

Fast determination of antibiotics in whole blood

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Abstract

There is a need for analytical methods capable of monitoring blood antibiotic levels in real time. Here we present a method for quantifying antibiotic levels in whole blood that does not require any sample pretreatment. The tests employ the enzyme penicillinase to assay for penicillin G, penicillin V and ampicillin using a flow-injected biosensor, the Enzyme Thermistor. Optimal flow rates, sample volumes and pH were determined to be 0.5 mL/min, 100 μ L and 7.0, respectively. Analysis of the antibiotics diluted in buffer gave a linear range of 0.17–5.0 mM. Calibration curves prepared using blood spiked with the antibiotics gave a linear range of 0.17–2.0 mM. Linear regression values for all of the calibration curves were 0.998 or higher. Assay cycle time was 5 min. The relative standard deviation value for 100 determinations of a mock blood sample spiked with penicillin G was 6.71%. Despite the elimination of sample pretreatment, no detectable clogging or signal drift was observed. The assay provides a fast, simple, reliable analytical method for determining antibiotic concentrations in blood without the need for any sample pretreatment. This is an important first step towards developing a device capable of real-time monitoring of antibiotic levels in whole blood. The technology has the potential to significantly improve the outcomes of patients undergoing critical care.

Keywords: Antibiotics, biosensor, blood, critical care, penicillinase

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Introduction

Antimicrobial resistance is rapidly becoming a major threat to global public health. The World Health Organization estimates that infectious and parasitic diseases are the second leading cause of death worldwide [1]. Multi-drug resistance has major economic consequences as well [2]. With few new antibiotics in the 'pipeline', health authorities will have to make do with the antibiotics that are currently available [3,4]. As a result, there is a global drive to reduce the development and spread of antibiotic resistance [5].

Unfortunately current diagnostic methods for identifying diseases are slow, which results in over-prescription of antibiotics, which increases the spread of antibiotic resistance [6]. Faster diagnostics have been developed, but these

methods are expensive, which is delaying their implementation [7,8]. Improved antibiotic testing regimens are urgently needed. One approach is to continuously monitor antibiotic blood levels. This has the potential to dramatically improve patient outcomes, especially for patients undergoing critical care treatment, such as trauma victims, patients with postoperative infections and those undergoing treatment of multidrug resistance. Current assay methodology is too slow for real-time antibiotic monitoring.

In this context, enzyme-based biosensor assays have several advantages [9]. First, enzyme specificity makes it possible to specifically identify compounds, which eliminates the need for time consuming analytical identification. Second, over 200 different β -lactamases have been identified to date [10], which provides a range of choices. Third, these enzymes are robust, highly active enzymes making them ideal for thermal biosensing [11]. Fourth, our assay detects primarily unbound, biologically active antibiotics and so provides a more accurate measurement of activity. In the case of antibiotics, bioavailability is particularly important because serum proteins bind antibiotics [12].

Our group has developed a flow-injected thermal enzyme biosensor, the Enzyme Thermistor. This device detects the heat generated by enzymatic reactions to quantify the presence of specific analytes [13,14]. To achieve real-time monitoring, it was necessary to eliminate sample treatment. Analysis of unpretreated blood is considered very difficult. As a result, few assays based on whole blood analysis have been published despite growing interest from medical practitioners. Here we present the development of a method that employs penicillinase to detect β -lactam antibiotics in whole unpretreated blood using the Enzyme Thermistor device.

Materials and Methods

Chemicals and materials

All chromatographic materials were purchased from Sigma (St Louis, MO, USA). Imipenem was bought from Fresenius Kabi (Uppsala, Sweden). Penicillinase (E.C. 3.5.2.6) from *Bacillus cereus* and penicillin G potassium salt were purchased from Sigma-Aldrich (St Louis, MO, USA). The penicillin V potassium salt and ampicillin were obtained from Sigma-Aldrich. The propylamino-derivatized controlled-pore glass beads (CPG), with a diameter of 125–140 μm and pore size of 50 nm, were obtained from Steinachglas (Steinach, Germany). All other chemicals were purchased from Sigma-Aldrich.

