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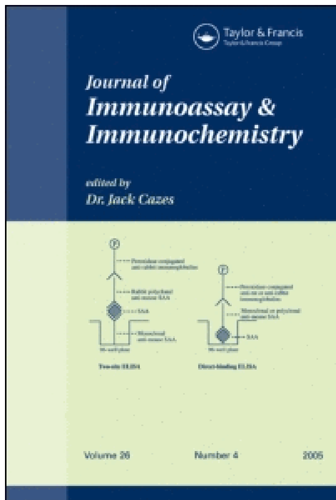

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A RAPID ENZYME IMMUNOASSAY FOR MEASUREMENT OF HBK IN BLOOD

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ABSTRACT

An enzyme immunoassay has been developed for the measurement of HBK (4-amino-2-hydroxybutylyldibekacin) a new semisynthetic aminoglycoside antibiotic. Antisera were raised in rabbits by immunization with HBK conjugated to bovine serum albumin (BSA). 3'-Eno-HBK conjugated to alkaline phosphatase (ALP) was used as an enzyme labeled antigen. The antibody-bound drug was separated from free using goat anti-rabbit IgG serum. The assay can be completed within one hr by co-incubating the first and the second antibody. The present immunoassay allows detection of 10 ng HBK per ml of serum, and is applicable for monitoring HBK level in blood. HBK concentrations in human sera were determined by the immunoassay during and after infusion and the levels were compared with those determined by high performance liquid chromatography (HPLC) and antimicrobial assay.

INTRODUCTION

HBK is 4-amino-2-hydroxybutylyl derivative at the 1-amino group of dibekacin (DKB), an aminoglycoside. HBK has

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strong activity against kanamycin-resistant bacteria producing kanamycin-phosphotransferase I, kanamycin-phosphotransferase II and kanamycin-nucleotidyltransferase(1). Like other aminoglycosides, HBK is potentially ototoxic and nephrotoxic at high doses. To achieve and maintain therapeutic HBK levels (ug/ml of serum order), frequent and rapid monitoring of blood levels is essential to make valid decisions concerning the dose and dosing intervals.

HBK has been measured by antimicrobial assay (2) or HPLC (3), however, these methods are unsuitable for monitoring because they are time consuming. A rapid and simple method for the measurement of HBK, therefore, is desired. The present paper deals with an enzyme immunoassay for HBK. By use of this assay, serum levels of HBK were determined during and after infusion.

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MATERIALS AND METHODS

Materials

HBK and other aminoglycosides were supplied from Meiji Seika Kaisha Ltd. Chemical structures of HBK and 3'-eno-HBK are illustrated in Figure 1. BSA was obtained from Sigma Chemical Co. Freund's complete adjuvant was obtained from Calbiochem-Behring Co. ALP (from beef mucosa) was obtained from Miles Laboratories Ltd. Second antibody (Goat anti-rabbit IgG serum) was obtained from Eiken Immunochemical Laboratories Ltd. All other reagents used in the present study were commercial grade.

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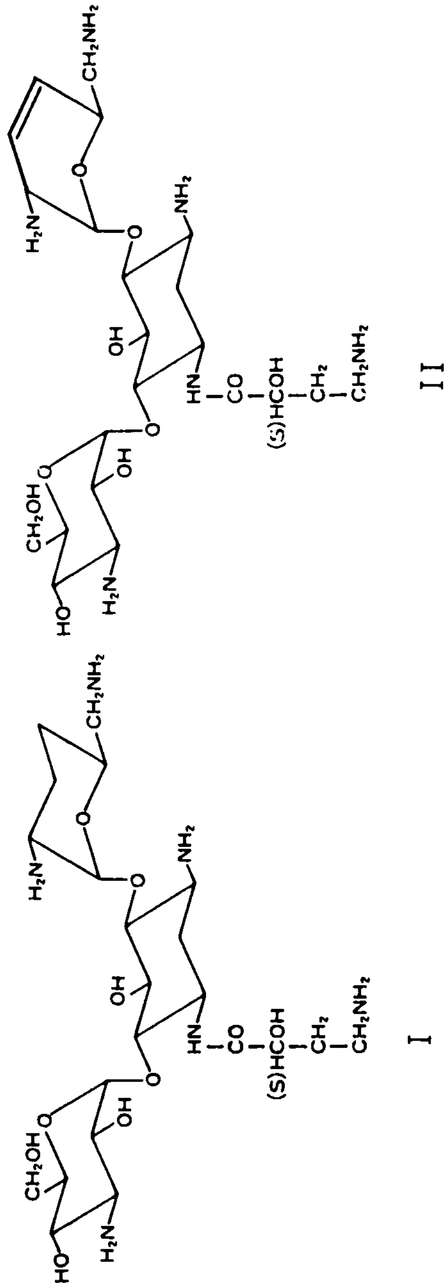

