DETECTION OF RESIDUAL PENICILLIN IN MILK BY SENSITIVE ENZYME IMMUNOASSAY

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A convenient and precise method by an application of a highly sensitive enzyme immunoassay of penicillin to detect the veterinary penicillin residue in milk was studied. The procedures of the assay to detect more than 0.01 $\mu\text{g/ml}$ of ampicillin in milk were developed. Five commercial milk samples were tested by the assay procedure, and no veterinary ampicillin residue was detected in any of them.

Keywords—penicillin; milk; enzyme immunoassay; immunoassay; ampicillin; detection of penicillin; residual antibiotics; veterinary drug

INTRODUCTION

The problem of veterinary drug residues in food producing animals is of great concern because of their health hazard potentials. Penicillin contamination in milk from mastitis preparation is one of the problems. Several methods to detect the antibiotic in milk have been developed over the years.¹⁻⁹ Still no completely acceptable method is available which can easily be applied under industrial conditions. The usual microbiological methods¹⁻⁵ for the detection of penicillin require too much time to effectively prevent contaminated dairy products from entering the market. The infusion of dye-markers⁶⁻⁹ results in production of colored milk which in turn acts as a strong detergent to the producer to include contaminated milk in his shipments. Though this method does not require much time, visual analysis of the dye-marker is not a specific assay of penicillin.

Recent development of a variety of immunological assay procedures¹⁰⁻¹⁶ in the past decade made possible for a highly sensitive, specific, precise, rapid and convenient measurement of the concentrations of drugs. In this report we describe an application of a highly sensitive enzyme immunoassay of penicillin¹⁶ for detection of the residual ampicillin, one of the commonly used

veterinary penicillin, in milk.

MATERIALS AND METHODS

β -D-Galactosidase from *Escherichia coli* was purchased from Boehringer Mannheim (W. Germany). 7- β -D-Galactopyranosyloxy-4-methylcoumarin, glycine and EDTA were bought from Nakarai Chemicals Ltd. (Kyoto). Complete Freund's adjuvant used was Difco Product (Detroit, Michigan). Sepharose CL-6B from Pharmacia Fine Chemicals, Ltd. (Uppsala, Sweden), BSA (Cohn Fraction V) and rabbit IgG from Sigma Chemical Co. (St. Louis, Missouri) were used.

Buffers—Buffer A; 0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl_2 and 0.1% NaN_3 . Buffer B; The phosphate-EDTA buffer, pH 7.4 (2120 mg of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 745 mg of $\text{EDTA} \cdot 2\text{Na} \cdot 2\text{H}_2\text{O}$ and 200 mg of BSA were dissolved in distilled water with a final volume of 200 ml). PBS; 0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl.

Preparations of Antisera—Antiserum specific to ampicillin was prepared by the method reported previously.¹⁶ Antiserum to rabbit antibody was produced in a goat by injections with rabbit IgG in complete Freund's adjuvant according to the method of Tachibana and Migita.¹⁷

Penicillinase — A crude penicillinase was isolated from a penicillin resistant strain of *E. coli* which was isolated from a feces of clinical source in Nagasaki University Hospital, according to the method of Citri.¹⁸⁾ Enzyme labelled ampicillin with β -D-galactosidase was prepared by the method reported previously.¹⁶⁾

Assay of β -D-Galactosidase Activity — The enzyme activity was measured by the method reported.¹⁴⁾ The amount of enzyme labelled ampicillin was expressed as units of β -D-galactosidase activity, defining 1 U enzyme activity as the amount that hydrolyzes 1 μ mol of 7- β -D-galactopyranosyloxy-4-methylcoumarin per min.

Preparation of Penicillin-free Milk — A solution of penicillinase from *E. coli* (10^4 unit) in 10 μ l of 0.02 M sodium phosphate buffer, pH 6.5, was incubated with 1 ml of fresh milk at 30°C for 1 h. The milk was then heated at 120°C for 15 min to inactivate the penicillinase, and used as a standard milk.