Instrumentation and column preparation

The single-channel Enzyme Thermistor device used in this study has been previously described [14]. The setup consists of

a peristaltic pump (Gilson Minipuls 2, Villiers-le-Bel, France) mounted with one tube for each channel, a pneumatic injection valve with a 100- μL sample loop (Valco Instrument Co. Inc., Houston, TX, USA) and the Enzyme Thermistor instrument (Omik Bioscience AB, Lund, Sweden). A schematic of the device is shown in Fig. 1. The working temperature was set to 25°C and the amplifier was set to 200 amplification scale.

The enzyme column was prepared by immobilizing 50 units of penicillinase onto CPG as previously described [15]. In brief, 700 mg CPG were glutaraldehyde activated in phosphate buffer at room temperature under reduced pressure for 30 min, and then at ambient pressure for an additional 30 min. The CPG were extensively washed, after which penicillinase (50 units) was added. The CPG were incubated for 2 h at room temperature, and then at 4°C overnight under agitation. Enzyme-bound CPG were washed and remaining sites were blocked by the addition of 1 mL coupling buffer containing 0.2 M ethanolamine. After 2 h incubation, the CPG were washed and then packed into a column.

Antibiotic determinations

Phosphate buffer (100 mM, pH 7.0) and a modified PBS (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 52 mM NaF and 3.4 mM EDTA, pH 7.4) were the only buffers used in these studies. All the solutions were preparing using de-ionized (18 M Ω) water from a Milli-Q system (Millipore, Bedford, MA, USA).

Antibiotic stock solutions were prepared for penicillin G, penicillin V and ampicillin in phosphate buffer. Antibiotic dilution series were prepared in buffer using the antibiotic

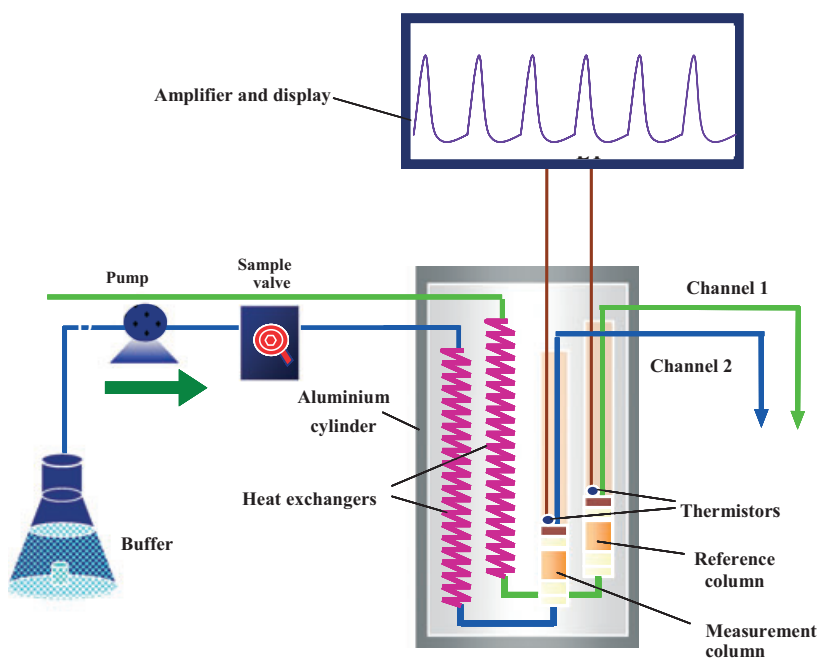


FIG. 1. Schematic for the flow-injected Enzyme Thermistor instrument.

stock solutions. The phosphate buffer was used as running buffer.

Blood samples (5 mL/tube) were taken in EDTA-coated tubes from the antecubital vein of a healthy volunteer and stored on ice after collection. Mock antibiotic blood dilution series were prepared using the antibiotic stock solutions. The modified PBS buffer was used as the running buffer.

A sample volume of 100 μ L and a flow rate of 0.5 mL/min were used unless otherwise stated. The calibration curve data were statistically analysed to determine the standard deviation values and relative standard deviation (RSD) values. The difference between groups was analysed using one-way analysis of variance, and in cases where there was significance, a Dennett test was applied. *p* values less than 0.05 were considered significant.

Results

Assay optimization

A series of experiments were performed to determine the effect of sample volume and flow rate on the sensitivity and linear range of the assay using penicillin G as the substrate (data not shown). The results showed that these parameters affected both the linear range and sensitivity of the assay. The results were highly reproducible under all conditions tested. For the purposes of this study, a flow rate of 0.5 mL/min and a sample volume of 100 μ L were chosen.

