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Short Communication

Determination of netilmicin in plasma by HPLC

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1. Introduction

Netilmicin is an antibiotic of the aminoglycoside group and is effective in the treatment of severe infections, both local and systemic. Its narrow therapeutic range means that therapy must be individualized by monitoring serum concentrations.

For the determination of aminoglycoside antibiotics by HPLC with fluorescence detection, it is necessary to perform a derivatization reaction; this can be implemented either pre- or post-column. Most techniques available until now have used post-column derivatization. In comparing both types of derivatization, pre-column (1–5) and post-column (6–9), in general the former has advantages over the latter such as the use of simpler equipment, the possibility of using derivatization reactions that do not have to be instantaneous and a reduced consumption of derivatizing reagent. Accordingly, pre-column derivatization was the technique of choice for the present work; it was carried out the *o*-phthaldialdehyde, which reacts with primary amines.

The aim of this work was to develop a high-performance liquid chromatography (HPLC) technique to determine netilmicin in plasma with pre-column derivatization.

2. Experimental

2.1. Reagents

Netilmicin, pure product (Schering C); *o*-phthaldialdehyde (Sigma); heptanesulphonic acid (Sigma); HPLC grade acetonitrile (Merck); trichloroacetic acid (Merck); methylene chloride (Carlo Erba); isopropanol (Merck); 2-mercaptoethanol (Sigma); acetic acid (Merck); boric acid (Panreac); and sodium hydroxide (Panreac) were used.

2.2. Apparatus

A Crison pH meter, model 501 (Barcelona, Spain), equipped with an Ingold 9811 combined glass electrode (Wilmington, MA, USA); a Microspin 12 high-speed centrifuge, Sorval Instruments, Du Pont (Stevenage, Herts, UK); an Ultrason Selecta ultrasonic bath, model 513; and a Supelco 5-8068 vacuum ultrafiltration system with a 0.45-μm membrane were used.

2.3. Chromatographic conditions

The technique used for the quantification of netilmicin in plasma samples was reversed-phase HPLC on a 300 × 4 mm i.d. column packed with 10-μm RP-18 with a liquid chromatograph (Varian model 5000), a fluorescence detector (Kontron, Mod. SFM-25) and a data station (Kontron, MT-1).

The gradient elution system comprised two phases: phase A was water–acetic acid–heptanesulphonic acid (0.1 M) (80:10:10, v/v/v) and B was acetonitrile. The linear profile adopted was: $t = 0$, %B = 40; gradient $t = 10$ min, %B = 50. After analysis of the sample, the mobile phase was allowed to flow under the initial conditions for 10 min to stabilize the column before injecting the next sam-

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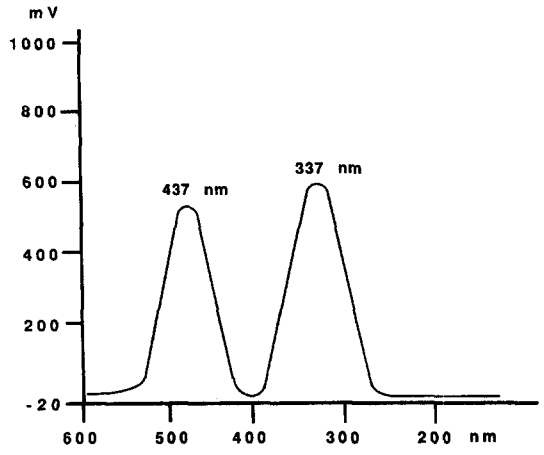

Fig. 1. Excitation (peak at 337 nm) and emission (peak at 437 nm) fluorescence spectra for the derivatization product of netilmicin.

ple. The flow-rate was 2 ml min^{-1}. The mobile phase was passed through a 0.45-μm and was degassed by ultrasound before use.

The detector was set at a response time of 2 s and the voltage was varied depending on the concentrations to be analyzed, ranging between 500 and 700 mV. The excitation and emission wavelengths were chosen according to those of the mobile phase; these were 337 nm and 437 nm, respectively, as shown in Fig. 1.

