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Homogeneous Enzyme Immunoassay for Netilmicin

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A newly developed homogeneous enzyme immunoassay for the determination of netilmicin in serum was evaluated and compared with a radioenzymatic assay. A total of 102 serum samples from patients treated with netilmicin were measured by both methods. This comparison showed an excellent correlation ($r = 0.993$). The enzyme immunoassay has proved to be precise, accurate, and specific. Because of its rapidity and the ease of performance, this method is a useful alternative to current assays for monitoring serum netilmicin concentrations.

Netilmicin, the 1-*N*-ethyl derivative of sisomicin, is a recently introduced semisynthetic aminoglycoside antibiotic. It is active against a large number of gram-negative bacteria, including some strains resistant to gentamicin and tobramycin (8). Although animal studies indicate that netilmicin may be less nephro- and ototoxic than gentamicin (2, 5), the therapeutic index remains relatively low. For this reason, and because of the dependency of netilmicin elimination on renal function, monitoring of serum levels is recommended to ensure optimal dosage and to reduce the risk of side effects (3).

Various techniques exist to measure aminoglycoside serum levels, including bioassay, radioenzymatic assay (REA), radioimmunoassay, and high-performance liquid chromatography (6). For gentamicin and tobramycin, non-radioactive enzyme immunoassays (EIA) have also been introduced (EMIT; Syva Corp., Palo Alto, Calif.). Owing to its accuracy, specificity, and rapidity, this technique offers a definite advantage for drug level determinations under clinical conditions (4, 7). Netilmicin can also be measured with the EMIT reagents for gentamicin after some minor modifications because of the cross-reactivity of the antibody (11). Recently, a specific EIA for netilmicin has been developed. We have evaluated the accuracy, reproducibility, and specificity of this new assay in comparison with an REA which has been previously used in our laboratory (12).

MATERIALS AND METHODS

A total of 102 serum samples from patients treated with netilmicin as the sole antibiotic were measured by EIA and REA. Of these blood samples, two-thirds were taken 1 h after drug administration or before the next dose to determine peak or trough level, respectively. All samples were stored at −70°C until assayed. Each of the assays was performed by one of the authors.

Reagents for the EIA method were supplied by Syva Corp. In this non-radioactive assay, netilmicin in the sample competes with netilmicin bound to glucose-6-phosphate dehydrogenase for a specific antibody and thus reduces antibody-induced inactivation of the enzyme. The remaining enzyme activity, measured as a change in absorbance at 340 nm due to the conversion of NAD^+ to NADH, correlates directly with the netilmicin concentration in the serum sample. This so-called homogeneous EIA does not need a step for separation of bound and free enzyme-labeled antigens.

The assay was performed according to the instructions of the manufacturer. With a pipetter-diluter, each 50-μl serum sample was diluted with 250 μl of buffer in a 2-ml disposable beaker with a conical bottom. After repeating this dilution step in a second beaker, which yielded a 36-fold-diluted working solution, 50 μl of antibody-substrate (reagent A) was added together with 250 μl of buffer, followed by 50 μl of enzyme-labeled netilmicin (reagent B), again with 250 μl of buffer. This mixture, resulting in a total volume of 0.9 ml for the assay, was immediately aspirated into a Stasar III spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a flow cell maintained exactly at 30°C. The absorbance rate between 15 and 45 s after filling the flow cell (ΔA) was recorded with a CP-1000 EMIT clinical processor, which also calculated the standard curves and the concentrations in the samples from the patients. The concentrations of the standards were 0, 1, 2, 4, 8, and 12 μg of netilmicin per ml and were assayed in duplicate.

The absorbance rates of the standards, corrected for the zero standard, yield a straight line when plotted on a special logit function paper supplied with each kit.

The REA was a modification (12) of the method of Broughall and Reeves (1). It uses kanamycin 6′-acetyltransferase prepared by sonication of an overnight culture of *Escherichia coli* W677/R5. The reaction mixture consisted of the following: 50 μl of sodium citrate buffer (pH 5.7) containing 1.2 μM $MgCl_2$, 0.3 μM dithiothreitol, and 12 nM [1-^{14}C]acetyl coenzyme A (Amersham Corp., Amersham, England) with a