Studies were also performed to determine the effect pH had on the assay (Fig. 2). Background values have been subtracted from each of the curves to eliminate pH-dependent thermal interference effects. The results show that penicillinase activity was highest at pH 6.5, which confirms previously reported results [16]. Linear regression values of 0.999136,

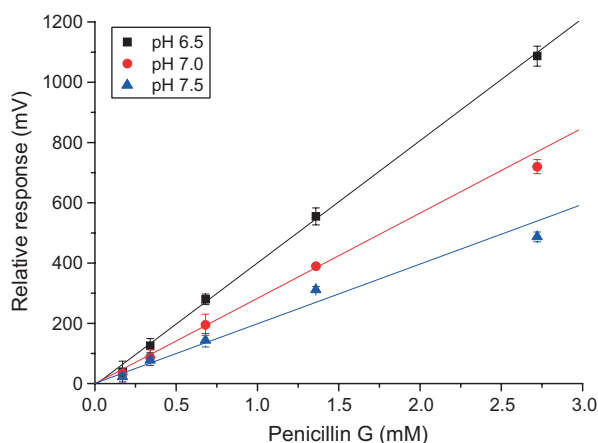


FIG. 2. Penicillin G calibration curves at various buffer pH values.

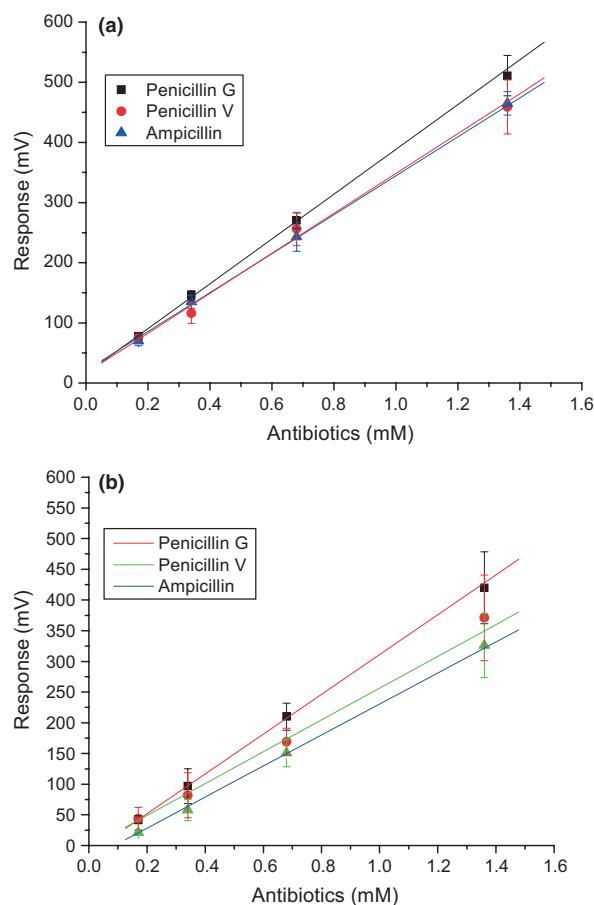


FIG. 3. Calibration curves for the antibiotics determined in buffer (a) and in whole blood (b).

0.99827 and 0.987737 were obtained for pH 6.5, 7.0 and 7.5, respectively. A neutral pH of 7.0 was chosen for the buffer calibration studies.

Antibiotic determinations

The ability of the assay to measure penicillin G, penicillin V and ampicillin in buffer and whole blood was investigated. The breakdown of the β -lactam antibiotics by penicillinase resulted in large, highly reproducible thermal signals. The linear range for all of the antibiotics diluted in buffer was 0.17–3 mM. The linear regression values were 0.999 or better in all cases (Fig. 3a).

