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A new bioassay method for quantitative analysis of tetracyclines

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Abstract A new method is described for quantitative analysis of tetracycline, based on the decrease in external pH of bacterial suspensions after the addition of a glucose pulse. The decrease in external pH of these suspensions was inversely proportional to the concentration of tetracycline. The correlation coefficient of standard response lines derived from the bioassay was 0.99. Tetracycline potency was determined in six tetracycline HCl samples by the sugar pulse bioassay and a turbidimetric method. The turbidimetric assay result varied from the glucose-pulse data by no more than 7 and 3% at 3 and 7 min, respectively. The procedure is rapid, precise and quantitative, and requires minimal preparation and use of media, with savings in laboratory resources and time.

Keywords Bioassay · pH response · Tetracyclines

Introduction

Tetracyclines are broad-spectrum bacteriostatic antibiotics produced by species of the genus *Streptomyces*. The mechanism of antibacterial action is inhibition of protein synthesis. They are active against many aerobic and anaerobic Gram-positive and Gram-negative pathogenic bacteria, mycoplasmas, rickettsias, chlamydias, and spirochetae, and some protozoa. They are especially used in the treatment of acne, brucellosis, urethritis and acute pelvic infections. Tetracyclines are the drugs of choice for treating rickettsial infections. Tetracycline or doxycycline (semisynthetic derivative) has been used with quinine in the management of chloroquine-resistant falciparum malaria. Tetracyclines are also the usual treatment of balantidiasis and they have been used in the treatment of severe amoebic dysentery and in *Dientamoeba fragilis* infections [1, 10, 12]. Tetracyclines are widely used to treat bovine mastitis. They are also used frequently in veterinary formulations to prevent and control disease, as well as in feed additives to promote weight gain and increase feed conversion efficiency [3].

Most of the methods developed for quantification of tetracyclines are based on inhibitory activity on a test organism. The agar diffusion technique and turbidimetric methods are used routinely for determining tetracycline potency [2, 7, 8, 11]. These assays require a significant amount of materials and media, and are labour intensive and time consuming.

Solé et al. [9] reported that the addition of glucose to resting cells of different bacteria produces a decrease in pH of the extracellular medium due to the rapid production of organic acids resulting from bacterial metabolism. It has been shown that formation of several polypeptides is induced in *Escherichia coli* during starvation, and that starvation protein synthesis begins within minutes of the onset of starvation and lasts 2–4 h [6]. Thus, non-proliferating cell suspensions might be used to quantify tetracycline potency. We have developed a new bioassay method for quantitative analysis of tetracycline, based on the measurement of pH response of bacterial suspensions of *Enterococcus hirae* (formerly *E. faecalis*) following addition of an aliquot of concentrated glucose solution.

Material and methods

Bacterial strains

The strains used in this study were *Staphylococcus aureus* ATCC 9144 and NCTC 7447, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778 and *E. hirae* ATCC 10541. All strains were stored on tryptone soya agar (TSA; Oxoid, Basingstoke, UK) slopes at 4°C.

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Assay medium

A complex medium containing (g l^{-1}): trypticase 9, glucose 20, yeast extract 5, sodium citrate 10, K_2HPO_4 1, KH_2PO_4 1 (pH 6.7–6.9 after autoclaving) was used as assay broth for the turbidimetric method.

Bacterial suspensions densities

Cell densities were assayed by McFarland's standards [4] and/or by measuring absorbances at 600 nm (A_{600}). The protein content was determined using the method of Lowry [5].

Preparation of tetracycline standards and samples

Tetracycline hydrochloride SQ9457 107 TC-5 (Bristol-Myers Squibb, New Brunswick, N.J.) was used as standard.

For the sugar-pulse assay a stock solution (1,000 μg ml^{-1}) of tetracycline was prepared in sterile phosphate buffer (pH 4.5) and kept in the dark at 4°C. Working standards at concentrations described in the text, also in sterile pH 4.5 phosphate buffer, were prepared from stock solution.

For the turbidimetric method [8], a stock solution (80 μg ml^{-1}) of tetracycline was prepared by dissolving the salt in 0.1 *N* HCl. An intermediate stock solution (0.8 μg ml^{-1}) was obtained by diluting 2.5 ml stock standard to 250 ml with sterile pH 4.5 phosphate buffer. Working standard solutions of tetracycline (0.12, 0.16, 0.24, 0.32 and 0.4 μg ml^{-1}) were prepared daily in sterile pH 4.5 phosphate buffer from intermediate stock solution.