2.4. Procedure

Before injection into the chromatograph, the samples had to be conditioned. For this purpose, 100 μl of a solution of 25 μg ml^{-1} gentamicin (internal standard) and 100 μl of trichloracetic acid were added to 100 μl of sample. This mixture was shaken for 30 s and centrifuged for 5 min at 3200 rpm. Then, 100 μl of 1 M NaOH was added to the supernatant and the mixture was for 30 s; 1 ml of monobasic potassium phosphate solution (previously adjusted to pH = 11 with 19 M KOH) and 2 ml of dichloromethane were added; the mixture was shaken for 10 s and centrifuged for 5 min at 3200 rpm. The aqueous phase was collected and 1 ml of derivatizing reagent was added; this mixture was shaken for 30 s, 500 mg of anhydrous sodium carbonate was added and the mixture was shaken again for 30 s. 500 μl of 2-propanol was added to extract the derivatized netilmicin. This extract was then shaken and centrifuged for 5 min to remove foreign particles; the supernatant 2-propanol extract was injected into the chromatograph.

The derivatizing reagent was prepared daily from 5 mg of *o*-phthaldialdehyde, 5 ml of borate buffer (0.1 M; pH 10.4), 0.5 ml of methanol and 0.3 ml of 2-mercaptoethanol.

Fig. 2 shows the structure of the reaction products with *o*-phtaldialdehyde.

3. Results and discussion

Borate buffer (0.1 M; pH 10.4) is used in the derivatizing reagent because the reaction has to be carried out under critical pH conditions. This solution of derivatizing reagent is stable at room temperature, but is readily oxidized by the action of light or air to produce a completely transparent yellow solution; accordingly, the vessel containing the solution must be protected by aluminium foil and suitably sealed.

Anhydrous sodium carbonate was added to the sample preparation to prevent the aqueous phase from mixing with the 2-propanol organic phase, since under normal conditions both compounds are miscible. The lower limit of detection was 0.1 μg ml^{-1}under these conditions.

Fig. 3 shows a chromatogram of netilmicin in plasma at a concentration of 5.0 μg ml^{-1}, with 25 μg ml^{-1} of gentamicin as internal standard.

Fig. 4 shows a chromatogram of a blank plasma sample with 25 μg ml^{-1}, of gentamicin as internal standard.

For studying the reproducibility of the analytical technique the RSD was calculated both within-day and between-day.

Calculation of the within-day RSD of the analytical technique was performed by measuring three replicates of each point on the calibration graph on the same day, and performing a simple linear regression between the peak-height ratios of netilmicin and gentamicin and the netilmicin concentration by applying the ANOVA test. The correlation coefficient (r) of the straight line was 0.994; the RSD was 9.22%.

To establish the statistical between-day RSD values, replicates of each point on the calibration graph were measured on 5 different days and a simple linear regression was performed with the data; the RSD was 14.23%.

The RSD values obtained were quite high, which is understandable in view of the complex treatment required by the sample. Although

