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Flow injection analysis of mercury(II) in pharmaceuticals based on enzyme inhibition and biosensor detection

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Abstract

An enzymatic amperometric procedure for measurement of mercury(II) in pharmaceuticals, based on the inhibition of invertase and on a glucose electrode was studied. Analytical parameters for measurements in batch and flow injection analysis (FIA) have been optimised. Mercury(II) was detected in the 10–60 ppb range with RSD $\leq 2\%$. A sample throughput of 6 h^{-1} for batch and 15 h^{-1} for FIA was obtained. The total mercury(II) from thimerosal (thiomersal, sodium ethylmercurithiosalicylate) in eye-drop samples was measured with the amperometric procedure after oxidative cleavage treatment. Results for both batch and FIA procedures correlated well with atomic absorbtion spectroscopy (AAS) data.

Keywords: Mercury; Pharmaceuticals; FIA; Biosensor; Enzyme inhibition

1. Introduction

A large number of pharmaceutical compounds contain mercury in organometallic or inorganic form as well as mercury as degradation by-product [1–3]. Several instrumental or titrimetic methods are commonly used for the determination of total or free mercury [4–16]. They generally require lengthy procedures, expensive and complex instrumentation and/or exhibit lack in sensitivity.

Electrochemical sensors and biosensors have, in some cases, the advantage of rapidity and sensitivity over the traditional techniques. Enzyme inhibition-based sensors recently appeared in the literature for the determination of environmental pollution [17–21]. Particularly, measurement of mercury(II) with amperometric [22], potentiometric [23] and thermal [24] detection has been proposed.

This paper proposes a method for measurement of mercury(II) in pharmaceuticals. As a model system mercury(II) coming from degradation of

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thimerosal in eye-drop samples was chosen. Thimerosal (thiomersal, sodium ethylmercurithiosalicylate) is an antibacterial agent commonly used as both topical antiseptic and antimicrobial preservative in pharmaceutical formulations, especially in ophtalmic solutions [3,25]. This method is based on the enzymatic scheme proposed by Amine et al. [26]. The procedures for batch analysis and flow injection analysis (FIA) were optimised and compared with atomic adsorption spectroscopy (AAS) data. Results were in agreement showing that the biosensor-based procedure can be used alternatively to AAS with advantages in terms of rapidity and cost.

2. Experimental

2.1. Apparatus and equipment

Amperometric measurements were carried out with an Amel (Milan, Italy) model 559 HPLC detector connected with a model 868 Amel recorder. The H_2O_2 sensor consisted of Pt electrode poised at $+650$ mV versus a built-in silver/silver chloride from Universal Sensors (Metairie, LA, USA). All experiments were performed at 25°C maintained by an Haake F3 Thermostath, (Berlin, Germany), in a double wall beaker.

A three-electrode cell type wall-jet from Metrohm (Switzerland), connected with a 15 cm tube to a Rheodyne syringe loading injector model 7125 from Supelco (Bellefonte, PA, USA) was used for FIA measurements. The working buffer was pumped through the cell by a Minipulse 3 peristaltic pump from Gilson Medical Electronics (Villiers le Belle, France).

2.2. Reagents

Cellulose acetate membranes, 100 molecular weight cut-off (m.w.c.o.), were prepared in laboratory as described in the literature [27]. Polycarbonate membrane 0.6 μm pore size was from Nucleopore, (Pleasanton, CA, USA). A 0.1 M phosphate buffer (sodium phosphate monobasic + potassium phosphate dibasic) pH 6.0 and 7.0 was used. Glucose oxidase (GOx) type V-S from *Aspergillus niger* (1200 U ml^{-1}, 4.9 mg ml^{-1} protein) was from Sigma Chemical (St. Louis, MO, USA) and invertase (from baker's yeast, 290 U mg^{-1}) was from Fluka Chemie (Buchs, Switzerland). A standard stock solution was prepared by dissolving 0.1354 g of crystalline mercuric chloride in 1 N HCl and diluting to 100.0 ml with the same solvent. This solution was prepared fresh on a biweekly basis. A dilution of 1:1000 was prepared in phosphate buffer to provide a working standard solution having a concentration of 1 ppm of mercury. This dilution was carried out daily just prior to the quantitative step. Pharmaceutical preparations containing a mercurial compound were from commercial sources.