Samples of tetracycline hydrochloride powder were purchased from chemical and pharmaceutical suppliers (Sigma-Aldrich, Alcobendas, Madrid, Spain; ICN Ibérica , Corbera de Llobregat, Spain). A stock solution and two test dilutions were prepared for each tetracycline sample as specified for working standard solutions of each method. Concentrations of test dilutions were assumed to be between 0.16 and 0.32 μg ml^{-1}.

Sugar-pulse bioassay

Strains were cultured 24 h before the assay by plating on TSA at 37°C (*S. aureus*, *B. subtilis* and *E. hirae*) or 30°C (*B. cereus*). Solid cultures were suspended in sterile 0.85% NaCl solution at concentrations described in the text.

Experiments were conducted on 8 ml samples of cell suspensions in 10 ml glass vials, which were magnetically stirred at room temperature. The pH was measured using an ORION SA720 pH meter equipped with a combined glass electrode (ROSS 81-03 sc). After insertion of the pH electrode the suspensions were vigorously mixed and exposed to different concentrations of tetracycline. After 2–4 min, a glucose-pulse was given and changes in external pH were recorded for 7 min. Glucose was added as an 80 μl aliquot of 100 mM sterile solution in distilled water (1 mM final concentration) [9]. All glucose-pulses were performed in duplicate, and the reported data are means of replicate samples. Glucose-pulses to tetracycline samples were performed after the working standards. A standard response curve was constructed with tetracycline concentrations and pH decrease of cell suspensions. The tetracycline equivalent for each sample dilution (T_1 and T_2) was obtained by interpolation from the standard curve. The potency of the original tetracycline sample was calculated by multiplying T_1 and T_2 by its respective dilution factor and averaging the two values.

Turbidimetric method

The turbidimetric assay was performed by the method described by Sancho et al. [8]. One day before the assay 20 ml assay medium in 20×200 mm glass tubes was inoculated with a loop from a 24-h stock culture of *E. hirae* ATCC 10541, and incubated 16–18 h at 37°C. The inoculum was prepared by transferring 13 ml overnight culture to 1 l assay broth. A volume of 1 ml of each working solution and each sample dilution was inoculated into each of two sterile glass tubes (20×200 mm). One to two tubes containing 1 ml pH 4.5 phosphate buffer were included as controls. Two tubes containing 1 ml 7% phenol (Panreac Química, Barcelona, Spain) water solution were used as blank tubes. A volume of 9 ml inoculum was added to all tubes. After incubation in a water bath at 37 ± 0.2°C for 2–4 h, growth was stopped by adding 1 ml 7% phenol solution to all tubes. Absorbances (*A*) at 530 nm were read. Readings were taken from the highest to the lowest concentration of working solutions. Sample dilution readings were inserted into the equivalent levels of working standards. A standard curve was prepared of log *A* against concentration using the average turbidity of each pair of tubes. The tetracycline equivalent for each sample dilution (T_1 and T_2) was obtained by interpolation from the standard curve. The potency of the original tetracycline sample was calculated multiplying T_1 and T_2 by its respective dilution factor and averaging the two values.

Results and discussion

Several experiments were carried out to optimise the glucose-pulse assay.

Bacterial species and culture conditions

Staphylococcus aureus ATCC 9144 and NCTC 7447, *B. subtilis* ATCC 6633, *B. cereus* ATCC 11778 and

E. hirae ATCC 10541 are used as test organisms in different microbiological methods for quantitative assay of tetracyclines [2, 7, 8, 11]. The pH response of suspensions of these bacteria to a glucose-pulse depended on the bacteria species. We did not detect any change in pH when glucose was added to *S. aureus*, *B. subtilis* and *B. cereus* suspensions. The addition of glucose to cell suspensions of *E. hirae* ATCC 10541 caused a rapid decrease in pH, as reported by Solé et al. [9]. The magnitude of the pH decrease depended on the age of the bacteria culture. pH changes in *E. hirae* suspensions (prepared with 24-, 48- and 72-h cultures on TSA incubated at 37°C) detected after 7 min of a glucose stimuli were 1.06, 0.68 and 0.56, respectively. Thus, cultures of *E. hirae* ATCC 10541 grown on TSA for 24 h at 37°C were used in all further tests.

Age of suspensions and cell densities

The response of *E. hirae* suspensions to a sugar stimulus decreased when the bacterial suspension was kept at room temperature. The decrease in pH of a non-proliferating suspension 7 min after a glucose-pulse was 0.65, whereas the same suspension kept for 2 h at room temperature gave a pH decrease of 0.39 pH units. These results indicate that suspensions should be used immediately after preparation.

