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Gas diffusion flow injection determination of thiomersal in vaccines

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ABSTRACT

A new simple gas diffusion flow injection method has been developed for the determination of thiomersal in biological samples. The method is based on cold vapor generation of monoatomic mercury from thiomersal reaction with acidic stannous chloride solution (0.6%) acting as reducing agent. The evolved mercury partially diffuses through a Teflon membrane into an acidic permanganate (2.25×10^{-4} mol L^{-1}) acceptor stream, where it is oxidized and re-converted to Hg(II). The resulting decrease in acceptor stream absorbance is sensitively monitored at 528 nm. Flow injection variable parameters such as reagents concentrations, injected volume, reactor length, temperature and flow rate were carefully investigated and optimized. The concentration–response relationship was linear over a concentration range of 1–30 mg L^{-1} with a correlation coefficient greater than 0.99 and a good reproducibility (RSD < 1.42%, $n = 6$) at 10 mg L^{-1}. A detection limit of 0.07 mg L^{-1} ($S/N = 3$) and a sampling frequency of 5 samples h^{-1} were obtained. The method was successfully applied for the determination of thiomersal in different types of vaccines and gave results in close agreement with those found by previously established HPLC method with no significant interference from vaccines matrices.

1. Introduction

Thiomersal also known as merthiolate, thimerosal or sodium ethylmercury thiosalicylate, is an organomercury compound, commonly used as antimicrobial preservative in cosmetics and several pharmaceutical products such as vaccines, nose and ear drops and ophthalmic solutions [1]. It is used to avoid microbiological contamination and spoilage and to ensure the safety and stability of products [2]. Thiomersal contains 49.6% (w/w) mercury which is metabolized in human body to thiosalycilic acid and ethyl mercury [3,4]. Considering the well known toxicity of mercury and some of its organic complexes, a controversial debate has associated thiomersal-containing vaccines with biologically plausible neurodevelopmental disorders [4]. Although not systematically studied and fully established, the presumed effect of childhood thiomersal exposure constitute a health risk that should not be neglected. For purposes of risk assessment there is need for accurate and careful control of thiomersal dosage in pharmaceutical products. At present, many analytical methods have been developed for stability studies and quantitative measurement of thiomersal. They include atomic absorption spectroscopy [5–9], high performance liquid chromatography (HPLC) [10–13], spectrophotometry [14,15] as well as electrochemical methods [16]. Most of these techniques, however, suffer from some disadvantages such as low specificity and the possible appearance of interfering substances.

Flow injection analysis (FIA) has proved to be a suitable technique for on line analysis because of its low reagent and sample consumption and to its higher precision and accuracy, due to reproducible controlled timing, as well as its simplicity and high sampling frequency. Bertocchi et al. [17] described a flow injection method using an enzymatic amperometric procedure for the measurement of thiomersal. However, this method suffers from two main limitations which are critical enzyme preparation and the need for an off-line oxidative cleavage treatment. More recently, Zhu et al. [18] used cathode glow discharge for cold vapour generation of mercury and reported its successful coupling with flow injection system and ICP-atomic emission spectrometry. This method allowed direct conversion of thiomersal to volatile mercury and its sensitive determination to the μg L^{-1} level. Coupling of flow injection with gas-diffusion separation module (FIGD) has proven useful in complex matrices and led to the development of more selective methods [19–24].

In this work, we aimed to develop a simple, selective and sensitive FIGD method for the determination of thiomersal in vaccines and immune serum. The method is based on the injection of thiomersal in a reductive stannous chloride stream, leading to the generation of mercury vapor which diffuses through the microporous membrane and is trapped in a permanganate stream where it is oxidized and converted to Hg(II). After being optimized, the

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proposed method was studied in terms of linearity, precision, sensitivity and recovery and its suitability verified by analysing different real samples.

2. Experimental

2.1. Reagent and solutions

All chemicals used in this work were of analytical-reagent grade and used without further purification. A standard thiomersal stock solution (100 mg L^{-1}) was prepared by dissolving the appropriate amount of solid thiomersal (Fluka, France) in distilled water. Hg(II) stock solution (1000 mg L^{-1}) was prepared from mercuric nitrate (Fluka, France) in sulfuric acid (Scharlau, France). Working standards (1.0–30 mg L^{-1}) of thiomersal and Hg(II) were freshly prepared by suitable dilutions of the respective stock solutions. Potassium permanganate (PROLABO, France) stock solution (10^{-2} mol L^{-1}) was stored in a sealed container and protected from light and oxygen to prevent its decomposition. Tin (II) chloride solution was prepared in 1.5 mol L^{-1} sulfuric acid (PROLABO, France). The working carrier solutions were prepared daily and degassed by sonication for 10 min before use.