FIGURE 1 Chemical structure of HBK(I) and 3'-Eno-HBK (II)

Preparation of antigen

HBK was coupled directly to BSA using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ethyl CDI) and the conjugate was used as an antigen. HBK sulfate (potency : 700 ug/mg) (118 mg) was dissolved in water (1 ml). To this solution were added BSA (100 mg) in water (2 ml) and then dropwise ethyl CDI (2.9 g) in water (3 ml) at 4°C, adjusted to pH 7.0 by the addition of 0.1N HCl and stirred overnight at that temperature. The reaction mixture was dialyzed against water for 2 days at 4°C and then lyophilized. The yield of HBK-BSA conjugate was 108 mg.

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Immunization

HBK-BSA conjugate (1 mg) was dissolved in 0.5 ml of saline. To this solution was added 0.5 ml of Freund's complete adjuvant, and the mixture was emulsified with a homogenizer. The emulsion (1 ml) was administered to each of five female Japanese white rabbits intradermally at multiple sites five times every 2 weeks and then subcutaneously once a month for 3 months. The rabbits were bled 10 days after the last immunization. Antibodies directed against HBK were raised in all of the rabbits.

Preparation of enzyme conjugate

3'-Eno-HBK, instead of HBK, was conjugated to ALP by the same method as described in preparation of antigen. ALP (420 ug) and 3'-eno-HBK (260 ug) were dissolved in water (1 ml) and adjusted to pH 7.0 by the addition of 0.01N HCl. To this solution was added dropwise ethyl CDI (900 ug) in water

(0.1 ml) at 4 °C and stirred overnight at that temperature. The reaction mixture was dialyzed against 0.01M phosphate buffered saline (pH 7.4) for 2 days at 4 °C. The enzyme conjugate was stored at 4 °C after diluting with the same buffer containing 1mM $MgCl_2$ and 0.1% NaN_3 (0.01M PBS) to 10 ml.

Enzyme immunoassay for HBK

The present enzyme immunoassay for HBK was based on the double antibody method. Antiserum which bound 35-50 % of 3'-eno-HBK-ALP conjugate at a final dilution of 1:200 was used in the assay. A series of standard samples containing unlabeled HBK was prepared in normal human serum. A 0.1 ml portion of the standard or serum sample from a patient was added to 10 ml of 0.01M PBS containing 0.1 % BSA to give a 101-fold dilution of each serum. The resulting standard or sample solution was used in the assay. The second antibody (goat anti-rabbit IgG serum) was diluted to 1:8 with 0.01M PBS containing 4 % dextran T-70, and was used in the assay. To each tube were added 0.1 ml of the HBK standard or sample solution, 0.1 ml of the enzyme conjugate solution (1 ug of ALP per ml), 0.1 ml of the antiserum and 0.1 ml of second antibody. The reaction mixture was incubated for 20 min at room temperature. To each tube was added 2 ml of ice-cold water and centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was aspirated, and to the precipitate was added 1 ml of a substrate solution (0.1M carbonate buffer containing 0.01M disodium p-nitrophenylphosphate, pH 10.0). After incubating for 15 min at 37 °C, 2 ml of 0.1N NaOH was added to stop the enzyme reaction. Enzyme activity was determined by measuring optical density at 405 nm. Standard curves used to determine HBK concentration was constructed by plotting

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HBK concentration in standard samples versus absorbance at 405 nm.

RESULTS

Standard curve

Since HBK conjugated directly to BSA was used as an immunogen, it was supposed that unlabeled HBK might hardly displace HBK conjugated to ALP. Therefore, we have made attempt to compare homologous and heterologous enzyme immunoassay by using HBK-ALP and 3'-eno-HBK-ALP as enzyme labeled antigens, respectively. Figure 2 shows typical standard curves for the enzyme immunoassay for HBK by using those two enzyme conjugates. The sensitivity has been significantly increased by using 3'-eno-HBK-ALP as an enzyme conjugate instead of HBK-ALP. Therefore, 3'- eno-HBK-ALP was used as an enzyme conjugate in the present enzyme immunoassay for HBK.

