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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NETILMICIN IN GUINEA-PIG AND HUMAN SERUM BY FLUORODINITROBENZENE DERIVATIZATION WITH SPECTROPHOTOMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic procedure for netilmicin determination in guinea-pig and human serum using pre-column derivatization with 1-fluoro-2,4-dinitrobenzene and UV detection is described. Linearity was established over the range 0.5–40 μg/ml using only 50 μl of serum. Accuracy and precision were good, with a mean coefficient of variation less than 5% and a mean relative error less than 4%. This procedure correlates well with an enzyme multiplied immunoassay technique and has a sensitivity similar to those of published fluorescence derivatization methods.

INTRODUCTION

Netilmicin is a semisynthetic aminoglycoside antibiotic obtained by modification of sisomicin [1]. It is active at relatively low concentrations, is widely used and has a broad spectrum of activity against both gram-positive and gram-negative bacteria, including strains resistant to other aminoglycosides [2–4]. Moreover, various preclinical and clinical data suggest that this antibiotic has less nephro- [5,6] and ototoxicity [7–10] than other aminoglycosides used in therapy. However, to ensure therapeutic efficacy associated with a low incidence of side-effects, monitoring of serum netilmicin levels is necessary. This is especially important in life-threatening infections, in patients with impaired renal function or when long-term therapy is required.

Netilmicin serum levels are typically determined by microbiological tests, radioimmunoassays (RIA) or non-isotopic immunoassays. The microbiological assays [11,12] are simple and inexpensive, but have several disadvantages, such as interference from other antimicrobial agents, low accuracy and prolonged analysis time (more than 6 h). RIA methods [13] are very sensitive, but depend on

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the specificity of the antibodies. Cross-reactions have been reported [14]. Furthermore, RIA requires the use of radiochemicals and the availability of adequate facilities. Non-isotopic immunoassays have recently been proposed for determination of netilmicin in serum, such as enzyme immunoassay (EIA) [15–17] and substrate-labelled fluorescent immunoassay (SLFIA) [18]. These methods are rapid, and concentrations lower than 0.5 μg/ml can be detected, but purified enzymes are required. The specificity of the enzyme preparations also affects this type of assay and, in some cases, normal constituents of serum may interfere with the assay [19].

High-performance liquid chromatography (HPLC) has also been used for determination of aminoglycosides: there are several methods available for testing gentamicin, tobramycin and amikacin, but few for netilmicin. All require either pre- or post-column derivatization for fluorescence detection [20–23]. The published methods involve complicated procedures and require specialized equipment.

Since our ototoxicity studies were conducted in guinea-pigs, we developed an HPLC method that would allow drug evaluation in a minimal amount (50 μl) of guinea-pig serum. In order to extend the validity of the assay, we also included samples of human serum.

EXPERIMENTAL

Materials and reagents

Demineralized water was used throughout. 1-Fluoro-2,4-dinitrobenzene (FDNB), acetic acid, sodium hydroxide and disodium tetraborate decahydrate were reagent grade from Merck (Darmstadt, F.R.G.). Acetonitrile, "LiChrosolv", was also from Merck. Netilmicin sulphate (SCH 20569) and purified gentamicin sulphate component C1a were supplied by Schering-Plough (Bloomfield, NJ, U.S.A.). Stoppered polypropylene "Eppendorf" tubes of 1.5 ml capacity, stoppered (with PTFE silicone discs), Reacti-Vials (Pierce, Rockford, IL, U.S.A.), and a Millex-HV4 Filter Unit, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.), for sample purification prior to instrumental analysis, were also used. Stock solutions of netilmicin and the internal standard in demineralized water were stored at 4 °C and were stable for at least 6 months. The working serum standards (0.5–40 μg/ml) were stored at −20 °C and were stable for at least 1 month. All antibiotic concentrations were calculated as free base. Borate buffer (0.1 *M*, pH 10) was prepared by dissolving disodium tetraborate decahydrate (3.81 g) in water, adding sodium hydroxide until pH 10 was attained, and finally making the volume up to 100 ml. Derivatizing reagent was prepared weekly by dissolving 250 mg of FDNB in 1 ml of acetonitrile. Enzyme multiplied immunoassay technique (EMIT) – Netilmicin Kit (Syva Company, Palo Alto, CA, U.S.A.) was used for comparative purposes.