Hepatitis B vaccine, anti-rabic immune serum (PAR) and anti-scorpionic immune serum (PAS) were kindly supplied by Pasteur institute (Tunisia), stored in the refrigerator at 4 °C and used as received without any pre-treatment. The suspension of hepatitis-B contains the following recipients: sodium chloride, di-sodium phosphate dihydrate, sodium phosphate dehydrate and purified water.

2.2. Apparatus

The flow-injection system used in this work to determine the thiomersal concentration in vaccine and serum samples is based on two-channels configuration. A variable-speed peristaltic pump (Gilson Minipuls, Anachem, Luton Bedfordshire, UK) was used for propelling sample plug, stannous chloride and permanganate reagents. A low pressure injection valve (Rheodyne 5020, Anachem, Luton, UK) fitted with variable volume Teflon loop was used for injection of standards and samples. The gas diffusion cell consisted of two identical methacrylate blocks each with S-shape grooves (channel dimensions: 240 mm × 1.5 mm × 0.2 mm) between which a micro porous PTFE membrane of 75 μm thickness, 0.1 μm pore size and 50% porosity (Nastro, Professional Gas System, Italy) was placed. The two blocks were pressed together by eight screws. A silicone rubber tubes (0.8 mm i.d.), PTFE joints, tubing and reaction coils (0.8 mm i.d.) were used for connections. The online detection was carried out at 528 nm, using a Beckman spectrophotometer equipped with 1 cm path length flow cell.

The analytical procedure involves cold vapor generation of monoatomic mercury via thiomersal injection into acidified stannous chloride donor stream (0.5% $SnCl_2$ in 1.5 mol L^{-1} H_2SO_4). The evolved mercury diffuses through the Teflon microporous membrane into an acidic permanganate acceptor stream (2.25×10^{-4} mol L^{-1} $KMnO_4$ in 0.3 mol L^{-1} H_2SO_4), where it is oxidized and re-converted to Hg(II). The variation in the absorbance of acceptor permanganate stream (expressed as the positive difference between initial absorbance of permanganate acceptor stream and its measured value after thiomersal injection) was continuously monitored at 528 nm and related to the concentration of thiomersal in the injected sample.

Chromatographic analysis was performed on a Beckman chromatograph equipped a quaternary pump, a diode-array UV-detector Gold 168 and a data station equipped with Gold Nouveau software. The separation was achieved on a reversed phase C18 column (Spherisorb ODS: 250 mm × 4.6 mm i.d., and 5 μm particle size) (Alltech Associates, USA) using a mobile phase containing 66:34 (v/v) methanol/water (0.5% phosphoric acid at pH = 2.5). The analysis was performed at a flow rate of 1 mL min^{-1} and thiomersal detection monitored at 226 nm.

Table 1
Evaluation of thiomersal sensitivity with regards to mercury species ($n = 6$).

Concentration (mg L^{-1})	Absorbance (Hg^{2+})	Absorbance (thiomersal)
1	0.052 ± 0.002	0.025 ± 0.001
5	0.075 ± 0.003	0.039 ± 0.001
10	0.112 ± 0.003	0.058 ± 0.002

3. Results and discussion

It has been reported previously that traditional cold vapor generation is not effective for generating volatile mercury directly from organomercury species and requires a prior oxidation step [20]. Therefore, it is important to verify and provide clear evidence on effective mercury vapor generation from thiomersal. Several trials were carried out by injecting inorganic mercuric ions, added as mercuric sulfate, and thiomersal standard samples of the same mass concentration. Comparative results of recorded analytical signals (Table 1) of multiple runs ($n = 6$) showed that inorganic samples gave a signal intensity almost twice of that obtained with thiomersal solution of the same mass concentration. This result is in close agreement with the molecular weights ratio ($M_{thiomersal}/M_{Hg} = 1.903$) and suggested that mercuric ions present in the thiomersal structure were almost quantitatively reduced to volatile monoatomic mercury, leading to a signal equivalent to that of inorganic mercury solution of the same molar concentration. In addition, the injection of thiosalicylic acid gave no significant modification of the blank signal providing further confirmation that the observed signal is solely related to mercuric ions.

