Limitations in the Analytical Use of Invertase Inhibition for the Screening of Trace Mercury Content in Environmental Samples

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The possibility of screening the mercury(II) content in real environmental samples based on inhibition of the activity of dissolved invertase has been examined. The extent of inhibition was measured with an amperometric glucose biosensor with glucose oxidase immobilized on a membrane. Data concerning the stability and reproducibility of measurements are provided. The effects of heavy metals on the inhibition of invertase, together with that of common anions such as chloride, nitrate and sulfate are reported. The determination of mercury using this procedure has been carried out in samples of natural and waste water samples of various origins already analyzed by ICP-AES, by spiking ppb levels of mercury(II). Differences in the inhibiting effect of the samples and in the recoveries were found and are discussed.

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Introduction

Inhibition of enzyme activity is a phenomenon widely investigated in biochemistry and life science. It is also of significant importance for chemical analysts, as a chemical interaction that can be potentially used for developing enzymatic biosensors for analytes, which do not act as substrates of enzymes. Such applications can be based on knowledge concerning various mechanisms of inhibition, as well as on the wide literature available on these processes for hundreds of enzymes of various classes.^{1,2} The analytical applications of these processes include the determinations of numerous organic analytes and inorganic species (metal ions, some anions).^{3,4} Most often, the inhibition of enzyme activity is primarily considered as being a convenient way to determine the total content of various species that affect the enzyme activity, such as, for instance, pesticides or heavy metals.

Mercury species, that inhibit the activity of numerous enzymes, were already targeted for the development of different enzyme biosensors based on inhibition, with urease and invertase usually being selected for this purpose. The analytical use of the inhibition of urease by Hg(II) has been proposed by different authors,⁵⁻⁷ however, in order to obtain sufficient detection limits, fluorometric detection is needed.^{5,6} Such methods can find only limited applications, for instance in water analysis, because of the presence of Cu, Ni and Cd ions at concentrations much higher than Hg(II), which also inhibit the urease activity. The use of invertase seems to be more

favorable in terms of selectivity, although the activity and the inhibition pattern of the enzyme have been reported to be strongly dependent on the pH.⁸ Metal cations, such as Cu(II), Zn(II), Cd(II) and Pb(II) inhibit the activity of invertase only in the ppm range, while Hg(I), Hg(II) and Ag(I), do so in the ppb range. The latter cation is likely to be of the insoluble form in natural samples. An analytical application of the inhibition of invertase was already pioneered in the late sixties, when the inhibition by Hg(II) and Hg(I) was reported in the submicromolar range of concentration, whereas Ag(I) was in micromolar range of concentration.⁸ Later, this procedure was employed for the determination of Hg(II) in water samples and soft drinks; however, the results were not verified by other methods.⁹

Another clear advantage of using invertase is the possibility of measure the enzymatic activity amperometrically. In fact, the low cost of electrochemical instrumentation is coupled in this case with the intrinsic sensitivity of the generated signal (*i.e.* directly proportional to the detected analyte). Determinations of Hg(II) and alkyl-mercury compounds based on the inhibition of invertase and amperometric detection with an enzyme glucose probe were introduced by Amine *et al.*¹⁰ This method was satisfactory employed for the determination of Hg(II) in pharmaceuticals in the concentration range from 10 to 60 ppb,¹¹ both in batch and flow-injection systems. The results obtained in an FIA set-up in eye-drop samples, containing practically no interferences for such an assay, correlated well with the AAS data. Recently, the same method was employed in a batch-injection system, using the amperometric detection of glucose with a Pt electrode modified with glucose oxidase, or with a copper modified glassy carbon electrode without a second enzymatic step in the determination of glucose.¹² It was

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reported that the bi-enzymatic procedure provides a better sensitivity of detection. Satisfactory results were also reported for the recovery of the determination of Hg(II) in three samples of wastes of non-specified origin. Determinations of 5 to 80 ppb Hg(II) based on the inhibition of invertase were also reported using a controlled-porous glass reactor containing glucose oxidase and catalase with thermometric detection, and applied in pharmaceutical preparations,¹³ which are usually samples with not very complex matrices.