Enzyme Immunoassay — Buffer B was used as the diluent and the incubation media in competitive enzyme immunoassay of ampicillin except otherwise stated. A mixture of ampicillin- β -D-galactosidase conjugate (enzyme conjugate, 30 μ U/50 μ l) and 100 μ l of known amounts of ampicillin dissolved in buffer B or the standard milk were incubated with 100 μ l of a 2000-fold diluted solution of rabbit antiserum to ampicillin [anti-(ampicillin)] in a final assay volume of 250 μ l at 25°C for 3 h. Then, 50 μ l of a 20-fold diluted goat anti-(rabbit IgG) serum and 50 μ l of a 300-fold diluted normal rabbit serum were added. After further incubation at 25°C overnight, 1 ml of buffer A was added and the antibodies bound conjugate was precipitated by centrifugation at $800 \times g$. The supernatant was removed completely and β -D-galactosidase activity of the precipitate was measured using buffer A solution of 0.1 mM 7- β -D-galactopyranosyloxy-4-methylcoumarin as the substrate.¹⁴⁾ Each assay was run at least in a triplicate.

RESULTS AND DISCUSSION

To obtain the optimum results, adequate con-

ditions to quantify penicillin contents in milk by enzyme immunoassay of penicillin were first investigated. When the enzyme activity of 30 μ U of the enzyme conjugate was assayed, the fluorescence intensity observed was approximately 80 times that of the reagent blank. This amounts of the conjugate was incubated with each 100 μ l of a 2000-, 4000- or 8000-fold diluted solution of anti-(ampicillin) and then bound conjugate was precipitated by the second antibody method according to the procedures described in "Materials and Methods" except the amounts of anti-(ampicillin) used. Values of the fluorescence intensities of bound conjugates assayed for their β -D-galactosidase activity were 12, 9, and 5 times, respectively, compared with that of the blank prepared by adding all of the reagents, except anti-(ampicillin). Thus, the 2000-fold diluted anti-

FIG. 1. Calibration Curve for Enzyme Immunoassay of Ampicillin in Milk (Open Circle) or in Buffer B (Closed Circle), and Ampicillin Contents in the Milk Preparations treated with Penicillinase (Open Triangle)

The enzyme activities of the bound conjugates are plotted against the logarithmic amounts of ampicillin. Bo: Enzyme activity of the bound conjugate without competition of ampicillin. B: Enzyme activity of the bound conjugate with competition of ampicillin.

(ampicillin) was chosen for use. Enzyme immunoassay of ampicillin was performed using 30 μ U enzyme conjugate and a 2000-fold diluted anti-(ampicillin) by a competitive binding procedure with double antibody method. A typical calibration curve of enzyme immunoassay of ampicillin which can determine 0.3–100 ng of the antibiotic is shown in Fig. 1.

Effect of 100 μ l of milk on enzyme immunoassay of ampicillin was then examined. As reported previously,¹⁶⁾ the penicilloic acid derivative of ampicillin, which was converted from ampicillin by means of a penicillinase treatment,

possessed 0.1% cross reactivity compared with that of ampicillin toward the anti-(ampicillin) as judged by enzyme immunoassay. Since the same anti-(ampicillin) is used in this study, contamination of less than 50 ng of the penicilloic acid derivative of ampicillin is expected not to disturb the assay of 0.3–100 ng of ampicillin by the present enzyme immunoassay. Thus, a standard milk free from penicillin was prepared from commercial milk by treating it with penicillinase followed by inactivation of the enzyme. Milk preparations containing graded amounts but less than 16 ng of ampicillin were also examined after the same

TABLE I. *Accuracy and Precision of Enzyme Immunoassay of Ampicillin in Milk Samples*

	Ampicillin		Recovery %	CV %	<i>N</i> ^{b)}
	Added (ng/tube)	Found (ng/tube)			
Variation, within-run	0.3	0.33 $\pm$ 0.14 ^{a)}	110.0	42.4	10
	1.0	1.01 $\pm$ 0.2	101.0	20.0	10
	5.0	5.20 $\pm$ 0.70	104.0	13.5	10
	10.0	11.1 $\pm$ 1.9	111.0	17.0	10
	100.0	97.2 $\pm$ 13.9	97.0	14.3	10
Variation, between-run	0.3	0.22 $\pm$ 0.13	106.6	40.6	6
	1.0	0.94 $\pm$ 0.32	93.6	32.0	6
	5.0	4.90 $\pm$ 1.5	98.0	30.6	6
	10.0	10.2 $\pm$ 2.1	101.7	21.0	6
	100.0	99.5 $\pm$ 18.9	99.5	18.9	6

a) Mean $\pm$ SD. b) Assay times.