Whole human blood samples were spiked with the concentrated antibiotic solutions prepared in the modified PBS buffer (see Materials and Methods section). These dilution series were used to prepare calibration curves (Fig. 3b). Apart from the addition of antibiotic, the blood samples were untreated. The linear regression values for the curves were 0.9991 or better for all three antibiotics. The linear range and slope of the resulting calibration curves was similar to those obtained in the buffer study. However, the calibration curves

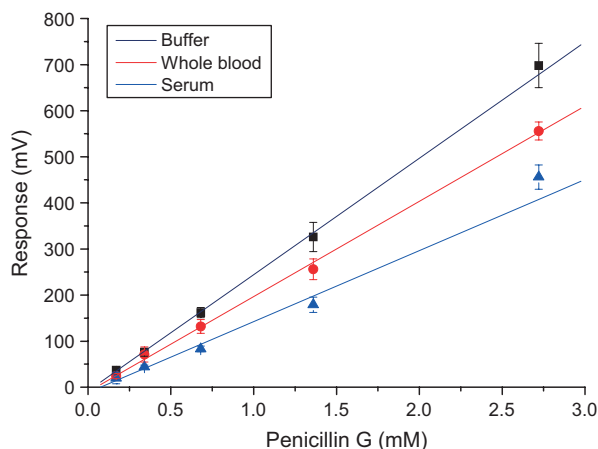


FIG. 4. Calibration curves for the penicillin G spiked samples.

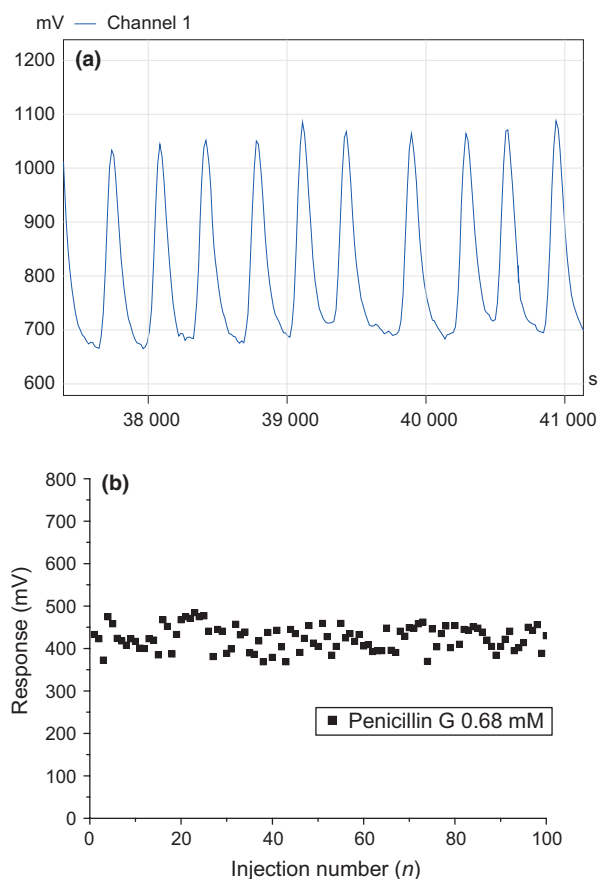


FIG. 5. Repeatability study using antibiotic-spiked whole human blood: (a) actual chart recorder readout and (b) graph of the 0.68 mM penicillin G data set.

for whole blood were slightly shifted downwards. Analysis of whole blood alone gave a response of 37 mV, which was lower than that obtained for buffer alone. To further investigate the reason for this reduction in signal, penicillin G spiked buffer,

serum and whole blood dilution series were prepared and analysed (Fig. 4). The linear regression values for these curves were 0.999, 0.994 and 0.999 for buffer, serum and whole blood, respectively.

Repeatability studies were performed at several different penicillin G concentrations. The RSD values for buffer samples spiked with 0.17, 0.34, 0.68 and 1.36 mM penicillin G were 6.60, 4.99, 4.32 and 3.46%, respectively. Typical chart recorder data from analysis of penicillin G in buffer is shown in Fig. 5a. Repeatability studies using whole blood spiked with 0.68 mM penicillin G were also carried out (Fig. 5b). The RSD value for 100 determinations was 6.71%. The recovery for the whole blood study was 87.8% of that found in the buffer study. No column clogging was observed during the 100 whole-blood sample injections. During the course of these studies, the reproducibility of the penicillinase columns was monitored over a period of several weeks. The calibration curves using 6-week-old enzyme columns that had been used for more than 500 determinations had the same linear range and reproducibility as the original calibration curves (data not shown).

Discussion

The choice of buffer pH was influenced by three factors: enzymatic activity, buffering capacity and blood pH variation. Penicillinase shows an activity optimum at pH 6.5 [16]. The average pH value of blood is 7.4. An intermediate pH of 7.0 was chosen because phosphate buffer has optimal buffering capacity at this pH and the enzyme shows good activity (Fig. 2). Buffering capacity is especially important when making blood measurements because blood pH can vary between pH 6.5 and 8.0 depending on the illness.