Despite the elevated number of publications in the enzyme inhibition-based methods area, the majority of the systems are not challenged by real samples.⁴ The aim of this work was to examine a batch system with dissolved invertase and a membrane based amperometric glucose biosensor for the determination of Hg(II) in samples of different natural water samples and wastes.

Experimental

Reagents and instrumentation

Glucose oxidase (*Aspergillus niger*; Type VII; E.C. 1.1.3.4) and invertase (*Baker's yeast* E.C. 3.2.1.26) were bought from Sigma Chemical Co. (St. Louis, MO, USA); all other reagents were of analytical grade from either Sigma or Carlo Erba (Milan, Italy).

Immobilon AV membranes were from Millipore (Bedford, MA, USA), polycarbonate membranes (0.3 porosity) were from Nucleopore (Pleasanton, CA, USA), and nylon membranes (60 mesh, 100 μ m thickness) were from a local shop in Rome.

The hydrogen peroxide probe consisted of a platinum electrode polarised at +650 mV vs. a built-in Ag/AgCl reference electrode (Universal Sensors, Metairie, LA, USA). The current output was monitored using an Amperometric Biosensor Detector (Universal Sensors).

Procedures

The immobilization of glucose oxidase on a nylon-polyethylenimine membrane was carried out as reported by Thompson *et al.*¹⁴ at room temperature, a 5 cm² nylon-net membrane was immersed in methylene chloride (CH₂Cl₂) for 10 min, and then in a 0.1 M triethyloxonium-tetrafluoroborate (TOTFB) prepared in CH₂Cl₂, for 5 min. The membrane was then washed three times with freshly prepared iced methanol and immersed in an aqueous solution of polyethylenimine (5% w/v) for 3.5 h at room temperature. After a three-cycle washing step in distilled water (D.W.), the nylon-net membrane with immobilized polyethylenimine was allowed to react with glutaraldehyde (1% v/v in 0.1 M carbonate buffer, pH 10.0) for 40 min. After several washing steps in deionized water, the membrane was incubated overnight at 4°C with a 5 mg/mL GOD solution prepared in a 0.1 M phosphate buffer, pH 6.85. It was finally washed with a 0.5 M glycine solution for 30 min. The membrane was cut to obtain a final superficial area of approximately 1 cm². Immobilization on Pall Immunodyne and Immobilon AV membranes was performed as in Palleschi *et al.*¹⁵ 0.5 mg of an enzyme was dissolved in 10 μ L of a phosphate buffer (0.1 M, pH 7.0), spread on one side of a 1 cm² membrane and allowed to dry for 1 h, followed by rinsing in a 0.1 M glycine solution for 30 min. When not in use, the membranes were stored in a 0.1 M phosphate buffer (pH 7.0) containing Kathon (0.1 % v/v) as an antibacterial.

The glucose probes were assembled by placing an enzymatic membrane and an polycarbonate membrane on an electrode. The membranes were then secured with an O-ring. The

electrode jacket was filled with 0.1 mol L⁻¹ potassium chloride (supporting electrolyte), then placed onto the platinum base sensor and screwed down until the tip of the platinum was firmly in contact with the enzyme membrane.

The procedure of batch determination, based on the inhibition of invertase, was the same as that reported in earlier papers.^{10,11} A selected amount of invertase was added to a buffered solution (2.5 mL final volume); then, after a fixed time of incubation with the inhibitor (2 min), a freshly prepared solution of sucrose (final concentration 10 mM) was injected, and the change in the signal of a glucose electrode was recorded (8 min recording). The percent of inhibition was calculated as the ratio of the amperometric signal obtained for inhibited enzyme and for a blank (measurement without inhibitor). All samples were diluted to 1:5 (0.5 mL in 2.5 mL) for the analysis.

