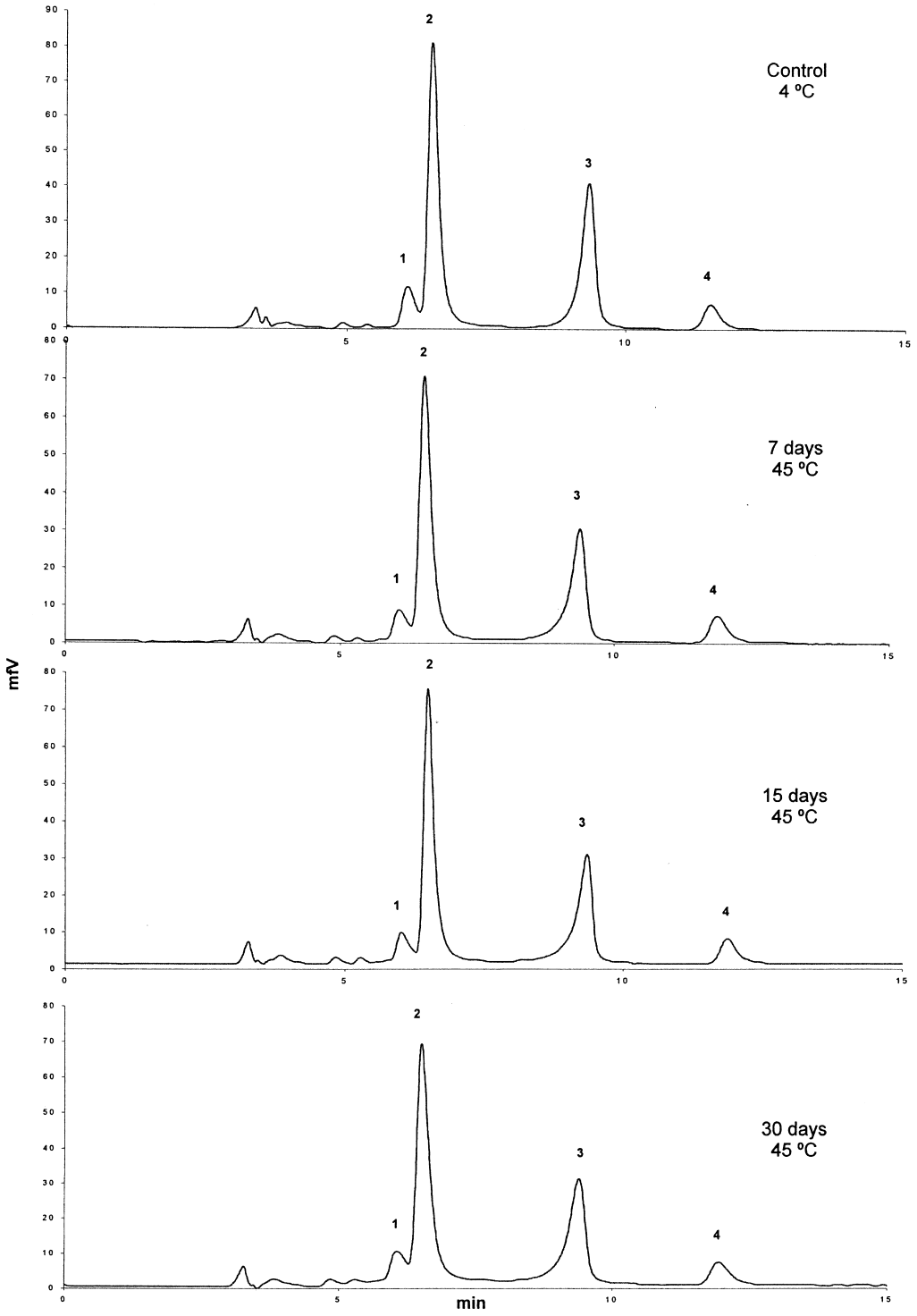

Fig. 2. Chromatographic profile of the vaccine supernatant incubated at different temperatures. Conditions as in Fig. 1. Peaks: 1=thiosalicylic acid, 2=salicylic acid (internal standard), 3=thimerosal, 4=dithiosalicylic acid.

Table 3
General behavior of the challenge microorganisms used in the preservative effectiveness test for samples of Cuban hepatitis B vaccine stored at 2–8°C during 5 years

Batch	Day	Test microorganisms[a]				
		1	2	3	4	5
1	0	$1.6\cdot10^6$	$2.9\cdot10^6$	$4.3\cdot10^5$	$1.0\cdot10^6$	$6.6\cdot10^5$
	14	<10	<10	<10	<10	<10
	28	<1	<1	<1	<1	<1
2	0	$1.0\cdot10^6$	$1.1\cdot10^6$	$1.2\cdot10^6$	$9.0\cdot10^5$	$4.2\cdot10^5$
	14	<10	<10	<10	<10	<10
	28	<1	<1	<1	<1	<1
3	0	$1.0\cdot10^6$	$7.5\cdot10^5$	$1.5\cdot10^6$	$9.0\cdot10^5$	$1.4\cdot10^6$
	14	<10	<10	<10	<10	<10
	28	<1	<1	<1	<1	<1
4	0	$1.0\cdot10^6$	$3.8\cdot10^5$	$9.8\cdot10^5$	$7.0\cdot10^5$	$4.0\cdot10^5$
	14	<10	<10	<10	<10	<10
	28	<1	<1	<1	<1	<1
5	0	$5.2\cdot10^5$	$2.2\cdot10^6$	$1.3\cdot10^6$	$1.2\cdot10^6$	$4.2\cdot10^5$
	14	<10	<10	<10	<10	<10
	28	<1	<1	<1	<1	<1

[a] Test microorganisms: 1=*Escherichia coli*, 2=*Pseudomonas aeruginosa*, 3=*Staphylococcus aureus*, 4=*Candida albicans*, 5=*Aspergillus niger*.

has been reported, but the concentration of non-degraded thimerosal in the samples stored up to 6 years at 4°C was approximately 74% suggesting that the vaccine formulation system (pH, buffers, adjuvant) probably prevent thimerosal degradation.