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Figure 3 shows a typical standrad curve for HBK. Ten replicates HBK free plasma samples (diluted 101-fold) were determined in order to obtain the statistically minimal detectable dose (MMD). The MMD value was estimated as 10 ng/ml by extrapolating 95 % confidence limit of free plasma samples to the standard curve. Practically, the MMD value is about 1 ug/ml of plasma because samples are diluted 101-fold prior to assay. The enzyme immunoassay can routinely measure HBK in the concentration range between 1 and 12 ug/ml using 100 ul of diluted serum. That concentration range is ideal for monitoring serum levels of HBK.

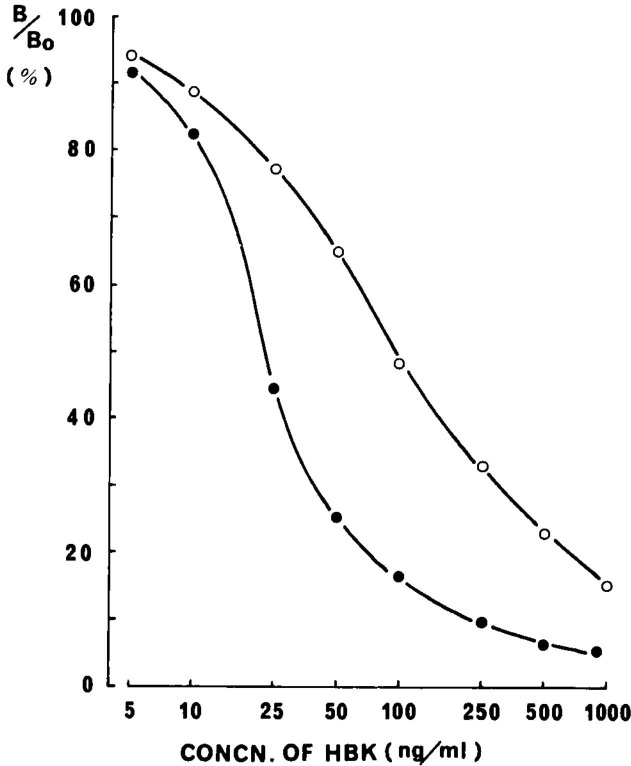

FIGURE 2
Standard curves for HBK using HBK-ALP(○—○) and 3'-Eno-HBK-ALP(●—●) as enzyme labeled antigens.

In most immunoassay, incubation of first and second antibody are separately performed. To justify co-incubation, the separate incubation and co-incubation were compared. In the separate incubation, the first incubation was performed for 16 hr at 4°C and the second one was done for 16 hr at 4 °C. Two standard curves are shown in Figure 4 and no significant difference in sensitivity was observed.

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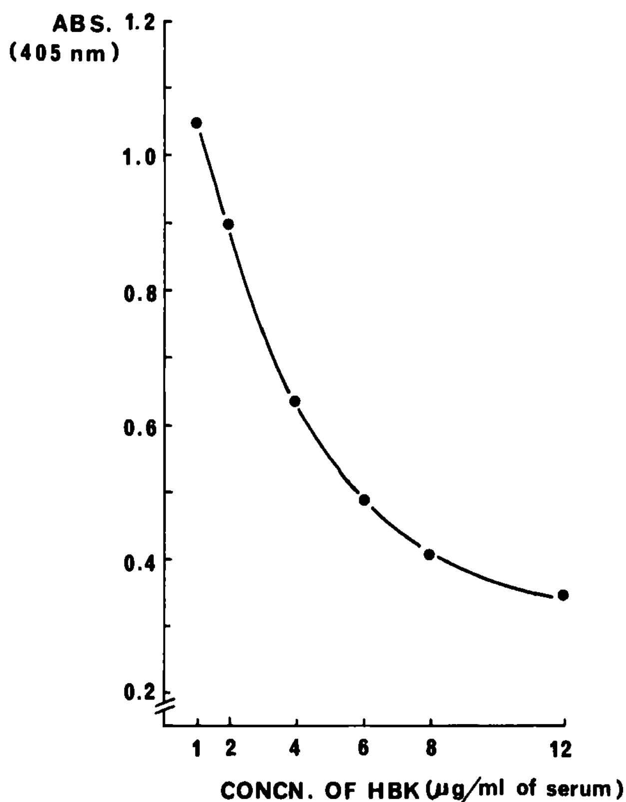

FIGURE 3

Standard curve for the determination of HBK in human sera.
Each standard solution was diluted to 101-fold prior to assay.