Based on observations of a numerous series of measurements, it was concluded that the best signal stability was obtained by washing the biosensor after each measurement of inhibition for 0.5 min with a 1 mM EDTA solution. It was also better to carry out two blank measurements before each inhibition; the mean of these two measurements was taken as a reference value for determining of the percent inhibition.

Results and Discussion

Optimization of batch measuring system with glucose biosensors

Detection of the inhibition of invertase is based on measurements of the D-glucose concentration produced during the catalysed hydrolysis of sucrose:

For this reaction, strong non-competitive inhibition by Hg(II) has been observed,¹⁶ as well as weaker inhibition by several other metal ions. The inhibition of invertase by metal ions has been reported to be significantly weaker in the case of an immobilized enzyme;¹⁷ hence, the use of a soluble enzyme is more advantageous for trace analysis. The non-competitive inhibition of invertase was also observed for glucose and fructose,^{18,19} which may be sources of the often observed changes in sensitivity of the determinations based on inhibition. Thus, a careful optimization of the assay conditions is required. Among the other inhibitors of invertase, there are glycoproteins,²⁰ polypeptides²¹ and alkaloids.²² Some effect on the invertase activity was observed under saline conditions,²³ and also at elevated carbon dioxide concentrations.²⁴

The glucose produced by the invertase was detected amperometrically using an hydrogen peroxide-based glucose electrode. Such a bi-enzymatic procedure can be used due to the much lower inhibition of glucose oxidase (GOD) by heavy metal ions. This has already been reported for covalent immobilization onto a membrane.²⁵

The sensitivity of the amperometric determination of glucose greatly influences the minimum detectable levels of the inhibitor. In fact, a sensitive measurement allows the use of a lower amount of enzyme, which is inhibited by a lower amount of the inhibitor. For this reason, no strategy to improve the selectivity vs. hydrogen peroxide (*e.g.* an additional internal or electropolymerised membrane) was adopted, because sensitivity, and not selectivity, is the main issue in this kind of measurement. In order to protect the enzyme from bacterial attack, a polycarbonate membrane of large porosity was placed

Fig. 1 Response to 0.2 mM glucose in consecutive calibration runs obtained for glucose amperometric biosensors with different membranes used for the immobilization of glucose oxidase. Measurements were carried out in a 0.1 M phosphate buffer of pH 6.2.

Fig. 2 Example of recording obtained for measurements of the inhibition of 2 units of invertase with Hg(II) together with two recordings for blanks. Measurements were carried out with a glucose biosensor with the Immobilon membrane in a 0.1 M phosphate buffer of pH 6.2.

externally. The employed biosensor should obviously provide sufficiently stable and reproducible signals in order to keep the sensitivity at the maximum level and to avoid frequent calibration. Three different types of membranes were tested for stability: Immunodyne, Immobilon AV and nylon-PEI. All of the membranes tested for the immobilization of GOD exhibited good sensitivity. Figure 1 shows the response to 2×10^{-4} M glucose after consecutive calibrations of Hg(II), carried out as described in the Experimental section. Each calibration curve consisted of 4 different concentrations of Hg(II) tested in duplicate (10, 20, 30, 50 ppb); one calibration curve per day was run with the electrodes left at 4°C in the working buffer when not in use. Considering a very similar sensitivity and stability, the Immobilon AV membrane and nylon-PEI membrane were then selected for further studies; in the latter immobilization, the additional activation with polyethyleneimine increases the binding efficiency of nylon without compromising the sensitivity. Figure 2 shows an example of the type of signals obtained using a Immobilon membrane based glucose biosensor, with 2 units of invertase in solution, for inhibition by Hg(II) in the 10 – 30 ppb range for 2 min and signal measurements after 8 min, because of sucrose addition to the reaction mixture.