All other chemicals, of analytical reagent grade, were from Sigma.

2.3. Enzyme immobilisation

A preactivated membrane, Immobilon Affinity Membrane (Millipore, Bedford, MA, USA) was used for enzyme immobilisation. The GOx enzyme was dialysed versus 0.1 M phosphate buffer pH 7.0 at 4°C overnight to eliminate preservatives from enzymatic solution. A total of 10 μl of the enzymatic solution was then placed onto 1 cm^2 Immobilon membrane and allowed to dry at room temperature for 2 h. Unreacted sites onto the membrane were blocked by soaking with 0.1 mol l^{-1} glycine for 10 min. Before use, the membrane was washed with 1 M potassium chloride to eliminate noncovalently bound enzyme.

2.4. Electrode assembling

The glucose sensor was assembled by placing the following membranes on the jacket provided with the electrode: the 100 m.w.c.o. cellulose acetate membrane (which protects the electrode from electrochemical interferences); the enzymatic membrane and the 0.6 μm pore size polycarbonate membrane which protects the enzyme from large molecules and prevents microbial attacks. These membranes were then secured with an O-ring. The electrode jacket was filled with a solution of 0.1 M potassium chloride, the electrode

was inserted into the jacket and screwed down until the tip of the platinum surface was firmly in contact with the cellulose acetate membrane.

2.5. Batch analysis procedure

The analysis procedure reported by Amine et al. [26] was modified for our purposes.

The reactions involved are the following:

$$\text{Sucrose} + H_2O \xrightarrow{\text{invertase}} \text{D-glucose} + \text{D-fructose} \quad (1)$$

$$\text{D-Glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconic acid} + H_2O_2 \quad (2)$$

Reaction (1) is catalysed by the enzyme invertase which is added in solution. Reaction (2) is catalysed by the biosensor assembled using glucose oxidase type V-S.

The output current due to the hydrogen peroxide oxidation at the Pt electrode is correlated to the concentration of sucrose and invertase present in solution. At a fixed concentration of invertase and sucrose, the decrease of the output current can be correlated to the inhibitor concentration.

Measurements were made in two steps: the electrode was immersed in a buffered solution of invertase [2 U (2.5 ml^{-1})], sucrose was then added to a final concentration of 5 mmol l^{-1} and the signal recorded for 6 min. In order to minimize the current signal from sucrose spontaneous hydrolysis, the i (current value) at 2 min was subtracted from i at 6 min (I_1). In the second step, the enzymatic solution was incubated with a known amount of mercury, sucrose was then added and the residual enzyme activity (I_2) measured as described above.

Fig. 1 shows the steps giving rise to the typical current–time curve observed in the absence (a) and presence (b) of inhibitor. The degree of inhibition, can be calculated as follows:

$$I\% = 100 \times \frac{I_1 - I_2}{I_1} \quad (3)$$

2.6. Sample pretreatment

Mercury is often present in pharmaceutical formulations as organic compound. For this reason it is important that the samples undergo an oxidative degradation to have mercury as free mercuric ion that is the form inhibiting the enzyme invertase. A series of cleavage reactions reported in literature has been tested [27–33]. Finally, the procedure reported by Thompson and Hoffman [28] has been adapted as follows. Representative aliquots of the mercurial compound (thimerosal), pharmaceutical products and standard $HgCl_2$, were diluted 1:4 with aqua regia (nitric acid:hydrochloric acid 1:3) into Pyrex vials and heated for 1 h on a steam bath. Upon completion of the heating step, the vials and their contents were cooled under tap water. This acidic solution was diluted as appropriate just prior the determination step with phosphate buffer to adjust the pH.

2.7. Flow injection analysis procedure

The scheme of the apparatus used for FIA measurements is shown in Fig. 2.