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Specificity

To test the specificity of anti-HBK serum for aminoglycosides, i.e., dibekacin (DKB), tobramycin (TOB), kanamycin (KM), bekanamycin (AKM), amikacin (AMK), gentamicin (GM), ribostamycin (RSM), fradiomycin (FRM) and 3'-eno-HBK, cross reactivity studies were conducted.

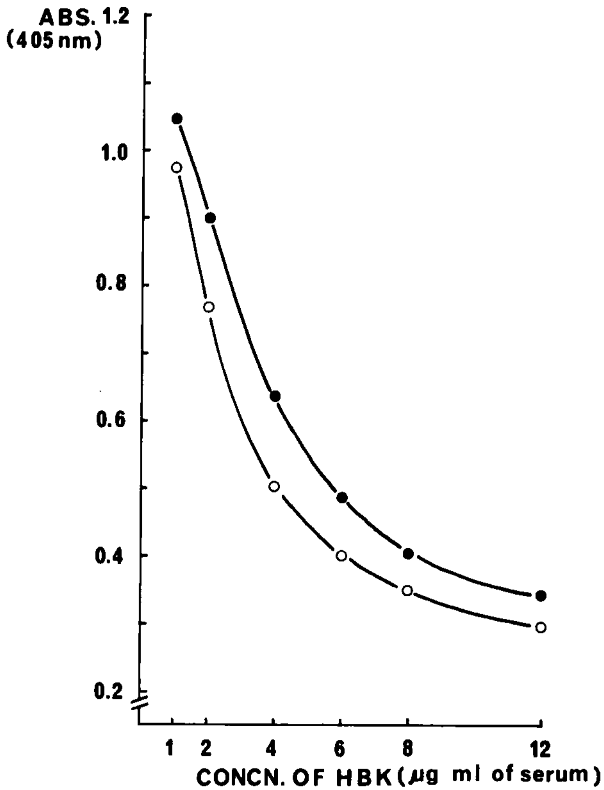

FIGURE 4

Standard curves for HBK by co-incubation of first and second antibody (●—●) and separate incubation of them (○—○).

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Cross-reactivity was expressed as the relative potency of each aminoglycoside compared to HBK, for the 50 % displacement of 3'-eno-HBK-ALP. As shown in Table 1, the percentages of cross-reactivity of the antiserum for DKB, TOB, KM, AKM, AMK and 3'-eno-HBK were 20.0, 18.8, 11.8, 12.2, 6.8 and 87.7 %, respectively. The other aminoglycosides test-

TABLE 1

Cross-reactity of Aminoglycosides in the EIA for HBK

Aminoglycoside	Cross-reactivity[a](%)
HBK	100
Dibekacin(DKB)	20.0
Tobramycin(TOB)	18.8
Kanamycin(KM)	11.8
Bekanamycin(AKM)	12.2
Amikacin(AMK)	6.8
Gentamicin(GM)	0
Ribostamycin(RSM)	0
Fradiomycin(FRM)	0
3'-Eno-HBK	87.7

[a]As determined by the relative amounts needed for 50% displasement of the enzyme labeled antigen (3'-Eno-HBK-ALP).

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ed in the assay showed no inhibition at the concentration of 320 ug/ml.

Recovery

Recovery experiments were performed by adding three different amounts of HBK to normal human serum and assaying them by the immunoassay. As shown in Table 2, the averages of ten replicates at three concentrations of HBK, i.e., 2.0, 4.0 and 8.0 ug/ml of serum, were 105.3 %, 95.2 % and 96.0 %, respectively, and the mean recovery was 98.8 %.

TABLE 2

Recovery and Reproducibility of the HBK EIA system

Sample (μg/ml)	Recovery (%) (n=10) Mean ± SD	Intra-assay variance (n=10) Found(μg/ml)		Inter-assay variance (n=10) Found(μg/ml)	
		Mean ± SD	CV(%)	Mean ± SD	CV(%)
2.0	105.3 ± 3.4	2.11 ± 0.07	3.3	2.07 ± 0.13	6.2
4.0	95.2 ± 2.2	3.81 ± 0.09	2.4	3.78 ± 0.20	5.3
8.0	96.0 ± 5.0	7.68 ± 0.40	5.2	7.70 ± 0.48	6.2
Average	98.8		3.6		5.9