The use of the same amount of invertase and measurements with a glucose biosensor with Immobilon and nylon membranes provides a similar response curves for inhibition with Hg(II), but with the earlier observed plateau of the signal exhibited for

Fig. 3 Calibration plots for the inhibition of invertase with Hg(II) obtained for (A) 2 units of invertase with a glucose biosensor with Immobilon (■) and nylon membranes (●) and (B) for 1.2 units of invertase with a biosensor with a nylon membrane used for the immobilization of GOD.

a biosensor with a nylon membrane. The results shown in Fig. 3A are the means for six calibration plots for each biosensor, and were taken as described in the Experimental section. Some statistical characteristics are reported in Table 1 for measurements run during two consecutive days.

An adjustment of the sensitivity can be made by changing the activity of the enzyme used in the measurement. As reported earlier in the literature,¹⁰ the inhibition effect of Hg(II) is not linearly related to the amount of enzyme; thus, the gain in the sensitivity could be remarkable at very low levels of the enzymatic activity, provided that the analytical signal is still satisfactory. Using 1.2 units of invertase and the nylon-PEI glucose biosensor, the curve plotted in Fig. 3B was obtained. Taking into account the obtained sensitivity of inhibition, this system was considered to be applicable for screening purposes.

Sensitivity to various inhibitors

The data about inhibition by different metal ions obtained in this bioassay format using 2 units of invertase and glucose amperometric biosensor with Immobilon membrane are collected in Table 2. The obtained values can be affected by different experimental factors; hence, their comparison to other literature data should take this into account. Undoubtedly, the strongest inhibitors are Hg(II) and Hg(I) species. The latter analyte is usually not considered because it does not occur in most analyzed samples. Only about half weaker is the inhibition observed for organometallic mercury compounds, such as methylmercury, ethylmercury and phenylmercury (Fig. 4). Among other examined metal ions, the strongest inhibition was exhibited by the silver(I) and vanadium(V) species.

It was also interesting to evaluate how the inhibition of invertase by Hg(II) may be affected by the presence of other macro- and micro-components, which can be potentially present in environmental samples, and which can alter the chemical

Fig. 4 Calibration plots obtained for the inhibition of 2 units invertase with organometallic mercury species with a glucose biosensor with the Immobilon membrane in a 0.1 M phosphate buffer of pH 6.2.

form of mercury(II) in natural samples. At the 10 ppb level of Hg(II), the effects of 10 to 500 ppm of chloride, sulfate and nitrate were tested; the obtained data are given in Table 3. In spite of the fact that strongest complexation of mercury(II) is exhibited by chloride, for which the largest effect was expected, the inhibiting effect of mercury(II) was decreased by any of the anions at a level of 0.5 g/l. This can not be explained, however, by a strong effect of the ionic strength, since all measurements are carried out in a 100 mM phosphate buffer. This should be rather attributed to interactions that alter the form of mercury(II) in analyzed solutions.

In the concentration range from 1 to 10 ppm, no effect was found of the presence in solution of a model surfactant, sodium dodecylsulfate, and humic substances on invertase inhibition with 10 ppb Hg(II).

Measurements in real environmental samples

The application of the optimized analytical procedure for the determinations in complex matrices is always a challenge, even if all of the factors affecting the results of determinations were predicted and taken into account during optimisation of the analytical procedure. This is particularly true for enzymatic systems and for inhibition based procedures, where the results depend on numerous physical and chemical factors.

The assay was initially tested in 9 different samples of various natural water samples, in which the content of 12 trace metals and also calcium, potassium and sodium, were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The measured contents of the elements that may contribute to the inhibition of invertase are listed in Table 4, together with the content of calcium, which is the main component contributing to the salinity of the analyzed samples. The origin of tested samples is shown together with the results of inhibition measurements in Table 5. Determinations were carried out by the addition of 0.5 mL of the analyzed sample to 2.0 mL of 0.1 M phosphate buffer with invertase and with the use of a glucose biosensor with the Immobilon membrane. Determinations were also carried out for samples spiked with 10 ppb Hg(II) to control the recovery. The content of mercury determined in tested samples spectrometrically was in the range of 0.09 to 0.20 ppb, well below the detection limit of the inhibition assay. Although no inhibition from Hg(II) was expected from any of the samples, all of them exhibited some effect on invertase activity (average of 3 measurements). If we define 2-times the standard deviation of the background signal as the detection limit of our assay, it turns out from the data of Table 1 that this corresponds to about