HPLC equipment and chromatographic conditions

The chromatographic system consisted of a Series 410 LC-Pump, a Model LC-95 UV–VIS variable-wavelength detector, and a laboratory computing integrator LCI-100 (Perkin-Elmer, Norwalk, CT, U.S.A.). A prepacked μBondapak C_{18} (10

μm particle size, 300×3.9 mm I.D., Waters Assoc., Milford, MA, U.S.A.) preceded by a guard column (C_{18}, 33×4.6 mm I.D., 5 μm particle size, Perkin-Elmer) was used for chromatographic separation. The samples were injected with a Model 7125 valve (Rheodyne, Cotati, CA, U.S.A.) equipped with a 50-μl loop. The mobile phase was prepared by mixing 300 ml of water with 700 ml of acetonitrile and 1 ml of acetic acid. This solution was passed through a 0.5-μm filter (Millipore) and degassed by helium bubbling. The flow-rate was maintained at 2.2 ml/min at a pressure of 11.8 MPa. Chromatography was performed at room temperature and the column effluent was monitored at 365 nm.

Extraction procedure and HPLC separation

Serum (50 μl) was dispensed into a polypropylene vial followed by the addition of 20 μl of internal standard (gentamicin C1a 1 mg/100 ml) and 50 μl of borate buffer. The mixture was vortexed for 15 s and 200 μl of acetonitrile were added. The tubes were vortexed again for 15 s and then centrifuged at 2000 *g* for 5 min. The supernatant was filtered with Filter Unit Millex-HV4, 200 μl were transferred into a Reacti-Vial, and 20 μl of derivatizing reagent were added. The vial was closed and placed in a water-bath at 80°C for 120 min. The sample was cooled rapidly to room temperature, filtered with Filter Unit Millex-HV4, and 50 μl were injected onto the chromatographic column. For calibration, an aqueous solution containing netilmicin was added to serum samples to provide concentrations in the range 0.5–40 μg/ml.

Under these conditions, the gentamicin C1a and netilmicin derivatives had retention times of 11.0 and 13.5 min, respectively. Guinea-pig and human serum contained a few endogenous peaks, which eluted in less than 8 min and therefore did not interfere with the analysis. No other peaks were observed in the chromatogram after the netilmicin derivative peak. More than 150 samples were injected with no appreciable loss of sensitivity or alteration of retention time. A gradual increase in back-pressure was eliminated by replacing the guard column. Methanol–water and acetonitrile–water were investigated as possible mobile phases. The best results were obtained with acetonitrile–water, as described above. Acetic acid was added to prevent column damage due to the repeated injection of alkaline samples [27,29].

Deproteinization and derivatization

Aminoglycosides are poorly soluble in non-polar solvents and their extraction from biological fluids is difficult. Because direct derivatization is obstructed by proteins, serum proteins were removed by precipitation [24–30]. This was done with acetonitrile after dilution with alkaline buffer to avoid co-precipitation of the aminoglycosides.

UV detection of aminoglycosides is severely limited. This was overcome by derivatizing the primary amino groups with FDNB to yield better chromophores. The 2,4-dinitrophenyl (DNP) derivative formed is stable and can be measured using UV detection at 365 nm [24,27,29]. The optimal conditions for the formation of derivatives were determined by investigating the effects of reaction time, buffers, FDNB concentration and temperature on derivatization of aqueous

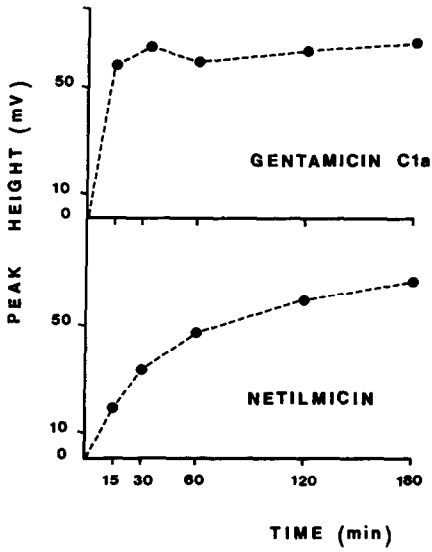

Fig. 1. Effect of the reaction time on the peak-heights of derivatized netilmicin (40 μg/ml) and gentamicin C1a (10 μg/ml).

netilmicin (40 μg/ml) and gentamicin C1a (10 μg/ml) solutions. The effect of reaction time on the peak-heights of derivatives was examined at 80°C (Fig. 1). The reaction was judged to be maximal by 15 min for gentamicin C1a, but was not maximal after 120 min for netilmicin. The formation of derivatives was also examined at 60°C and 100°C. At the lower temperature the reaction was found to proceed too slowly, whereas at the higher temperature the experimental error was somewhat larger and netilmicin derivatization was still not complete. A reaction time of 120 min at 80°C was selected in order to keep the time for the total assay within acceptable limits. The derivatizing reaction was tested using different concentrations of FDNB in acetonitrile over the range of 0.25–1 g/ml. The reaction was not enhanced by increasing the FDNB concentration. The stability of the derivatized products was studied. Aqueous solutions of gentamicin C1a (10 μg/ml) and netilmicin (40 μg/ml), which had been derivatized for 120 min and stored at 4°C for 24 and 48 h, showed increases of peak-heights ranging from 6.3% to 10.0% with no change in the chromatographic profile. The effect of various buffer systems on FDNB derivatization was also studied. Different buffer systems at concentrations of 0.1 *M* and at pH 10 were tested: borate buffer gave the best result with netilmicin, and both borate and phosphate buffers gave similar results with gentamicin C1a.