3.1. Optimization of FIA parameters

Considering that the performance of the proposed method will be determined by the kinetics and efficiencies of thiomersal reduction in stannous donor stream, mercury vapour diffusion through the porous membrane and finally its dissolution and re-conversion to mercuric ions under the action of permanganate anions, the effects of main operational parameters were studied and optimized. The optimization was carried out using the univariate method with a standard thiomersal solution of 5 mg L^{-1}.

Classically, $NaBH_4$ and $SnCl_2$ are the most popular reductants used to generate cold vapor of mercury. Nevertheless, when $NaBH_4$ is used, a parasitic generation of hydrogen may occurs and lead to possible interference during spectrophotometric measurements. Therefore, stannous chloride was adopted as the most appropriate reductant for this study.

First, the influence of $SnCl_2$ present in the acidic donor stream on the method sensitivity was investigated by varying its concentration between 0.3% and 1%. As shown in Fig. 1, the analytical signal reached a maximum at $SnCl_2$ concentration of 0.6%. Above this concentration a significant decrease in the analytical signal was observed. According to Amini et al. [25], the decrease in the analytical signal at a $SnCl_2$ concentrations higher than 0.6%, could be attributed to the reduction of H_2SO_4 resulting in lower acidity of the reaction medium.

Accordingly, the concentration of $SnCl_2$ in the donor stream was fixed at 0.6% for all subsequent measurements.

When permanganate concentration in the acceptor stream was varied, a significant increase of the analytical signal was recorded as the permanganate ranged from 1.25 to 2.25 10^{-4} mol L^{-1}. Above

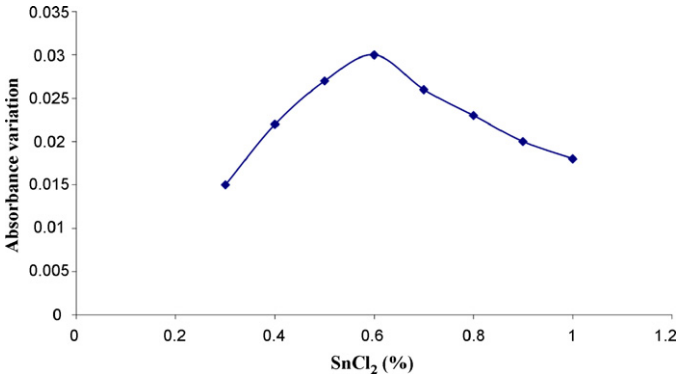

Fig. 1. The influence of $SnCl_2$ concentration "in the donor stream" on the detector response.

this concentration, a dramatic decrease of sensitivity is observed and could be attributed to possible reaction of permanganate with Mn^{2+} to form solid brown manganese dioxide. This later could block membrane micropores entrance and partially prevent mercury vapor diffusion, leading to the observed signal decrease. Therefore, a permanganate concentration of 2.25×10^{-4} mol L^{-1} was chosen for further measurements (Fig. 2).

In order to proceed with the final system, the FIA physical variables were studied under the above optimum chemical variables.

It was found that best results in term of sensitivity were obtained when the flow rate of the acceptor stream is lower than the donor stream. Under these conditions the mercury vapor diffusing through the Teflon membrane was accumulated in smaller volume of the acceptor stream thus improving the sensitivity of the method. The effect of overall flow rate (acceptor and donor stream) was studied over the range 0.05–0.25 mL min^{-1}. The flow rates of streams were maintained equal to reduce the number of system parameters being optimized in the present study. The sensitivity was observed to decrease with increasing the flow rate. This is attributed to slow diffusion of mercury vapour through the membrane and further dissolution into the acceptor stream, since rapid flow rates cause the sample zone to flow too quickly through gas-diffusion module. Very low flow rates caused a significant decrease in sample throughput, while the increase in sensitivity was less pronounced for flow rates below 0.07 mL min^{-1}. An increase of the flow rate to 0.14 mL min^{-1} will enhance the sample throughput to 10 samples h^{-1}, however, this enhancement of the throughput will encountered by a loss of sensitivity by approximately 30%. Therefore, a flow rate of 0.07 mL min^{-1} was selected as optimal, making compromise between sensitivity and sampling rate.