Table 1 Statistical data about stability and reproducibility of measurements of blanks and invertase inhibition by 10 ppb Hg(II) using 2 units of invertase and nylon based glucose biosensor

Consecutive day of measurement	Blank measurement		Inhibition by 10 ppb Hg(II)	
	Mean current/nA	RSD, %	Mean inhibition, %	RSD, %
1	2.45	6.7	26.8	28
2	2.80	6.2	27.4	22

Table 2 Inhibition of invertase measured for 2 units of enzyme, 2 min inhibition time and signal measurements 8 min after the addition of sucrose in a 0.1 M phosphate buffer of pH 6.0

Inhibitor	Concentration	Inhibition, %
Ag(I)	1 ppm	99.5
V(V)	10 ppm	66
Cd(II)	100 ppm	58
Cu(II)	100 ppm	47
Ni(II)	100 ppm	34
Pb(II)	100 ppm	12
Co(II)	100 ppm	9
Fe(III)	100 ppm	9
Mn(II)	100 ppm	6
Cr(III)	10 ppm	8
	100 ppm	46
Cr(VI)	10 ppm	15
	100 ppm	2
Hg(II)	5 ppb	15
	10 ppb	31
	20 ppb	63
Hg(I)	10 ppb	55

a 13% decrease in the signal, and to a concentration of 4 ppb on the calibration curve of Fig. 3A. At this concentration level, we have by definition a 50% maximum error. Among the samples tested, six out of nine were below this detection limit, two were just above and one sample (industrial channel) strongly inhibited the invertase activity (44%). The measurement of the inhibition after spiking with 10 ppb of Hg(II) the samples gives information on the ability of the enzyme to be inhibited in such matrices. Eight of the nine samples were still inhibited to some degree with recoveries ranging from 32 to 91%. The recovery of 80% or higher in spiked samples was considered to be satisfactory. From the obtained data it is evident that the direct inhibition effect should take into account the effect on the activity by all components of the natural samples. Moreover, the recoveries clearly demonstrate that Hg(II) does not inhibit invertase to the same extent in samples of different origin. The direct use of the assay rarely allows one to obtain semi-quantitative data, but could rather be used to give a yes/no answer in some case concerning Hg(II) presence for those samples giving an initial inhibition above the detection limit, and exhibiting satisfactory results of the recovery test.

One of the suggestions given concerning inhibition-based methods to overcome problems due to the lack of selectivity is to carry out an additional separation step before the analysis.⁴ For this reason, another series of determinations was carried out for another set of natural water samples, where additionally solid-phase extractions (SPE) with C18 columns were carried out as a sample pretreatment. In this series of measurements, a

Table 3 Effect of the presence of common anions on the relative decrease of inhibition % measured for the inhibition of 2 units of invertase by 10 ppb Hg(II)

Anion	Relative decrease of inhibition at various level of anion in the sample, %		
	10 ppm	100 ppm	500 ppm
Chloride	0	10	56
Nitrate	0	10	40
Sulfate	0	0	32

Table 4 Element analysis of examined water samples by ICP-AES

Sample No.	Determined content of elements				
	Ca, ppm	Fe, ppb	Hg, ppb	Mn, ppb	Cu, ppb
1	37	50	0.15	29	0.3
2	75	3	0.12	< 0.5	4.5
3	77	521	0.16	77	2.2
4	77	673	0.18	80	3.2
5	44	17	0.19	45	< 0.3
6	81	45	0.09	24	3.8
7	122	361	0.09	343	1.7
8	62	44	0.18	100	1.9
9	89	497	0.20	155	1.7