RESULTS

Linearity, precision and accuracy

Samples of spiked guinea-pig and human serum, each containing one of seven different concentrations of netilmicin, were analysed. The peak-height ratios (netilmicin/internal standard) were linearly related to netilmicin concentration

over the range 0.5–40 μg/ml. The equation for the straight line was $y=0.0013+0.0354x$, with $r=0.99$. The accuracy and precision of the method are shown in Table I. It is evident that, over the range 0.5–40 μg/ml, the relative errors ranged from 0.4% to 12.0% (mean 3.2%). The precision had a coefficient of variation (C.V.) ranging from 1.8% to 10.9% (mean 4.8%).

Limit of quantitation and recovery

The lowest concentration of netilmicin determined in serum was 0.5 μg/ml. As shown in Table I, the accuracy (relative error, 12%) and the precision (C.V., 10.9%) were quite good. Typical chromatograms of blank guinea-pig serum and guinea-pig serum spiked with 5.0 μg/ml of netilmicin are shown in Fig. 2. Typical chromatograms of blank human serum and human serum spiked with 9.5 μg/ml of netilmicin are shown in Fig. 3. The analytical recovery of netilmicin added to serum was determined by comparing the peak-heights from serum samples containing 1.0 μg/ml and 40 μg/ml of netilmicin with those obtained from the same concentration of aqueous solutions. The recovery of netilmicin was 81% (S.D.=6%; $n=4$) and 78% (S.D.=8%; $n=4$), respectively. The recovery of internal standard was 104% (S.D.=1%; $n=4$).

Stability and day-to-day reproducibility

Serum samples containing 5.0 and 20.0 μg/ml of netilmicin and stored at −20°C were analysed in duplicate on days 1, 2, 3, 12, 15, 18, and 23. As can be seen in Table II, the mean values of netilmicin concentrations were 5.10 ± 0.21 (C.V.=4.2%) and 19.46 ± 0.86 (C.V.=4.4%), respectively. Thus, the stability and reproducibility are quite good.

Comparison of HPLC and EMIT methods

Inter-method correlations were carried out by analysing guinea-pig serum samples spiked with different concentrations of netilmicin. The samples were assayed in duplicate by both HPLC and EMIT methods. Fig. 4 demonstrates corresponding values from HPLC and EMIT methods. A correlation coefficient of 0.99 was obtained. The equation for the straight line, $y=0.070+0.936x$, further confirmed

TABLE I

HPLC STANDARD CURVE OF NETILMICIN IN SERUM: ACCURACY AND PRECISION

Concentration added (μg/ml)	Number of samples	Observed concentration (μg/ml)	C.V. (%)	Relative error (%)
0.5	5	0.56	10.9	12.0
1.0	5	1.06	3.8	6.0
2.5	5	2.46	8.3	1.6
5.0	5	5.02	2.4	0.4
10.0	5	10.16	4.0	1.6
20.0	5	19.93	1.8	0.4
40.0	5	40.18	2.6	0.5

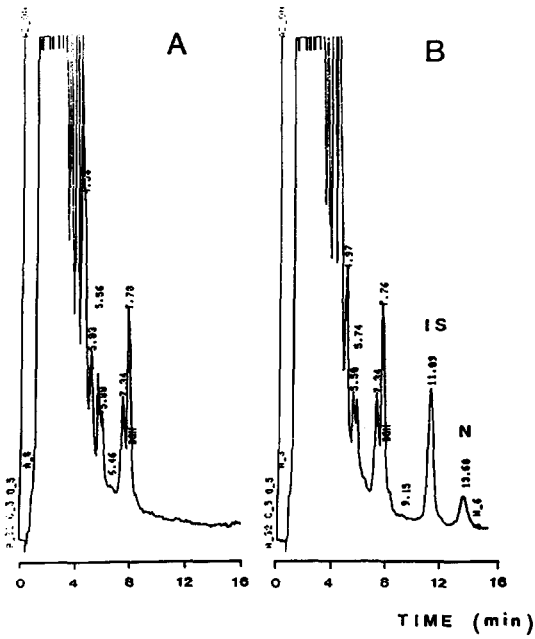

Fig. 2. (A) Chromatogram obtained from 50 μl of blank guinea-pig serum. No internal standard was added. (B) Chromatogram obtained from 50 μl of guinea-pig serum, spiked to a concentration of 5.0 μg/ml of netilmicin (N) with gentamicin C1a as internal standard (IS). Attenuation 0.008 a.u.f.s.

