

## Improved Spectrophotometric Pharmacopoeial Method for accurate Quantitation of Thiomersal content in *Haemophilus influenzae* type B (Hib)-TT Conjugate Vaccine

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### Abstract

Vaccines remain one of the most effective ways to prevent infectious diseases globally. Accurate measurement of thiomersal content is an important parameter to ensure safety of vaccines though these vaccines are less in use. In the present study a simple, accurate and highly effective spectrophotometric improved method has been developed for determination of thiomersal in Hib-TT conjugate vaccine by using 0.01% and 0.005% concentration of dithizone for making stock and comparing these with 0.001% dithizone concentration mentioned in Indian Pharmacopoeia (2014). All the concentrations were evaluated at three different wavelength i.e. 538nm, 540 nm and 542nm. When all the three concentrations compared, the dithizone concentration 0.01 % has been found to be more accurate, linear and giving reproducible results. Among the three wavelengths, the best result has been obtained at wavelength 538nm. The improved method is accurate, specific, and easy to carry out, consumes little time and requires a few microliters of sample and therefore, improved method is recommended than the present pharmacopoeial methods which underestimate the concentration of thiomersal and fails the otherwise passed Hib-TT conjugate vaccine.

**Keywords:** Spectrophotometer, Improved method, Thiomersal, Dithizone, Vaccines.

### 1. Introduction

Preservatives are defined as compounds that kill or prevent the growth of accidental contamination of microorganisms, particularly bacteria and fungi. These are used to prevent microbial growth in the event of accidental contamination of vaccine, which might occur with repeated puncture of multi-dose vials of vaccines. In some cases, preservatives are added during manufacturing to prevent microbial growth; however with advancement in manufacturing technology, the need to add preservatives during the manufacturing process has decreased markedly [1].

Mercury is a naturally occurring element found in the earth's crust, air, soil, and water. Two types of mercury to which people may be exposed are methyl mercury and ethyl mercury [2]. Methyl mercury is the type of mercury found in certain kinds of fish. At high exposure levels methyl mercury can be toxic to people. In the United States, federal guidelines keep as much methyl mercury as possible out of the environment and food, but over a lifetime, everyone is

exposed to some methyl mercury. Thiomersal is an ethyl mercury-based preservative that has been in use for decades in vaccine vials [3]. There is no evidence of harm caused by the low doses of thiomersal in vaccines, except for minor reactions like redness and swelling at the injection site. Ethyl mercury based thiomersal is cleared from the human body more quickly than methyl mercury, and is, therefore, less likely to cause any harm [4]. However, in July 1999, the Public Health Service agencies viz. American Academy of Pediatrics, and vaccine manufacturers agreed that use of thiomersal should be reduced or eliminated in vaccines as a precautionary measure [5,6].

The Influenza (flu) vaccines are currently available in both less thiomersal-containing (for multi/single dose vaccine vials) and more thiomersal-free versions. In India both the types of vaccines are available however the accurate measurement of thiomersal in vaccines as per existing method of Indian pharmacopoeia (2014) has been found to be difficult, therefore, the present study has been undertaken to find out improved method for accurate determination of the

concentration of thiomersal used in Hib-TT conjugate vaccine [7, 8].

## 2. Materials and method

### 2.1 Reagents and Chemicals

All the reagents used in the study were of analytical grade like Dithizone from fisher scientific, Thiomersal from Loba Chemi, Sodium Hydroxide Pellets and Acetone from Merck.

Sodium Hydroxide solution (50% w/v), Solution of dithizone in acetone (0.001% w/v, 0.01% w/v and 0.005 % w/v) and thiomersal Stock solution (500 µg/ml) were used in this study.

### 2.2 Samples

Two different Batches of Hib-TT conjugate vaccine from one of the Indian manufacturers were used.

### 2.3 Equipment

An Agilent UV-Vis spectrophotometer (Model-Carry100) for measurement of absorbance and Sartorius make Balance for weighing chemicals were used.