The calibration curves for penicillin G, penicillin V and ampicillin were highly reproducible. As would be expected, the thermal signal from penicillin G was slightly higher than for penicillin V and ampicillin, because penicillin G is the true substrate of penicillinase [16]. The spiked whole blood samples were prepared using concentrated antibiotic solutions to minimize dilution effects. Initially, the assay results were highly variable. This was subsequently determined to be the result of clogging caused by blood coagulation after storage (despite the use of heparin-treated collection tubes) as well as of residual metabolic activity. Addition of NaF and EDTA to the PBS buffer eliminated these effects. The NaF inhibited residual metabolic activity, which reduced the thermal noise signal (data not shown). Addition of EDTA effectively inhibited coagulation, which dramatically improved the buffer flow uniformity. Combined, these additions dramatically improved the repeatability of the measurements. It is important to note that these additions were

only required when samples were stored before analysis and would not be needed when making direct on-line measurements.

Interestingly, the blood calibration curves were shifted upward compared with the buffer curves. Further analysis revealed that whole blood injected alone gave rise to an interference response signal of 37 mV. The magnitude of the whole blood response corresponded to the upward shift seen in the whole blood calibration curves. Furthermore, analysis of serum showed a similar, but less pronounced shift. Our hypothesis is that the thermal signal resulted from mixing of the highly concentrated blood and serum samples with the running buffer. The interference signal is highly reproducible and therefore easily compensated for when making concentration calculations. Apart from the calibration curve shift, the linearity and sensitivity obtained from the buffer and whole blood calibration curves were similar.

Antibiotic levels can be as high as 1 mM immediately after injection, but fall rapidly within the first hour as a result of clearance by the kidneys. Typical blood concentrations are in the 0.2–0.5 mM range [17], which lies within the linear range of our assay. Many factors influence clearance rates including the degree of bacterial loading, type of antibiotic resistance, as well as the general health status of the patient. Hence, the half-life of antibiotics in blood varies considerably, which makes it difficult to maintain optimal antibiotic levels. Real-time antibiotic monitoring in the 5–10 min range would make it possible to more accurately treat critically ill patients where time is of the essence.

The repeatability for detecting penicillin G in whole blood samples is adequate for clinical use, but if one considers the fact that no sample pretreatment has been performed, the results are exceptional. Nevertheless further improvement is needed. Previous studies in our group have shown that the linear range and speed of the assay can be extended by reducing the sample volume [18]. Reducing sample volume would have the added advantage of allowing faster pH equilibration of the blood samples, which would further improve assay accuracy and repeatability. No clogging was observed after injection of over 100 untreated whole blood samples. The system has exceptional long-term stability, with minimal signal reduction after being used to make more than 500 measurements over a 6-week period. The linear range, repeatability and speed of the assay are adequate for real-time monitoring of antibiotics in human blood.

Conclusions

Antimicrobial resistance to antibiotics is an ever increasing global healthcare threat. Real-time antibiotic monitoring would improve treatment and patient outcomes, as well as reducing the spread of resistance. Current testing is too slow to meet

the demand of patients undergoing critical care, such as trauma victims, patients with postoperative infections and those receiving treatment of multiresistance. Here we present our efforts to develop an assay and instrument for the real-time monitoring of antibiotic levels in unpretreated whole human blood. It is also important to note that the system required minimum recalibration (once daily). To our knowledge, this is the first assay to be developed that is capable of measuring antibiotics in whole blood without any sample pretreatment whatsoever.

The sensitivity and repeatability of the assay are adequate for use in clinical applications. The ability to sequentially inject more than 100 unpretreated whole blood samples with highly reproducible results (6.71% RSD) attests to the robustness of the assay. No detectable clogging was observed. After 6 weeks of use and over 500 injections, no significant loss of sensitivity or reproducibility was detected, which attests to the long-term stability of system. The assay system fulfils the basic requirements needed to develop real-time monitoring of antibiotics in blood. The system is convenient to operate, has a rapid response time and adequate sensitivity for clinical use. The use of reusable immobilized enzyme columns provides an economical alternative to single test kits for point of care and outpatient testing.

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Transparency Declaration

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