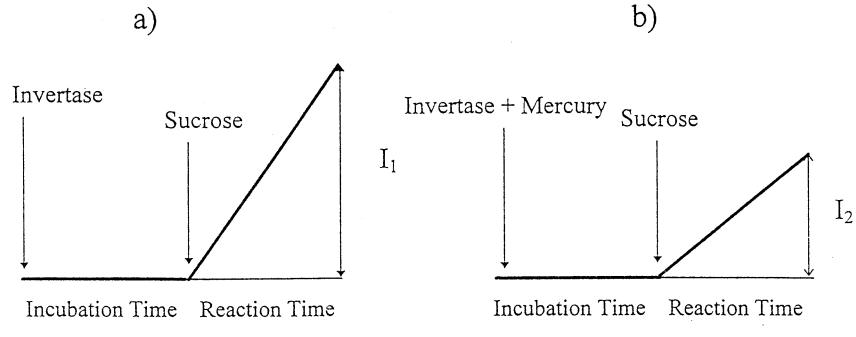

Fig. 1. Typical current–time curves obtained in the absence (a) and in the presence (b) of inhibitor.

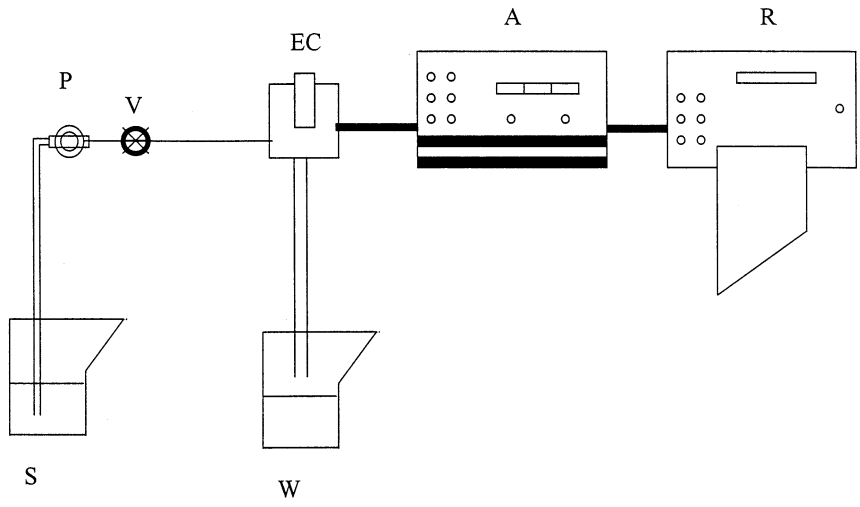

Fig. 2. Scheme of the FIA system apparatus: A = amperometer; EC = electrochemical cell; P = peristaltic pump; R = recorder; S = sucrose; V = valve; W = waste.

A carrier consisting of buffered sucrose solution was passed through the electrochemical cell by the peristaltic pump until a stable background current was reached. The injection valve loop (20 μl) was connected to the electrochemical cell with a 15 cm teflon tube (inner diameter 0.3 mm). The loop was filled with invertase and, after the injection, the transient peak current was recorded (I_1). The loop was then filled with the enzymatic solution plus sample or standard and incubated for 2 min; the peak current was taken as I_2. Eq. (3) was used for calibration curves and sample measurements. A flow rate of 0.2 ml min^{-1} was chosen as the best compromise to have a fast analysis time and a good signal.

3. Results and discussion

3.1. Batch analysis

Amine et al. [26] reported to be necessary an incubation time of 10 min for maximum value of inhibition. However, using 2 min of incubation time, good sensitivity with shorter analysis time was obtained.

Type V-S GOx was selected for this work because it has two advantages over with the type VII GOx enzyme used by Amine et al.: a higher specific activity and a lower amount of invertase present as impurity (0.006% vs. 0.17% of invertase, calculated as % of GOx activity). This resulted in better sensitivity of the glucose probe for glucose because of the higher current output and in a considerable decrease of the background current due to the limited sucrose hydrolysis.

In the experimental conditions described mercury(II) was determined in the 5–80 ng ml^{-1} range with a linearity in the 5–10 ppb range. A calibration curve obtained with standard solutions of mercury(II) is reported in Fig. 3. Each value represents the mean of ten determinations. Intraday relative standard deviation (RSD) was $\leq$ 2.5% while interday RSD was $\leq$ 4.0%.