Content of other heavy metals in all samples: Zn, 4 – 230 ppb; Co, below 1.0 ppb; Pb, up to 4 ppb; Cr, up to 1.9 ppb; Cd, below 0.3 ppb; Ti, up to 1.0 ppb; Ni, up to 3.3 ppb; V, up to 1.0 ppb.

glucose biosensor with a nylon membrane and 1.2 units of invertase was used in order to obtain a higher sensitivity of inhibitor determination (see Fig. 3B). For each natural sample, the determination was carried out without and with the SPE step. Determinations were carried out for samples without Hg(II) addition and in samples spiked with 2, 5 and 10 ppb Hg(II). The results obtained for tap water from Rome, river water, sea water and also for untreated and treated urban waste water samples are given in Table 6. The inhibition was below, or of the same level, as the detection limit for all samples apart from seawater (detection limit was $\sim 1.0 \pm 0.5$ ppb in this case; see Fig 3B). The use of the SPE pretreatment with the C18 sorbent in most cases did not significantly affect the results of a measurement of inhibition. The recovery values were fair for screening purposes for tap and river water samples as well as for treated urban waste, whereas evidently lower results were found for remaining samples, seawater and untreated urban waste, which can be attributed to strong salinity or an undefined effect of other contaminants in untreated waste samples, respectively. An SPE pretreatment with C18 columns showed some effect on the assay in the case of seawater samples and tap water spiked with 2 ppm Hg(II). Practically, substances that can be adsorbed on C18 do not affect the results of determinations based on the inhibition of invertase.

Conclusions

Attempts for the direct determination of trace levels of mercury(II) in various environmental samples based on the inhibition of invertase and the detection of glucose with an amperometric enzymatic biosensor, with a few exceptions, gave

Table 5 Recovery results of inhibition measurements in water samples spiked with 10 ppb Hg(II) using 2 units invertase and glucose biosensor with Immobilon membrane for GOD immobilization

Sample No.	Sample origin	Inhibition, %	Recovery, %
Satisfactory results			
5	Deep well water	6	91
6	Pond in Warsaw downtown I	15	80
9	Industrial channel	44	86
Non-satisfactory results			
1	Mineral water I	7	49
2	Mineral water II	10	29
3	River water I	22	32
4	River water II	11	40
7	Pond in Warsaw downtown II	8	57
8	Lake close to power plant	6	0

Table 6 Results of inhibition measurements in natural water samples and wastes without and with spiking with Hg(II) using 1.2 units invertase and a glucose biosensor with a nylon membrane for the immobilization of GOD

Sample	Determined	Recovery in spiked samples, %		
	I, %	2 ppb	5 ppb	10 ppb
Tap water	7	70	90	110
Tap water after C18	6	100	105	103
River water	16	73	122	77
River water after C18	17	63	79	67
Seawater	23	75	57	42
Seawater after C18	5	72	55	67
Urban waste untreated	5	40	15	11
Urban waste untreated after C18	5	40	15	11
Urban waste treated	8	94	81	100
Urban waste treated after C18	9	103	77	94

results at the level of the detection limit of the method (1 to 5 ppb, depending on activity of invertase used for each determination). Results concerning determination of the metal ions content in the examined samples with the ICP-AES method indicate that inhibition effect in natural samples can not be attributed only to the metal content in the samples. Preliminary treatment of samples with C18 columns has in certain cases an effect on the results of the determination. In principle, the procedure can be used for monitoring mercury(II) species, in matrices that have been demonstrated not to hinder the inhibiting effect. Thus, the determinations of total mercury in a particular site of sampling (*e.g.* from a lake or a wastewater treatment plant) is required before using the assay procedure. Each measurement of invertase inhibition should thus be accompanied by measurements of the recovery in spiked samples, because only its satisfactory value indicates that there is no strong effect of high salinity, or the presence of strongly complexing ligands.

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