### 2.4 Method

Three sets of thiomersal standard series of different concentration i.e. 125 µg/ml, 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, in duplicate was prepared from thiomersal stock solution (500 µg/ml). 0.1 ml of vaccine sample was taken in duplicate from each set of reconstituted vaccine vial claiming 50 µg/ml of thiomersal and 0.1 ml of thiomersal standards of different concentration i.e. 125 µg/ml, 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml in different test tubes. Distilled water was added to vaccine sample and thiomersal standard series tubes to make a final volume of 1ml in each of the test tube. Another test tube, with 1ml of distilled water was kept as blank. 1ml of acetone was added to each tube. Then 1ml of freshly prepared dithizone in acetone 0.001% w/v was added to first set whereas 1ml of freshly prepared dithizone in acetone 0.01% w/v and 0.005% w/v was added to second and third set respectively. 0.1 ml of sodium hydroxide solution 50% w/v was added to all the test tubes which were vortexed for 15-20 seconds. At last absorbance was measured at wavelength 538 nm, 540 nm, and 542 nm.

## 3. Results

The absorption spectra obtained from different thiomersal standard series i.e. 125 µg/ml, 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml is as shown in Figure 1. The peak of the standard series of thiomersal absorption spectra lies between 538nm to 542nm. Therefore, the wavelengths 538nm, 540nm and 542nm have been used for further analysis of the samples.

Overall absorption and linearity of thiomersal standards at concentration 0.01% w/v have been found to be better than the linearity at concentration 0.001% w/v and 0.005% w/v, in terms of coefficient of correlation ( $R^2$ ) which is greater than 0.99. Also among the three wavelengths, 538

nm shows the best result at concentration 0.01% w/v dithizone in acetone (Figure 2)

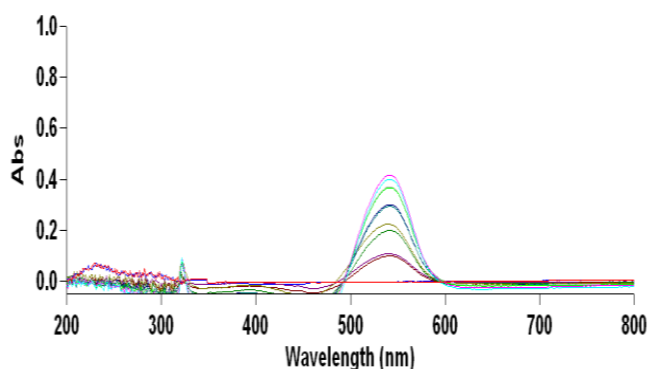
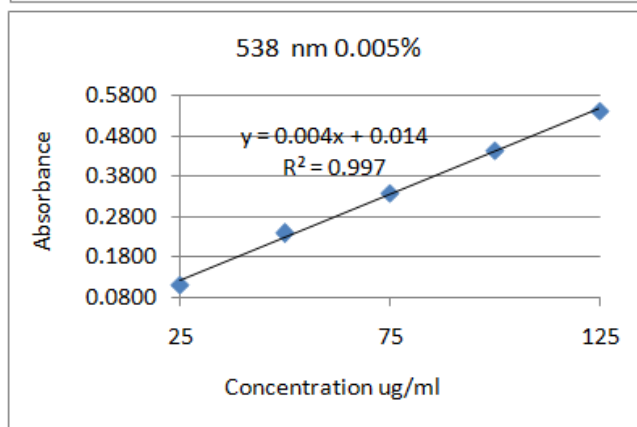
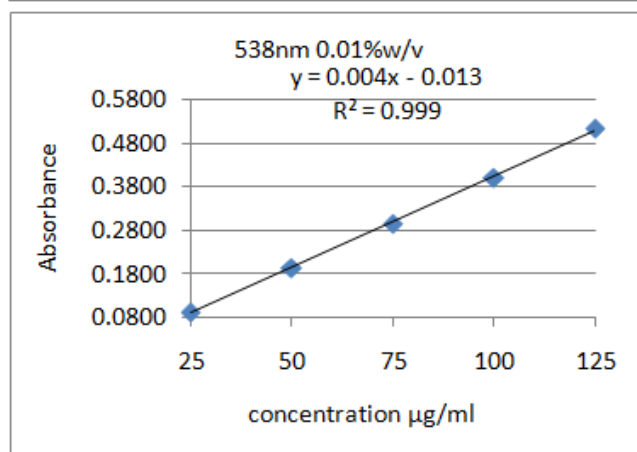
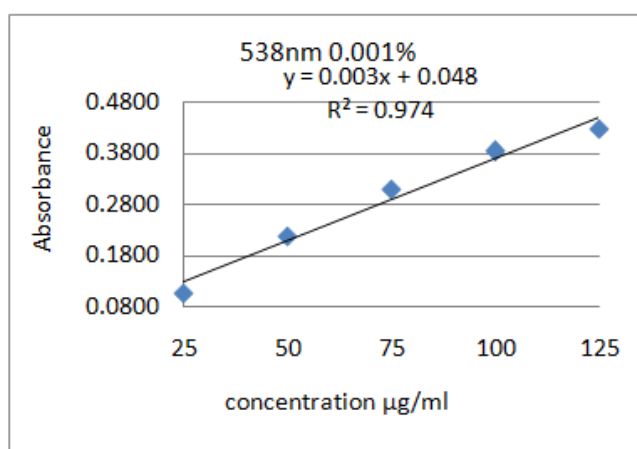