Since invertase is reported to be inhibited by some mercurial compounds like 4-mercurybenzoate, methyl and ethylmercury [34], the inhibition effect of thimerosal itself was tested. The enzyme was slightly inhibited by thimerosal in comparison with mercury(II), in the entire range of concentrations investigated. In fact, an inhibition of 5% at a concentration of 100 ng ml^{-1} and 37% at 10 μg ml^{-1} with a maximum value of 45% at 50 μg ml^{-1} was found.

Thimerosal was then processed to protic acid cleavage and oxidated to mercuric ion as de-

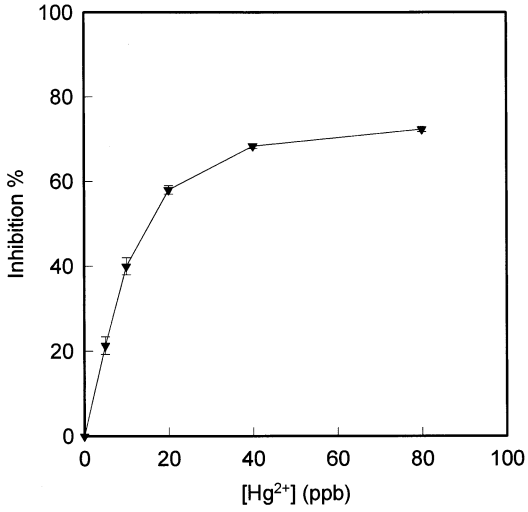

Fig. 3. Mercury(II) calibration curve obtained with batch method.

scribed in the experimental section. The standard solution was subject to the same treatment. Identical calibration curves were obtained with treated and not-treated standard; moreover a 100% mercury recovery from thimerosal was obtained indicating that the degradation procedure selected was successful and no matrix effect occurred.

The total mercury(II) content from thimerosal in eye-drop samples was then measured.

3.1.1. Sample analysis

Table 1 shows the results of five collirium samples analysed with the proposed method and compared with an atomic absorption spectrometry reference procedure. Amperometric data were calculated by interpolation of the calibration curve obtained for standard mercury(II) solutions.

Recovery study was also performed by addition of a mercury(II) standard solution to the samples (Table 1). Results obtained with the two different procedures correlated well with relative errors (RE) $< 5\%$. Only sample no. 3 gave a RE of 13% showing 88% recovery. This result may be attributed to the incomplete degradation of EDTA present in the formulation that was still partially complexing mercury(II) (no EDTA was present in the other samples). No matrix effect was observed also in this sample after increasing the samples degradation step from 30 min to 1 h.

3.2. Flow injection analysis

In order to reduce the analysis time and for a partial automation of the method, studies for the development of a FIA procedure were carried out. The best substrate concentration and amount of invertase have been evaluated for the FIA system.

The current and inhibition values obtained with a fixed amount of enzyme (0.2 U) and inhibitor (50 ppb) were investigated. An increase of substrate concentration resulted in an increase of the peak current but in similar $I\%$ value; in fact it ranged from 65 to 56% varying sucrose concentration from 5 to 50 mmol l^{-1}. A sucrose concentration of 10 mmol l^{-1} has been selected for further experimental work as the best compromise between a high current output and a low substrate requirement. Using this substrate concentration

Table 1
Total mercury(II) analysis and recovery studies performed with the biosensor-invertase batch method and with an AAS reference method

Sample[a] no.	Measured (ppb)	Added (ppb)	Found (ppb)	Recovery %	Batch mercury(II) content (ppm)	AAS mercury(II) content (ppm)	RE %
1	38	20	58	100	48	48	0
2	40	20	63	105	50	52	4
3	32	10	37	88	40	46	13
4	35	20	53	96	44	46	4
5	5	10	14	93	0.1	0.1	9

[a] Samples were diluted 1:1000 for the analysis.