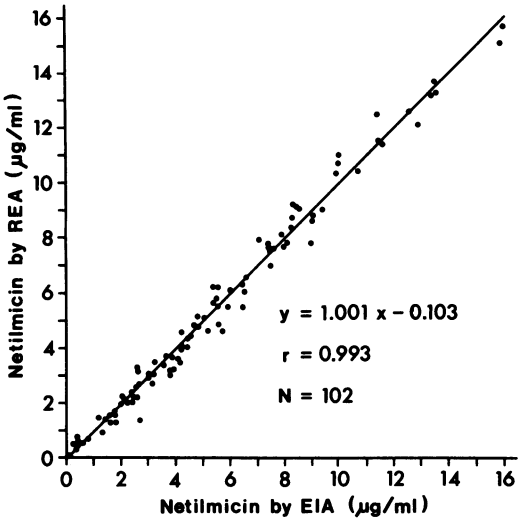

FIG. 1. Linear regression analysis of a comparison of serum netilmicin concentrations determined by EIA and REA.

specific activity of 9.4 μCi/mM, 50 μl of enzyme suspension, and 50 μl of serum. The assay tubes were incubated for 45 min at 35°C in a shaking water bath. After incubation, 50 μl of the reaction mixture was transferred onto a phosphocellulose disk, and after the disks were washed for 20 min in ice-cold 0.5 mM Tris-hydrochloride (pH 7.4), they were dried and then counted in a β-scintillation counter. All samples were assayed in duplicate. Standards were prepared from netilmicin supplied by Schering Corp., Bloomfield, N.J. The standard curves were strictly linear within the assay range. To achieve a counting error of less than 1%, we counted the samples for 2 to 20 min in the β-scintillation counter. This resulted in overall assay times of 6 to 12 h for a series of 20 serum samples.

RESULTS

A total of 102 serum samples from patients receiving netilmicin were measured by EIA and REA. The comparison between the two methods is shown in Fig. 1. The netilmicin concentrations ranged between 0.4 and 16 μg/ml. The equation for the linear regression line was $y = 1.001 - 0.103$ with a correlation coefficient of 0.993. Within-day and between-day reproducibilities were determined by analyzing three samples containing 1.5, 6, and 10 μg of netilmicin per ml 20 times on the same day and 12 to 18 times over a period of 2 months by both methods (Tables 1 and 2). The reproducibility of the standard curves is shown in Fig. 2, where the mean values and standard deviations of 12 curves are displayed. To evaluate the accuracy, 10 serum blanks were spiked with known amounts of netilmicin at 1.5 to 12 μg/ml and measured by both methods on 2 subsequent days. The mean recoveries ± standard deviations determined by EIA were 103.0 ± 3.2% on day 1 and 103.1 ± 4.7% on day 2. The corresponding recoveries determined by REA were 103.1 ± 4.7% and 103.9 ± 7.6%, respectively. The slightly elevated recoveries determined by both methods may have been due to a small spiking error during the preparation of the samples (J. A. Seck, Syva Corp., personal communication). To evaluate the accuracy below 1 μg/ml, 10 additional serum samples with netilmicin concentrations between 0.4 and 0.9 μg/ml were analyzed. Applying only a single dilution step, we could achieve a mean recovery of 93.3 ± 4.8% even in this range. The minimal netilmicin concentration which could be measured by the REA method with a within-day reproducibility of less than 5% was 0.25 μg/ml. The specificity of the EIA assay was assessed by adding known amounts of different antibiotics to human serum (up to 250 μg/ml) and was calculated as the percent response compared with the percent response to the equivalent netilmicin concentration. Cross-reactions were as follows: about 130% with sisomicin, which is structurally very similar to netilmicin, 100% with gentamicin, and only about 0.6% with tobramycin. No

TABLE 1. Reproducibility of the EMIT EIA for netilmicin

Netilmicin concn (μg/ml)	No. of samples	Mean concn ± SD (μg/ml)	CV (%)[a]
Within-day			
1.5	20	1.58 ± 0.06	3.7
6.0	20	6.22 ± 0.21	3.4
10.0	20	9.60 ± 0.23	2.9
Between-day			
1.5	12	1.57 ± 0.07	4.5
6.0	18	6.20 ± 0.15	2.3
10.0	12	9.87 ± 0.38	3.8

[a] CV, Coefficient of variation.

TABLE 2. Reproducibility of the REA for netilmicin

Netilmicin concn (μg/ml)	No. of samples	Mean concn ± SD (μg/ml)	CV (%)[a]
Within-day			
1.5	20	1.49 ± 0.04	2.7
6.0	20	6.23 ± 0.12	1.8
10.0	20	10.06 ± 0.22	2.2
Between-day			
1.5	11	1.48 ± 0.07	4.7
6.0	12	5.96 ± 0.12	2.1
10.0	11	9.95 ± 0.24	2.4

[a] CV, Coefficient of variation.