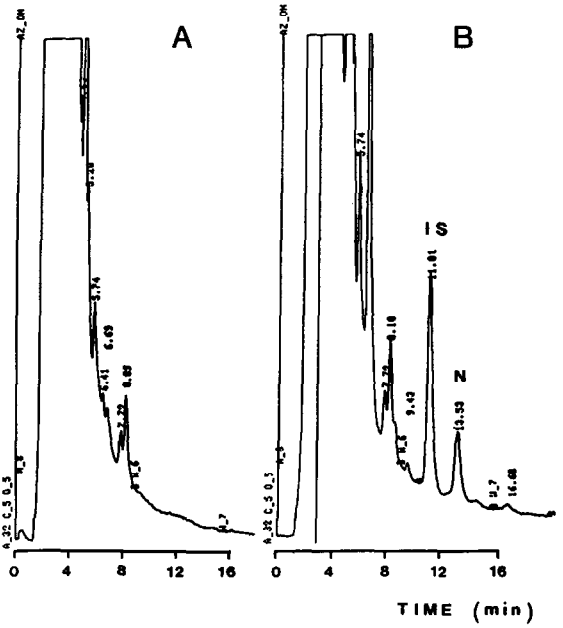

Fig. 3. (A) Chromatogram obtained from 50 μl of blank human serum. No internal standard was added. (B) Chromatogram obtained from 50 μl of human serum spiked to a concentration of 9.5 μg/ml of netilmicin (N) with gentamicin C1a as internal standard (IS). Attenuation 0.008 a.u.f.s.

TABLE II

HPLC ASSAY: DAY-TO-DAY REPRODUCIBILITY

Day	Netilmicin concentration (μg/ml) estimated in serum spiked with	
	5.00 μg/ml	20.00 μg/ml
1	4.93	19.52
2	5.02	19.75
3	5.31	20.12
12	4.94	18.20
15	5.06	19.22
18	5.35	19.89
23	–	19.49
Mean	5.10±0.21	19.46±0.86
C.V. (%)	4.2	4.4

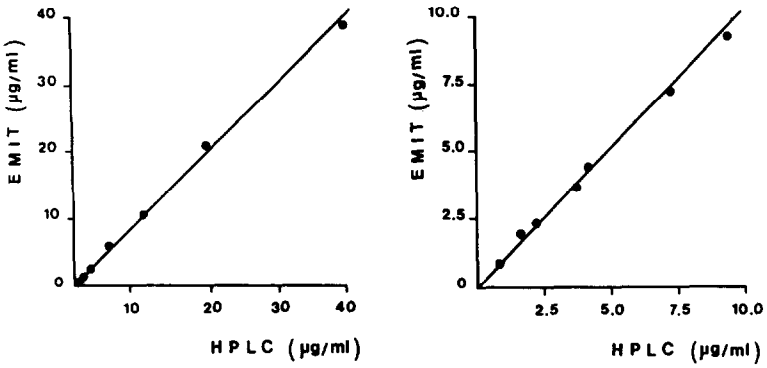

Fig. 4. Linear regression analysis of netilmicin concentration in guinea-pig and human serum as determined by EMIT and HPLC procedures. Left: guinea-pig serum, $y=0.070+0.936x$; $r=0.99$. Right: human serum, $y=0.113+0.984x$; $r=0.99$.

the uniformity between the methods. Human serum samples spiked to different therapeutic concentrations of netilmicin were similarly assayed. The correlation between the two methods was also good (Fig. 4, $r=0.99$) and the equation was $y=0.113+0.984x$.

DISCUSSION

The reported method is suitable for the routine assay of netilmicin with standard HPLC equipment. The derivatization procedure with FDNB is simple. The DNP derivative is stable and can be measured using UV detection at 365 nm. The sensitivity is similar to that of fluorescence derivatization methods [20,21]. The lowest netilmicin concentration that can be determined in 50 μl of serum samples with acceptable precision was found to be ca. 0.5 μg/ml. Use of gentamicin C1a

as internal standard minimizes variations in detector response or any chromatographic conditions; it also compensates for injection and sample preparation errors. Chromatography takes less than 20 min and thus allows daily analysis of several samples. The method was developed to measure netilmicin levels in small samples obtained from guinea-pigs treated with the aminoglycoside. However, the method was shown to be capable of measuring netilmicin in human serum and can therefore be used for clinical samples. The HPLC assay presented here correlates well with a commercially available EMIT assay, and is thus feasible for monitoring netilmicin level in the therapeutic range. Serum volumes of as little as 50 μl provide a further advantage for the multiple sampling associated with pharmacokinetic analyses or for monitoring pediatric patients.

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