3.4. Effectiveness of the antimicrobial preservative

Table 3 shows the general behavior of the test microorganisms used in the preservative efficacy test for a batch of the Cuban hepatitis B vaccine stored at 2–8°C during 5 years. An abrupt decrease in microorganism concentration was observed 14 days after the beginning of the test. At day 28 there was no detected test microorganisms in 1 ml of samples. A similar response was reached for all batches tested (data not shown). These results demonstrate that the preservative activity in the Cuban hepatitis B vaccine is not affected by the storage time, even after 5 years at 2–8°C

4. Conclusion

A HPLC method for the determination of non-degraded thimerosal in recombinant hepatitis B vaccine was applied satisfactorily. Tleugabulova and González achieved a similar result in 1996 [22].

In contrast to the response from the spectrophotometric assay, the data obtained from the RP-HPLC method showed that the thimerosal concentration decreases during the long-term stability study, even though all samples with different storage times are in agreement with the quality specification for the preservative concentration despite the time, indicating its good stability in the vaccine. The thimerosal concentration in the batches stored up to 6 years was around 74%. The non-degraded thimerosal concentration was unchanged in the samples stored at 37 and 45°C for 30 days. On the other hand, the results from the antimicrobial preservative effectiveness test applied to the vaccines demonstrated that thimerosal effectivity during the storage time of the vaccine was

satisfactory despite the fall in concentration of intact thimerosal.

Acknowledgements

We thank Dina Tleugabulova for critical comments on the manuscript.

References

[1] M.J. Akers, Pharm. Technol. May (1984) 36.
[2] S.P. Denyer, R.M. Baird, in: Guide To Microbiological Control in Pharmaceuticals, Ellis Horwood, 1990, p. 293.
[3] G. Edsall, J.A. McComb, L.H. Wetterlow, J. Ipsen, New Engl. J. Med. 267 (1962) 687.
[4] M. Pittman, JAMA 181 (1962) 25.
[5] A. Galazka, Stability of Vaccines. Expanded Programme on Immunization, WHO/EPI/GEN/89.8.
[6] A.M. Rabasco, I. Caraballo presented at the VIII Jornadas Farmaceúticas Españolas, Seville, 3–6 December 1991.
[7] K.P. Shrivastaw, S. Singh, Biologicals 23 (1995) 65.
[8] M.P. da Silva, J.R. Procopio, L. Hernández, Anal. Chim. Acta 283 (1993) 326.
[9] B.J. Meakin, Z.M. Khammas, J. Pharm. Pharmacol. 31 (1979) 653.
[10] S. Penzanti, M. Casini, Farmaco Ed. Prat. 35 (1980) 92.
[11] S. Penzanti, M. Casini, Anal. Abstr. 39 (1980) 3E66.
[12] J.S. Fleitman, I.W. Partridge, D.A. Neu, Drug Dev. Ind. Pharm. 17 (1991) 519.
[13] M.J. Reader, C.B. Lines, J. Pharm. Sci. 72 (1983) 1406.
[14] A.M. Rabasco, I. Caraballo, M. Fernández-Arévalo, Drug Dev. Ind. Pharm. 19 (1993) 1673.
[15] R.C. Meyer, L.B. Cohn, J. Pharm. Sci. 67 (1978) 1636.
[16] J.T. Carstensen, C.T. Rhodes, Clin. Res. Reg. Affairs 10 (1993) 177.
[17] J. Boehlert, Drug Dev. Ind. Pharm. 10 (1984) 1343.
[18] L.I. Bebawy, N.E. Kousy, Anal. Lett. 30 (1997) 1379.
[19] G. Szepesi, M. Gazdag, K. Mkhalyfi, J. Chromatogr. 464 (1989) 265.
[20] M.W. Dong, P.V. Passalacqua, D.R. Choudhury, J. Liq. Chromatogr. 13 (1990) 2135.
[21] W.E. Weiser, Pharm Technol. Anal. Validation (1998) 20.
[22] D. Tleugabulova, I. González, J. Chromatogr. A 729 (1996) 219.
[23] Manual for the Production and Control of Vaccines, Tetanus Toxoid, BLG/UNDP/77.2, Rev. 1, 78–79, Appendix T.16, World Health Organization, Geneva.
[24] United States Pharmacopeia 23, The National Formulary 18, Microbiological Tests, 1681, 1995.
[25] Norma Cubana 26-206:1991. Medicamentos. Vacuna Antihepatitis B de Antígeno de Superficie Viral ADN Recombinante, Especificaciones, 1991.
[26] I. Caraballo, A.M. Rabasco, M. Fernández-Arévalo, Int. J. Pharm. 89 (1993) 213.