Table 2
Current and inhibition values obtained with sucrose 10 mmol l^{-1} and mercury(II) 50 ppb

Invertase (U)	Current (nA)	Inhibition % [mercury(II) 50 ppb]
1.6	12.1	9
0.8	7.1	10
0.4	5.2	16
0.2	2.8	50
0.1	0.8	73

the effect of the amount of enzyme on the degree of inhibition has been studied (Table 2). As expected, $I\%$ was inversely related to the enzyme loading. A total of 0.2 U were chosen as the best compromise between a high inhibition and a good current signal.

A calibration curve has been attained with all parameters optimised using FIA system (Fig. 4). Mercury has been detected in the range 10–80 ppb with an interday RSD $\leq$ 1.5%. Linearity was found in the 10–60 ppb range. The analysis time was 4 min and the sample throughput was increased to 15 samples h^{-1}.

Glucose oxidase membranes were stable for months giving a signal of 50% of the initial current value after 3 months of use. The invertase solution was prepared daily, no changes in the activity were observed during this period.

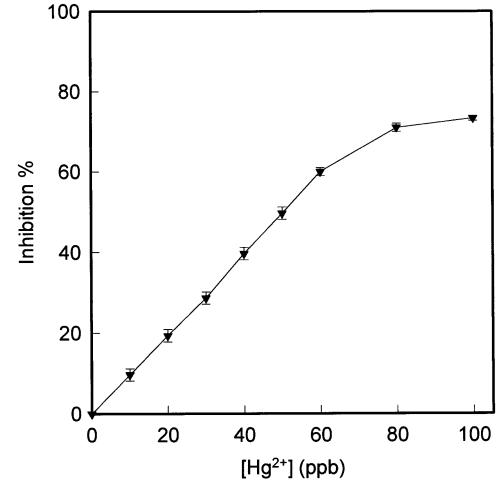

Fig. 4. Mercury(II) calibration curve obtained with FIA method.

3.2.1. Samples analysis

Table 3 shows the total mercury(II) content and the recovery studies for ten eye-drop samples analysed with the FIA system and compared with the AAS method. The recovery ranged from 98 to 106% showing no matrix effect. Mercury content measured with the FIA procedure was in good agreement with data from AAS procedure (RE $\leq$ 4%).

Table 3
Recovery studies obtained with the FIA system in 10 eye-drop samples and comparison between FIA and AAS mercury determination

Sample[a] no.	Measured (ppb)	Added (ppb)	Found (ppb)	Recovery %	FIA mercury(II) content (ppm)	AAS mercury(II) content (ppm)	RE %
1	25	25	49	98	49	50	2
2	24	25	51	104	48	47	2
3	25	25	50	100	50	52	4
4	24	25	48	98	49	50	2
5	24	25	52	106	49	49	0
6	25	25	49	98	50	49	2
7	24	25	49	100	49	50	2
8	25	25	52	104	50	51	2
9	26	25	50	98	52	53	2
10	25	25	51	102	50	51	2

[a] Samples were diluted 1:1000 for the analysis.

4. Conclusions

An amperometric procedure for determination of mercury(II) based on invertase inhibition and glucose sensor has been improved and transferred into a FIA system. Good sensitivity and reproducibility have been obtained in the 10–60 ng ml^{-1} range of mercury(II). Analysis of mercury(II) in eye-drop samples after oxidative cleavage resulted to be well-correlated with AAS determination.

The rapidity, sensitivity and easy handling operability of the method make it as a good alternative to traditional methods for mercury(II) analysis in pharmaceutical samples.