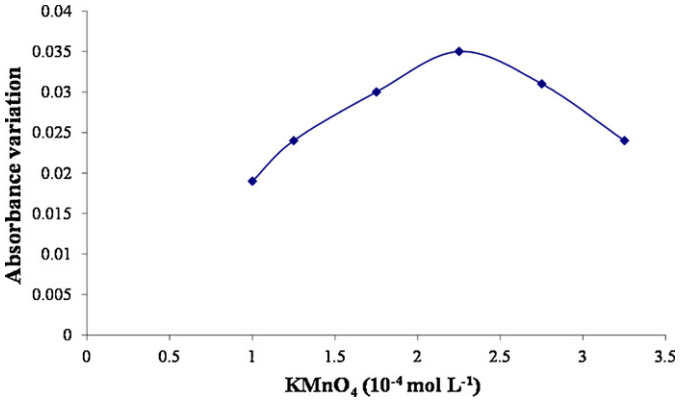

Fig. 2. The influence of $KMnO_4$ concentration "in the acceptor stream" on the detector response.

Table 2
Studied operating parameters and their optimal values.

Parameters	Range studied	Optimal value
$SnCl_2$ (%)	0.3–1%	0.6%
$KMnO_4$ (mol L^{-1})	$1.0–3.25 \times 10^{-4}$	2.25×10^{-4}
Flow rate (mL min^{-1})	0.05–0.25	0.07
Volume injection (μL)	80–400	180
Temperature of water bath (°C)	70–95	90
Reaction coil length (cm)	10–37.5	25

However, higher flow rates could be employed when faster and less sensitive analysis is required.

The effect of the sample injection volume on the detector response was studied in the range of 80–400 μL. The method sensitivity was found to increase upon increasing the sample injection volume up to 300 μL and stayed fairly constant afterward. Nevertheless, a loss of sensitivity by 20% when using a smaller injection volume instead of 300 μL, however, injection volume of 180 μL was selected as compromise between sample rate and sensitivity.

The influence of working temperature on the analytical response was also investigated by immersing connecting tubes (50 cm) and the gas diffusion bloc in a constant temperature water bath, controlled within ±1 °C in the range 70–95 °C. As expected, the method sensitivity was significantly improved (≅2 folds) by increasing the temperature from 70 to 90 °C. The observed increase of sensitivity upon increasing temperature, can solely be attributed to faster reaction kinetics and possibly faster mercury diffusion. A working temperature of 90 °C was chosen as optimum due to the low reproducibility of absorbance measurements at higher temperatures due to the formation of bubbles in the system.

Finally, the analytical signal increased with increasing the length (from 10 to 37.5 cm with an i.d. of 0.8 mm) of the acceptor stream reaction coil up to 25 cm. Above this value, the sensitivity decreased slightly because of the occurrence of dispersion phenomena leading to peak broadening. Therefore, a 25 cm reactor length was selected as optimal for further experiments.

The results of the investigations carried out to optimize the operating parameters of the FIA system are shown in Table 2. Based on these findings, all further measurements were performed under the above mentioned optimal conditions.

3.2. Linearity

The linearity of the developed method was investigated by recording the detector responses to three sets of standard thiomersal solutions in the range 1–30 mg L^{-1}, over three different days. Calibration graphs obtained with every set of standards (Fig. 3) showed almost similar and linear relationships between the recorded peak height of the flow injection signal and thiomersal concentration. The equation of the line ($\Delta A = 0.0033$ [thiomersal] + 0.022) was used for the determination of thiomersal in real samples. Least squares analysis gave regression coefficient higher than 0.999. Cochran's C test was applied for checking the homogeneity of variances and Fisher test was used to examine the significance of the slope and the results were always satisfactory ($p < 0.05$).

3.3. Precision and sensitivity

The intra- and inter-days precision of the proposed method was evaluated as the relative standard deviation (RSD) of 6 repeated determinations of 10 mg L^{-1} standard solution of thiomersal. The precision was found to be satisfactory with an average of intra- and inter-days RSD values of 0.88% and 1.42%, respectively. The limit of detection (LD), calculated as three times the noise at blank level, was determined by six replicate injections of a reagent blank and

Table 3
Recoveries obtained from various biological samples.

Samples	Amount spiked (mg L^{-1})	Amount found (mg L^{-1})[a]	Recovery (%)	R.S.D. (%)
PAS	0	10.98 ± 0.10		0.94
	2	13.03 ± 0.15	102.5	1.12
	5	16.01 ± 0.14	100.1	0.85
PAR (lot 1)	0	10.10 ± 0.07		0.68
	2	12.14 ± 0.17	102	1.41
	5	15.18 ± 0.19	101.6	1.23
PAR (lot 2)	0	5.10 ± 0.06		1.12
	2	7.15 ± 0.07	102.5	1.05
	5	10.09 ± 0.09	99.8	0.91
Vaccine (hepatitis B)	0	1.00 ± 0.02		1.62
	2	3.03 ± 0.04	101.5	1.35
	10	10.97 ± 0.13	99.7	1.15

[a] The presented concentrations are the average of six determinations.