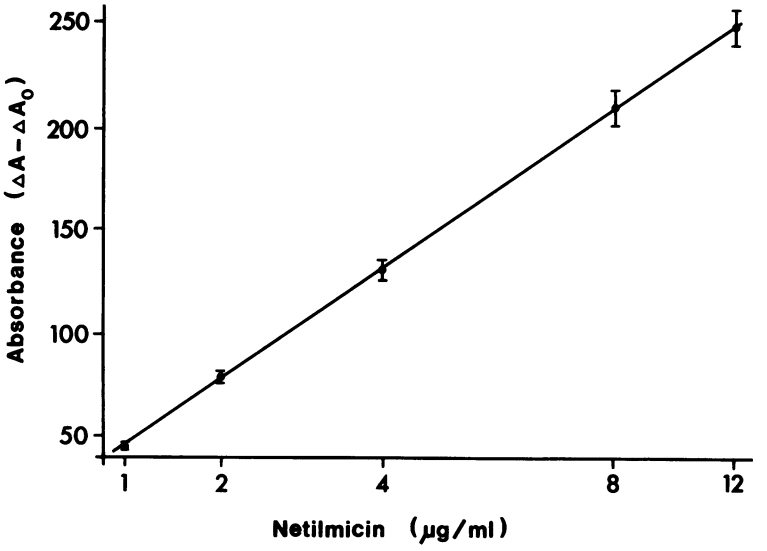

FIG. 2. Absorbance rates (means ± standard deviations) of 12 netilmicin standard curves determined by EIA.

cross-reaction was detected with neomycin (32 μg/ml), amikacin (50 μg/ml), kanamycin (100 μg/ml), cefoxitin (250 μg/ml), cefoperazone (250 μg/ml), cefacetril (250 μg/ml), carbenicillin (250 μg/ml), ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), tetracycline (50 μg/ml), sulfamethazole (145 μg/ml), or trimethoprim (30 μg/ml).

DISCUSSION

The EIA method evaluated in this study compared very well with the REA and was an accurate and precise assay for determinations of netilmicin in serum. The coefficients of variation for both assays were below 5% for both the interassay and the intraassay reproducibilities. One of the main advantages of the EIA is its rapidity, since one sample including a standard curve can easily be run within 20 to 30 min and any additional samples need only about 3 min for a duplicate determination, whereas the REA needs several hours, depending on the duration of β-counting. If a concentration is outside the standard curve or if a result is questionable, it is possible to rerun the sample immediately. The lowest concentration of the standard curve for the EIA is 1 μg/ml. However, serum levels between 1 and 0.4 μg/ml can be measured with sufficient accuracy, using only one predilution step, as has been demonstrated for gentamicin (9). This might be important in determining serum trough levels for patients with normal renal functions. Careful mixing of the reagents and an exact timing between pipetting the solution and aspirating it into the photometer flow cell is important for precise and reproducible results. Automation of this step may therefore further improve performance characteristics. As the EIA requires only 50 μl of sample, it is also suitable for use in pediatric medicine. By altering the dilution procedure it would be possible, although impractical, to use a serum sample as small as 9 μl. Compared with the radioimmunoassay, a popular assay for measuring aminoglycoside antibiotics, the EIA does notrequire separation of free and bound label. In addition, one does not have to deal with radioactive waste, which is an increasing problem.

The cross-reactions of the antibody with gentamicin and sisomicin have no clinical importance, as patients are not simultaneously treated with two aminoglycosides. On the other hand, it should be possible to measure sisomicin with this assay after some minor modifications. The antibody has shown no cross-reaction with β-lactam antibiotics, which are very often administered concomitantly with aminoglycosides. The acetylating enzyme kanamycin 6'-acetyltransferase, which is used in the REA method, also reacts with other aminoglycoside antibiotics and is therefore routinely used to measure these drugs.

Because the range of drugs measured in therapeutic drug monitoring is steadily increasing, laboratories involved in this kind of work should limit the number of analytical techniques to reduce costs and ensure effective quality controls (10). EIA fit well into this concept, since a wide variety of clinically important drugs can rapidly be measured with the same equipment and similar assay protocols.

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