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References

[1] P.A. Steenkamp, P.P. Coetzee, Fresenius J. Anal. Chem. 346 (1993) 1017–1021.
[2] J.R. Procopio, M. Pilar Da Silva, M. Del Carmen Asenzio, M. Teresa Sevilla, L. Hernandez, Talanta 39 (12) (1992) 1619–1623.
[3] C.C. Fu, M.J. Sibley, J. Pharm. Sci. 66 (5) (1977) 738–739.
[4] G.A. Drash, in: H.G. Seiler, A. Sigel, H. Sigel (Eds.), Dekker, Handbook on Metals in Clinical and Analytical Chemistry, New York, 1994, 479–493.
[5] S. Brown, J. Savory, M.R. Wills, in: A.J. Pesce, L.A. Kaplan (Eds.), Mosby, Methods in Clinical Chemistry, St. Louis, 1987, pp. 405. [6] D. Mealor, A. Townshend, Talanta 15 (1968) 747–758.
[7] J. Maslowska, J. Leszczynska, Talanta 32 (1985) 883–886.
[8] R. Benesch, R.E. Benesch, J. Am. Chem. Soc. 73 (1951) 3391–3396.
[9] J.E. Page, J.G. Waller, Analyst 74 (1949) 292–296.
[10] M. Korolczuk, Fresenius J. Anal. Chem. 357 (1997) 389–391.
[11] S.H. Omang, Anal. Chim. Acta 63 (1973) 247–253.
[12] J.S. Fleitman, I.W. Partridge, D.A. Neu, Drug Dev. Ind. Pharm. 17 (4) (1991) 519–530.
[13] P.C. D'Haese, G.F. Van Landeghem, L.V. Lamberts, M.E. De Broe, Mikrochim. Acta 120 (1995) 83–90.
[14] H. Morita, H. Tanaka, S. Shimomura, Spectrochim. Acta 1 (50B) (1995) 69–84.
[15] M. Pilar da Silva, J.R. Procopio, L. Hernandez, J. Chromatogr. A 761 (1997) 139–146.
[16] T.N. Shekhovtsova, S.V. Muginova, N.A Bagirova, Anal. Chim. Acta 344 (1997) 145–151.
[17] M. Bernabei, S. Chiavarini, C. Cremisini, G. Palleschi, Bios. Bioel. 8 (1993) 265–271.
[18] C. Cremisini, S. Di Sario, J. Mela, R. Pilloton, G. Palleschi, Anal. Chim. Acta 311 (1995) 273–280.
[19] A. Amine, M. Alafandy, J.M. Kaufmann, M. Novak Pekli, Anal. Chem. 67 (1995) 2822–2827.
[20] D. Liu, A. Yin, K. Chen, L. Nie, S. Yao, Anal. Lett. 28 (8) (1995) 1323–1337.
[21] S. Fennouh, V. Casimiri, C. Burstein, Bios. Bioel. 12 (2) (1997) 97–104.
[22] J.C. Gayet, A. Haouz, A. Geloso-Meyer, C. Burstein, Biosens. Bioelectron. 8 (1993) 177–183.
[23] C. Tran-Minh, Ion-Sel. Elec. Rev. 7 (1985) 41–75.
[24] B. Mattiasson, B. Danielsson, C. Hermansson, K. Mosbach, FEBS Lett. 85 (2) (1978) 203–206.
[25] S.W. Lam, R.C. Meyer, L.T. Takahashi, J. Parent. Sci. Tech. 35 (5) (1981) 262–265.
[26] A. Amine, C. Cremisini, G. Palleschi, Mikrochim. Acta 121 (1995) 183–190.
[27] M. Mascini, F. Mazzei, D. Moscone, G. Calabrese, M. Benedetti, Clin. Chem. 33 (1987) 591–593.
[28] D.R. Thompson, T.J. Hoffmann, J. Pharm. Sci. 64 (11) (1975) 1863–1866.
[29] B.J. Meakin, Z.M. Khammas, J. Pharm. Pharmacol. 31 (1979) 653–654.
[30] O. Szakacs, A. Lasztity, Z.S. Horvath, Anal. Chim. Acta 121 (1980) 219–224.
[31] P.Y.T. Chow, T.H. Chua, K.F. Tang, B.Y. Ow, Analyst 120 (1995) 1221–1223.
[32] G. Dorr, D. Deinzer, Fresenius J. Anal. Chem. 357 (1997) 117–120.
[33] B.A.I. Razagui, J.S. Haswell, J. Anal. Toxicol. 21 (1997) 149–153.
[34] J. Keesey, in: J. Keesey (Ed.), A revised biochemical reference source, Boehringer-Mannheim, Indianapolis, 1987, pp. 164.