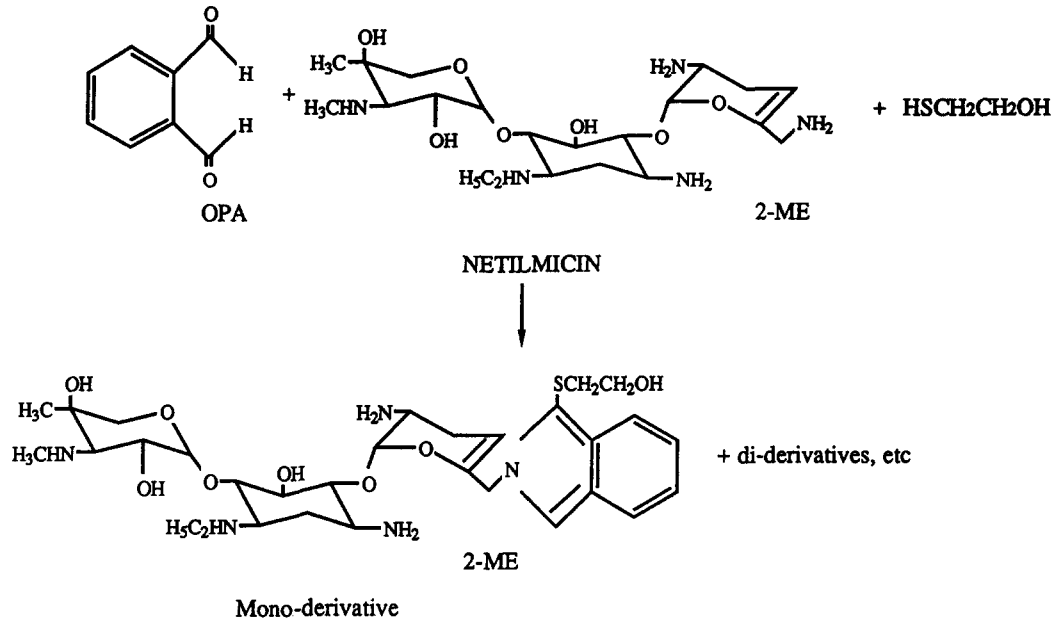

Fig. 2. Structure of the reaction products with *o*-phthaldialdehyde.

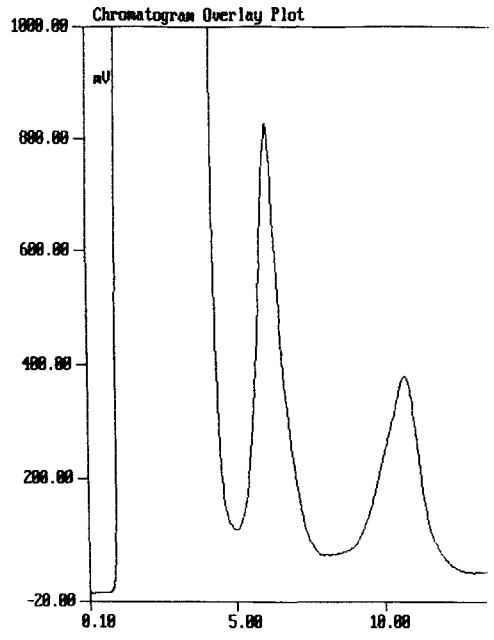

Fig. 3. Chromatogram of netilmicin in plasma at a concentration of 5.0 μg ml^{-1}, with 25 μg ml^{-1} of gentamicin as internal standar.

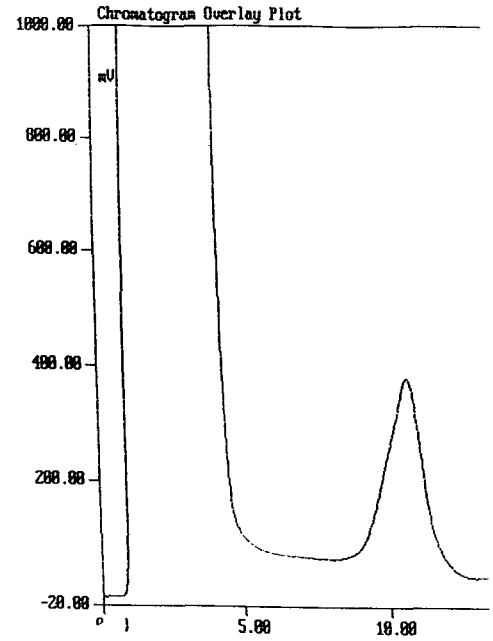

Fig. 4. Chromatogram of a blank plasma sample, with 25 μg ml^{-1} gentamicin as internal standard.

there was good linearity for the analytical technique, because the between-day RDS values obtained were above 10% it was appropriate to obtain a daily calibration straight line to minimize possible errors in evaluating the plasma samples. In this way, all the samples analyzed on one day were evaluated with respect to the same calibration straight line.

In conclusion, the proposed method for the determination of netilmicin in plasma by HPLC has good linearity and acceptable reproducibility.

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