Figure 1: The absorption spectra of thiomersal standard series



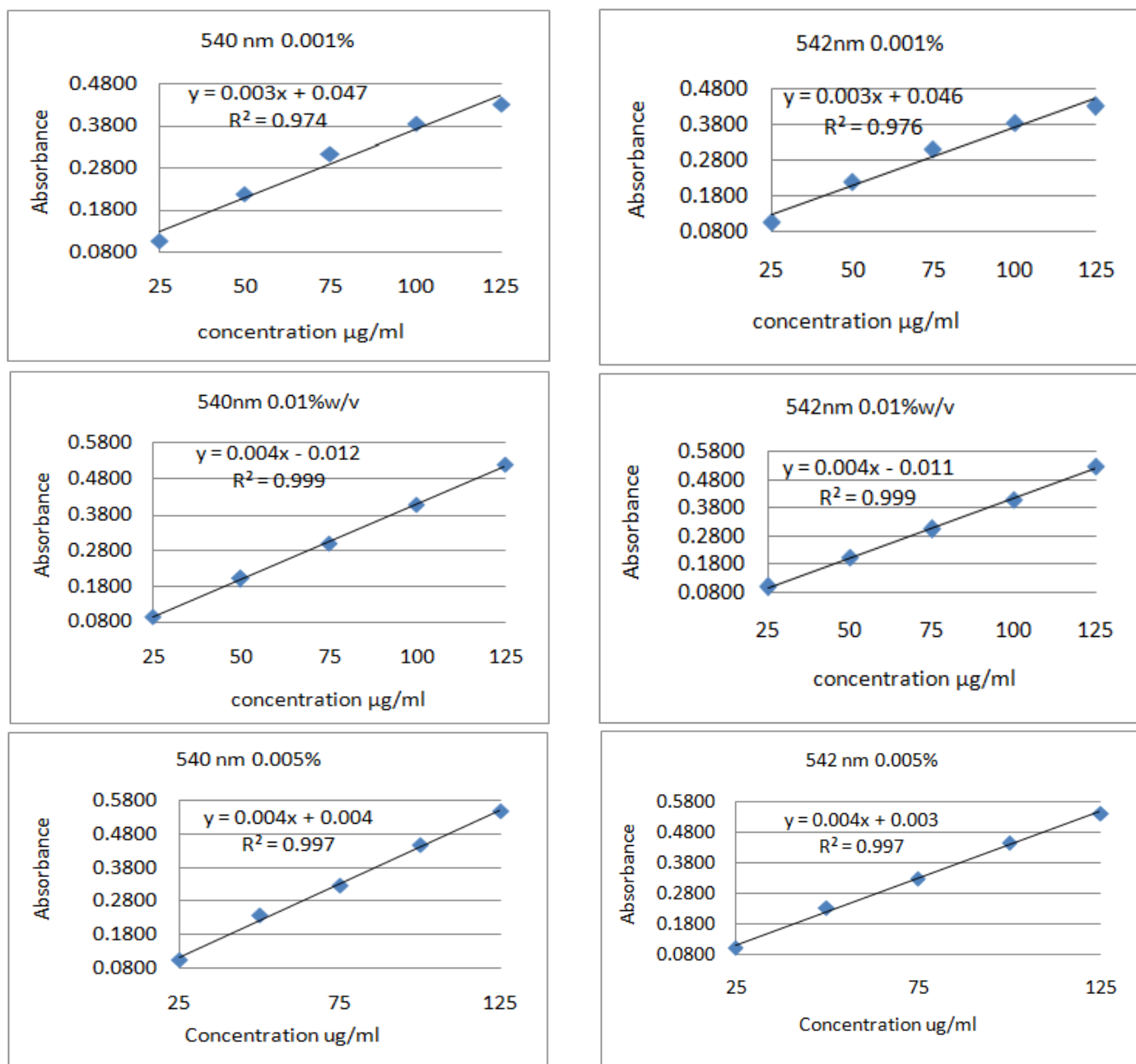


Figure 2: Shows regression line, R<sup>2</sup> slope and intercept of thiomersal standard series with three different dithizone concentration i.e. 0.001% w/v , 0.01% w/v and 0.005% w/v at wavelengths 538 nm, 540 nm, 542 nm respectively

Table 1: Concentration of thiomersal (mean of 03 replicates) in Hib TT conjugate vaccine batch 1 and batch 2 with three different wavelengths and three different dithizone concentration in acetone

Hib TT conjugate vaccine	Performance	0.001% Dithizone in acetone			0.01% Dithizone in acetone		
		538nm (µg/dose)	540nm (µg/dose)	542nm (µg/dose)	538nm (µg/dose)	540nm (µg/dose)	542nm (µg/dose)
Batch 1	Mean	35.39	37.05	35.69	<b>48.33</b>	44.63	44.34
	S.D	2.15	0.44	0.82	<b>1.07</b>	0.97	0.86
Batch 2	Mean	35.27	35.91	35.32	<b>48.71</b>	46.52	44.98
	S.D	1.80	1.31	1.28	<b>0.54</b>	0.90	0.66

Hib TT conjugate vaccine	Performance	0.005% Dithizone in acetone		
		538nm (µg/dose)	540nm (µg/dose)	542nm (µg/dose)
Batch 1	Mean	36.23	37.91	35.97
	S.D	0.33	0.74	2.59
Batch 2	Mean	36.44	37.15	38.15
	S.D	0.59	1.66	0.50

The printed value of thiomersal indicated on the label of tested vaccine batch was 50 µg/dose and the concentration of the thiomersal in Hib TT conjugate vaccine as per the Indian Pharmacopoeia (2014) should be between 85%-115% of the labelled claim i.e. in the range of 42.50 µg – 57.5 µg per dose. Testing of both the batches of vaccines revealed that the concentration of thiomersal in 1<sup>st</sup> batch tested with 0.001% w/v, 0.005% w/v and 0.01% w/v concentration of dithizone in acetone at wavelength 538nm has been found to be 35.39±2.15/µg, 36.23±0.33/µg and 48.33±1.07/µg respectively