To determine the optimum bacterial concentration that induces a pH change after a glucose-pulse, non-proliferating suspensions of *E. hirae* were prepared at cell densities corresponding to 0.5, 1, 2, 4, 6 and 10 McFarland standards [4]. Suspensions at a cell density similar to McFarland standard 4 gave the highest pH decrease of the standards studied (data not shown). Further experiments demonstrated that comparing the turbidity of *E. hirae* suspensions with McFarland standards was not accurate enough to standardise the assay conditions. Small differences in cell densities of *E. hirae* suspensions measured as A_{600}, which could not have been detected by visual comparison of cell suspensions with McFarland standards, gave different pH decreases after a sugar-pulse. Cell suspensions with A_{600} values of

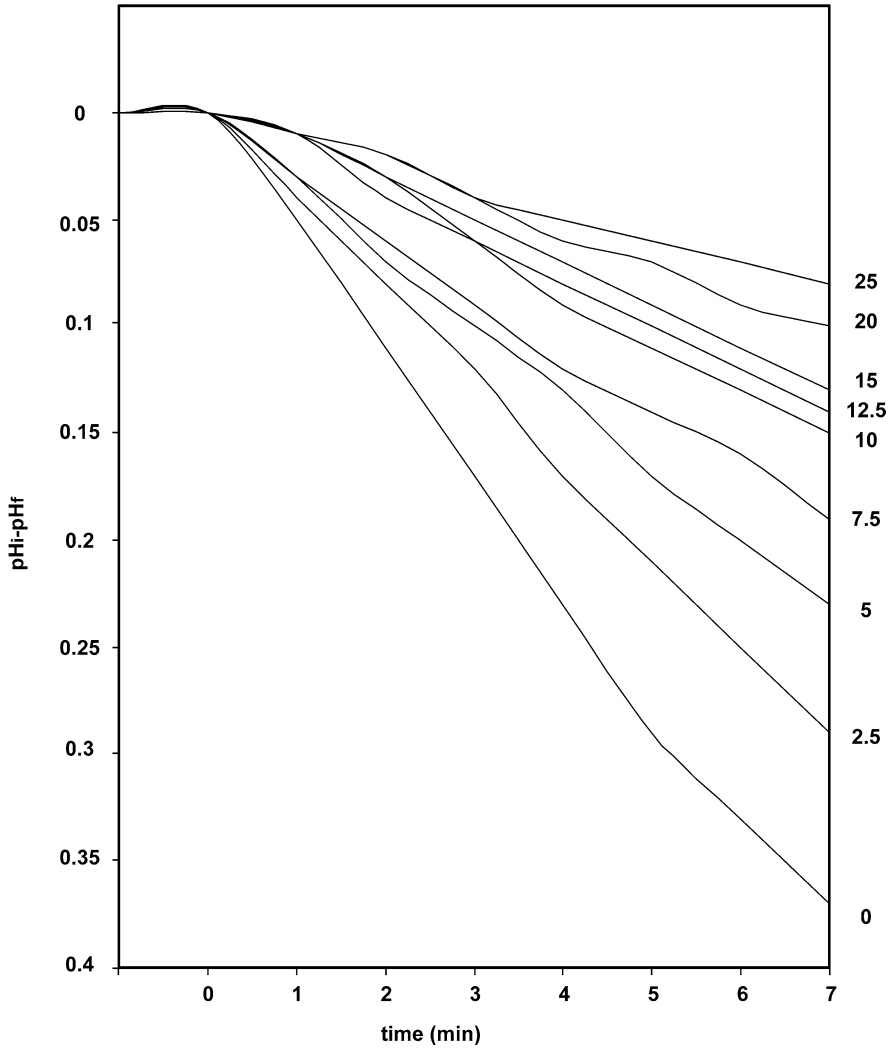

Fig. 1 pH decrease in *Enterococcus hirae* suspensions treated with different amounts of tetracycline (μg ml^{-1}) following a glucose pulse

0.54, 0.63 and 0.69 showed pH decreases of 0.17, 0.31 and 0.39, respectively, 7 min after a glucose-pulse.

Assay sensitivity and linearity

We first studied the response of cell suspensions (McFarland standard 4) to different amounts of tetracycline, ranging from 0 to 25 μg ml^{-1} (Fig. 1). Our results indicated that the rate and magnitude of pH fall of these suspensions depended on the concentration of tetracycline; the less antibiotic added the greater the decrease observed. We detected no pH decrease when no sugar was added to bacterial suspensions treated with 25 μg ml^{-1} tetracycline or less. We observed a very good correlation between the decrease in pH of cell suspensions and the concentration of tetracycline. pH responses were linear with tetracycline concentration in the ranges 10–25 μg ml^{-1} and 0–10 μg ml^{-1}, with correlation coefficients of −0.996 and −0.987, respectively. Thus our method can be used to quantitate tetracycline in samples containing 0–25 μg ml^{-1} antibiotic.