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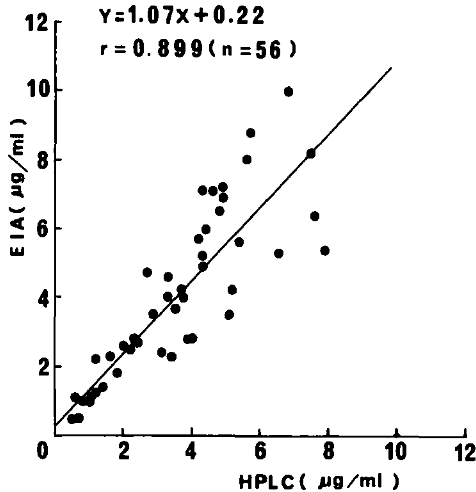

FIGURE 5

Correlation between HBK levels in patient serum samples determined by high performance liquid chromatography(HPLC) and enzyme immunoassay(EIA).

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Reproducibility

The reproducibility in the enzyme immunoassay for HBK was estimated from the coefficient of variation (CV) in intra-assay and inter-assay system. To determine intra-assay CV, ten replicates of HBK-containing serum were measured in the same assay at three different concentrations, i.e., 2.0, 4.0 and 8.0 ug/ml of serum. As shown in Table 2, the intra-assay CV values at each concentration were 3.3 %, 2.4 % and 5.2 %, respectively, and the mean value was 3.6 %. These samples containing above amounts of HBK were also measured ten times in the different assay to determine

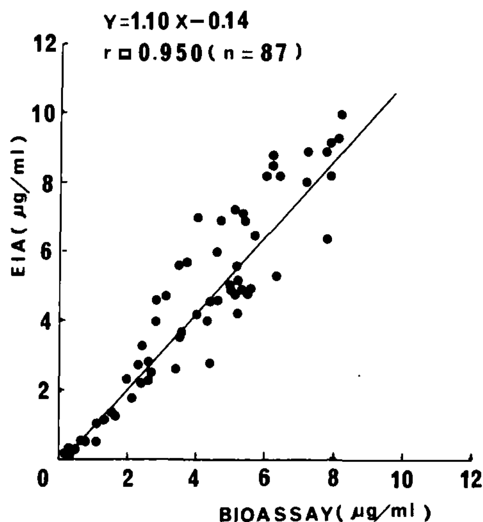

FIGURE 6

Correlation between HBK levels in patient serum samples determined by antimicrobial assay and EIA.

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inter-assay CV. The inter-assay CV values at each concentration were 6.2 %, 5.3% and 6.2 %, respectively, and the mean value was 5.9 %.

Comparison with antimicrobial assay and HPLC.

In order to compare the serum level of HBK determined by the enzyme immunoassay with that determined by antimicrobial assay and HPLC, 87 sera from patients infused HBK (dose: 100 mg, 75 mg) were measured by each method. Figure 5 shows the correlation of serum levels of HBK determined by the enzyme immmunoassay and HPLC. The regression equation was Y=1.07X + 0.22 and the correlation coefficient was

0.899. Figure 6 shows the correlation of serum levels of HBK by the enzyme immunoassay and antimicrobial assay. The regression equation was Y=1.10X - 0.14 and correlation coefficient was 0.950. Therefore, there was no statistically significant difference in the measurement of HBK among these three methods.

DISCUSSION

Although a lot of radioimmunoassay methods for the measurement of aminoglycosides have been reported (4, 5, 6), non-isotopic immunoassay methods are fewer (7). We have developed a rapid and simple enzyme immunoassay to measure serum levels of HBK using hapten heterologous method; the affinity of the antiserum is much higher to HBK-ALP than to HBK, therefore, we employed 3'-eno-HBK-ALP as an enzyme labeled antigen to reduce the affinity to the enzyme labeled antigen. Thus, the sensitivity was raised comparing with homologous assay system.

The antiserum used in the assay cross-reacts with such aminoglycosides as DKB, TOB, KM, AKM and AMK. These cross-reactivities, however, were practically negligible on measuring sera from patients receiving HBK, because it is unusual that the patients are received more than a kind of aminoglycosides.

In most immunoassay, incubation is performed overnight or more. Incubation period was shortened to 20 min by co-incubating the first and the second antibody and the assay can be completed within one hr in the present enzyme immunoassay. This assay system is applicable for monitoring serum levels of HBK.

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