TABLE II. *Ampicillin Contents (ng/0.1 ml) in Commercial Milk Samples with Recoveries of the Graded Amounts of Ampicillin added to the Samples measured by Enzyme Immunoassay*

Sample Ampicillin added	A	B	C	D	E
0.00	0.14 $\pm$ 0.28 ^{a)}	0.08 $\pm$ 0.70	0.04 $\pm$ 0.10	0.00 $\pm$ 0.00	0.12 $\pm$ 0.14
0.3	0.10 $\pm$ 0.11	0.30 $\pm$ 0.39	0.31 $\pm$ 0.39	0.19 $\pm$ 0.15	0.26 $\pm$ 0.23
1.0	1.22 $\pm$ 0.64	1.35 $\pm$ 1.00	0.87 $\pm$ 0.38	0.88 $\pm$ 0.12	1.15 $\pm$ 0.22
5.0	4.65 $\pm$ 2.44	4.25 $\pm$ 0.31	5.03 $\pm$ 0.65	4.70 $\pm$ 2.02	5.70 $\pm$ 1.76
<i>N</i> ^{b)}	4	4	4	3	3

a) Mean $\pm$ SD. b) Assay times.

treatments.

Calibration curve for enzyme immunoassay of ampicillin dissolved in 0.1 ml of the standard milk instead of buffer B is also shown in Fig. 1, together with the curve of ampicillin contents measured for the milk preparations. The milk preparations inactivated by penicillinase, contained almost no detectable ampicillin. The ampicillin free-milk prepared was found to be useful as a standard milk.

Experimental results for reproducibility and accuracy of enzyme immunoassay of ampicillin under this condition are summarized in Table I.

Recovery rates of their assay values were arranged over 93 to 111% for both within-run and between-run experiments. Precision of the assay was fairly good. CV values obtained under the within-run experiments, were less than 20% for ampicillin samples of 1.0–100 ng/tube, and more than 40% for the sample of 0.3 ng/tube. While, those values obtained under the between-run experiments, were larger than those of the corresponding within-run experiments.

Ampicillin contents in commercial milk samples were assayed by enzyme immunoassay. Known amounts of ampicillin were added to every milk sample and their recoveries were measured also to confirm the accuracy of the assay values of the ampicillin contents in commercial preparations. The results are summarized in Table II.

It was judged that all milk samples tested did not contain detectable amount of ampicillin. Recovery of 5 ng of ampicillin added to each sample was only larger for sample E than 5.2 ng which was the average recovered value in within-run study as shown in Table I. Ampicillin content in sample E measured was 0.12 ng/ml. The calculated values of the content from the difference between found and added amounts of ampicillin in milk E were 0.00, 0.15 and 0.70 ng for the samples that were added with 0.3, 1.0 and 5.0 ng ampicillin, respectively. All of these values assayed or calculated, however, are within the values of their standard deviations as shown in Table I. Consequently, it is concluded that five

milk samples tested contained no detectable ampicillin.

As reported previously,¹⁶⁾ penicillin G showed 10% cross reactivity to that of ampicillin using this anti-(ampicillin) serum. Thus, penicillin G which is one of the most widely used veterinary penicillin, can be assayed by the same procedure though its sensitivity is 10% of that of ampicillin. The *Bacillus subtilis* assay methods for detection of penicillin in milk specified by the Association of Official Analytical Chemist include both a disc and a cylinder technique.¹⁹⁾ The disc assay is sensitive to approximately 0.05 unit (1 U=0.6 µg) of penicillin G/ml. While the cylinder assay, which is more complex to use is suitable for concentration of less than 0.025 unit/ml. Sensitivity of the assay methods depend upon the cell concentration of the test organism in agar-seeded plate which should be kept at 4°C before the test. Days of storing the agar-seeded plate in the cold room cannot be too long because of a decrease in sensitivity of the test.⁸⁾ The microbiological methods used in the quantitation of penicillin content in milk need more precise processes than that for the detection.²⁰⁾ The present enzyme immunoassay method can detect and quantify ampicillin easily with many milk samples without any pretreatment. Sensitivity of the assay of ampicillin in milk is 0.01 µg/ml and, more than 0.5 µg/ml, amounts of ampicillin content can be quantified with accuracy. Precision of the data can be easily confirmed by the recovery experiment.

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