We explored the possibility that this method could be used for quantification of small amounts of tetracycline. On the basis of our previous results, *E. hirae* suspensions with a cell density of $A_{600} = 0.7$ (0.12 mg ml^{-1} cell protein) were used to test the sensitivity of the assay. Table 1 shows the pH decreases for bacterial suspensions of five independent experiments treated with 0.125, 0.175, 0.25, 0.325 and 0.4 μg ml^{-1} tetracycline. The sensitivity of the bioassay is similar to traditional microbiological assays described in the literature [2, 7, 8, 11].

The linearity between the decrease in pH of cell suspensions and tetracycline concentration in the range 0.125–0.4 μg ml^{-1} was studied 3, 5 and 7 min after the sugar pulse. An incubation time of 3 min was sufficient to obtain a good correlation coefficient (Fig. 2).

Comparison with turbidimetric method

To compare the glucose-pulse assay with a turbidimetric method [8], tetracycline concentrations were determined in six tetracycline samples by both methods (Table 2). Both assays used *E. hirae* ATCC 10541 and the same range of tetracycline concentrations (from 0.125 to 0.4 μg ml^{-1}), as standard solutions. A fresh bacterial suspension and calibration curve were performed for each tetracycline sample. No turbidimetric assay result varied from the glucose-pulse data at 3 and 7 min by more than 7% and 3%, respectively. Thus the glucose-pulse method is an accurate alternative to the turbidimetric assay and minimises sample handling and analysis time.

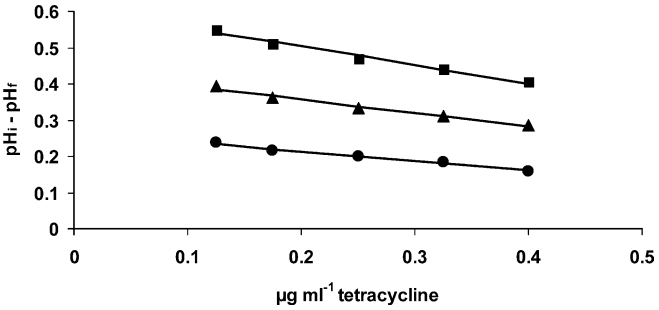

Fig. 2 Calibration curves of initial pH reading (pH_I) minus final pH reading (pH_f) vs tetracycline concentration. Time after glucose pulse: *filled circles* 3 min, *filled triangles* 5 min, *filled squares* 7 min. $r = -0.99$ in all cases

We have developed a new bioassay method for determining the tetracycline potency of chemical samples. The simplicity of our method makes it an ideal system for automation of tetracycline quantification. The procedure is rapid, precise and quantitative and requires minimal preparation and minimal use of media, with savings in laboratory resources and time. A similar approach may be applied to the quantitative determination of other antimicrobial substances.

Table 2 Tetracycline concentration (mg g^{-1}) measured by glucose-pulse and turbidimetric assays

Sample	Glucose-pulse bioassay		Turbidimetric method
	3 (min)	7 (min)	
1	856	943	925
2	909	840	866
3	956	949	968
4	893	907	883
5	917	929	905
6	914	900	890

Table 1 pH response of suspensions of *Enterococcus hirae* ATCC 10541, without tetracycline treatment (control) and with different tetracycline concentrations, after 3, 4, 5, 6 and 7 min of glucose pulse. The results are expressed in ΔpH ± SD, from five independent experiments

Time (min)	Control	Tetracycline concentration (μg ml^{-1})				
		0.125	0.175	0.250	0.325	0.400
3	0.383 ± 0.08	0.237 ± 0.05	0.216 ± 0.05	0.199 ± 0.05	0.185 ± 0.05	0.159 ± 0.06
4	0.494 ± 0.09	0.317 ± 0.07	0.288 ± 0.07	0.264 ± 0.07	0.249 ± 0.07	0.225 ± 0.06
5	0.605 ± 0.10	0.393 ± 0.08	0.361 ± 0.08	0.332 ± 0.08	0.311 ± 0.08	0.285 ± 0.07
6	0.713 ± 0.09	0.478 ± 0.09	0.438 ± 0.09	0.402 ± 0.09	0.377 ± 0.09	0.345 ± 0.09
7	0.820 ± 0.10	0.550 ± 0.10	0.510 ± 0.10	0.470 ± 0.10	0.440 ± 0.10	0.405 ± 0.10

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