Table 4
Determination of thiomersal in biological samples using the proposed FIA and HPLC methods.

Samples	Proposed FIA method (mg L^{-1}) mean ± SD[a]	HPLC method (mg L^{-1}) mean ± SD[a]	*t*-test[b]	*F*-test[c]
PAS	10.98 ± 0.12	10.92 ± 0.14	0.65	0.11
PAR (lot 1)	10.10 ± 0.13	10.03 ± 0.21	0.88	0.42
PAR (lot 2)	5.10 ± 0.06	5.03 ± 0.07	1.17	0.53
Vaccine (Hepatis B)	1.00 ± 0.01	1.01 ± 0.03	1.34	0.78

[a] The presented concentrations are the average of six determinations.
[b] Tabulated *t*-value for significance level $p = 0.05$ and $n = 6$ is 2.228.
[c] Tabulated *F*-value for significance level $p = 0.05$ and $f_1 = f_2 = 5$ is 5.0.

was found to be lower than 0.07 mg L^{-1}. Apparently, this value is higher than that found with HPLC and ICP but is one to two order of magnitude lower than methods based on spectrophotometric [15] and microbiological [26] methods.

3.4. Application and accuracy

The proposed method has been applied for the determination of thiomersal in four real biological samples (Hepatitis-B vaccine, immune serum PAS and two immune serums PAR1 and PAR2). The accuracy of the proposed assay was evaluated by spiking real samples with known amounts of thiomersal standards (Table 3). The calculated mean recoveries ranged from 99.7% to 102.5%, indicating that thiomersal could be analysed in biologicals with good accuracy.

The accuracy of the proposed method was further assessed by comparing the found values with those obtained with use of previously published chromatograhic procedure [12] (Table 4). As can be seen, the values obtained with both methods were in close agreement. Furthermore, the obtained data were compared statistically by student's *t*-test and variance ratio *F*-test. The experimental values were below the theoretical values in either test, indicating that there was no significant difference between the compared methods. Therefore, the proposed method is precise and accurate and could be applied to the determination of thiomersal vaccines and serums without any significant matrix-induced errors.

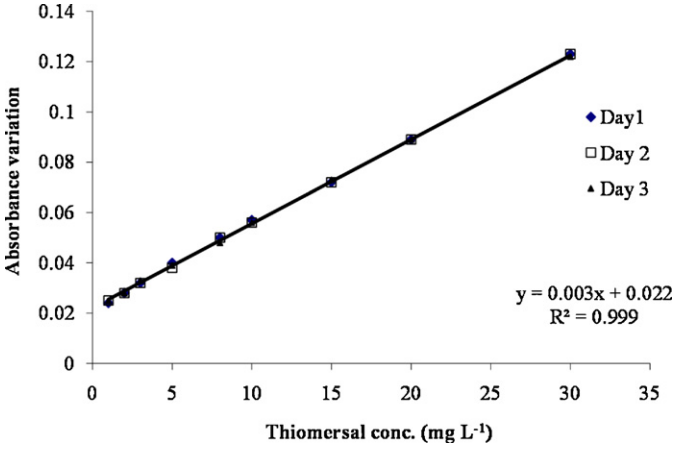

Fig. 3. Calibration curve of thiomersal at 528 nm using the optimum FI operating conditions.

4. Conclusion

A simple flow injection gas-diffusion method for the analysis of thiomersal was developed based on cold vapor generation of volatile mercury and its subsequent reaction with permanganate in the acceptor stream. The proposed method has proved to be selective and sensitive without any statistically significant difference when compared to established HPLC method. Moreover, it showed high precision (0.88–1.42%) and a reasonably low limit of detection (0.07 mg L^{-1}). The relatively low sample throughput (5 samples h^{-1}) can be circumvented by increasing the flow rate when lower sensitivity is permitted. An increase of the flow rate to 0.14 mL min^{-1} will enhance the sample throughput to 10 samples h^{-1}, however this enhancement of the throughput will encountered by a loss of sensitivity. Furthermore, this system offers interesting assets such as simplicity, low cost and flexibility and may be considered as a suitable alternative to existing methods.

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