whereas the concentration of thiomersal in 2<sup>nd</sup> batch 0.001% w/v, 0.005% w/v and 0.01% w/v concentration of dithizone in acetone at wavelength 538nm came out to be 35.27±1.80/ µg, 36.44±0.59/ µg and 48.71±0.54/µg respectively (Table 1). It is evident from the results that the recovery of thiomersal is highest at 0.01% w/v dithizone in acetone at 538 nm than all other combinations. Results of test performed at 0.1% w/v dithizone were less than that of 0.01% w/v of dithizone at 538 nm and these were not reproducible (result not shown).

#### 4. Discussion

Hib-TT conjugate vaccines without thiomersal are more in use than vaccine with thiomersal but in India both the types of vaccine are available. The method to test thiomersal in vaccine is not available in any pharmacopoeia except Indian Pharmacopoeia. It is evident from the present study that the concentration of thiomersal recovered with dithizone concentration 0.01% w/v is accurate and passes the label claim criteria whereas dithizone concentration 0.001% w/v (as per the Indian pharmacopoeial requirement), [9] and 0.005% w/v fails to recover the same. As a result both the batches of vaccine though having concentration of thiomersal in recommended range as per improved method in the present study but otherwise fail as per IP method. Thus, it is recommended to use 0.01% dithizone concentration in the method for accurate determination of thiomersal content in Hib-TT conjugate vaccines.

#### Conflict of interest statement

The authors have no financial interest in any vaccine manufacturer, including any arising from employment, consultancies, honoraria, stock ownership, patents, grants or royalties.

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