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Note

Determination of gentamicin in serum using liquid column chromatography

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Gentamicin (GTM) is an aminoglycoside antibiotic used in the treatment of serious infections. Maximum therapeutic efficacy occurs for peak levels in the range 5—10 mg/l [1]. Overdosing may result in renal impairment [2—4], whereas sub-therapeutic doses are not only ineffective but may lead to the development of antibiotic resistance [5]. GTM has been reported to have a serum half-life in patients with normal renal function in the magnitude of 2 h [2]. As a consequence serum concentrations determined 8 h after the medication have to be very low, and values above 1.5—2.0 mg/l indicate tissue accumulation of GTM. It is well established that monitoring serum GTM levels is the most effective ensurance for adequate therapy [5]. Until very recently these analyses were performed only by using microbiological (MB) methods. These methods are often time consuming due to the bacterial growth rate. However, the growth rate may be increased by using special test-strains [6] and higher incubation temperatures [7] allowing the plates to be read after 4—6 h. Furthermore the method is cheap and simple involving only the normal equipment of a bacteriological laboratory. However, faster methods would be an improvement. Radioimmuno assays have been used in the routine monitoring of serum GTM levels, but require the use of radiochemicals. High-performance liquid chromatography [8—13] has been reported to be an adequate technique. GTM is treated with reagents making the derivate detectable by means of fluorometry. Recently, a homogeneous enzyme immuno assay (EMIT®) has been described as a fast and specific analysis adequate for the routine monitoring of serum GTM level.

This paper reports on a method using high-performance liquid chromatography (HPLC) for the separation procedure with quantitation by means of ultra-violet (UV) absorption.

EXPERIMENTAL

Reagents

Methylene chloride, acetonitrile and concentrated borate buffer (pH 10) were all of analytical reagent grade from E. Merck (Darmstadt, G.F.R.). Benzene sulphonyl chloride (BSC) was of synthetic grade (E. Merck) and distilled once before use (1 mmHg, 108°C). Other commercial chemicals and solvents were of analytical grade.

Stock solutions (1 g/l) of GTM and of the internal standard, netilmicin, were prepared in distilled water. When kept in a refrigerator at 4°C they were stable for at least six months.

Extraction procedure

To a 500-µl serum sample were added 60 µl of sodium hydroxide (1 *N*), 500 µl of phosphate buffer (1/15 *M*, pH 7.4) and 12.5 µg netilmicin as internal standard. Acetonitrile (3 ml) was added and the tube was shaken vigorously for 10 sec resulting in a precipitation of the serum proteins. The specimen was then centrifuged for 3 min at 1000 *g* after which the supernatant was transferred to another tube containing 1500 µl of phosphate buffer (1/15 *M*, pH 7.4) and methylene chloride (3 ml). The contents were mixed for 5 min at 20 rpm on a rotary mixer, and centrifuged. The buffer phase (upper layer) was transferred to another tube using a pasteur pipette moistened with distilled water. A volume of 50 µl sodium hydroxide (1 *N*) and 600 µl BSC solution in acetonitrile (1%, v/v) was added. The mixture was reacted at 75°C for 10 min in an open system. After reaction, the tube was filled with nitrogen, stoppered and placed in the freezer for 10 min to cool the reaction mixture to 0°C. Borate buffer (3 ml, pH 10) and methylene chloride (3 ml) were then added. The mixture was extracted for 5 min at 20 rpm, centrifuged and the methylene chloride (lower layer) was transferred to a tapered tube and evaporated to dryness (40°C) under a stream of nitrogen. The residue was dissolved in 100 µl of the mobile phase.

Liquid chromatography

A liquid chromatograph (Pye Unicam, Cambridge, Great Britain) type LC3 equipped with an UV detector LC3 was used. The column (25 cm × 4.6 mm I.D.) was filled with LiChrosorb RP-18, particle size 10 µm. The mobile phase was acetonitrile—methylene chloride—water—methanol (80 : 10 : 8 : 4) with a flow-rate of 4.0 ml/min. The detection was carried out at 230 nm.

Calculations

The serum concentrations were read from standard curves constructed from chromatograms of serum samples containing varying, but known amounts of GTM, giving concentrations of up to 10 mg/l. The peak height ratios between GTM and netilmicin were plotted against the concentrations.

Enzyme immuno assay

The analyses were carried out using the instruments and the reagent kits as recommended by the Syva Corporation (Palo Alto, CA, U.S.A.).

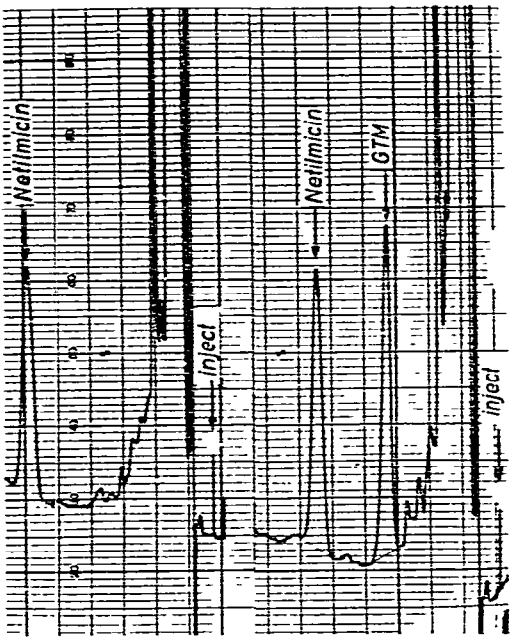

Fig. 1. Chromatograms of serum samples containing 0 (left) and 7.5 mg gentamicin per l (right). Gentamicin and netilmicin appear 3.5 and 5.5 min respectively, after the injection.

RESULTS

Fig. 1 illustrates chromatograms of serum samples containing 0 (left) and 7.5 mg GTM per l (right), respectively. Under the conditions described GTM and netilmicin appeared 3.5 and 5.5 min respectively, after the injection. A calibration graph constructed on the basis of serum samples containing various concentrations of GTM in the range from 0 to 10 mg/l, demonstrated linearity between the GTM concentrations and the ratio between the peak heights of GTM and netilmicin (Table I).

TABLE I

CORRESPONDING VALUES BETWEEN THE GTM SERUM CONCENTRATION AND THE PEAK HEIGHT RATIO (R) OF GTM TO NETILMICIN

Concentration GTM added (mg/l)	R
0.0	0.00
2.5	0.40
5.0	0.79
7.5	1.20
10.0	1.58

TABLE II

ACCURACY OF GTM DETERMINATIONS

Number of samples, 10.

Concentration added (mg/l)	Calculated concentration* (mg/l)	Coefficient of variation (%)
2.5	2.6 ± 0.13	5.0
5.0	5.1 ± 0.14	2.7
7.5	7.6 ± 0.39	5.1
10.0	9.9 ± 0.20	2.0

*Mean ± S.D.

TABLE III

RANDOMLY SELECTED GTM SAMPLES DETERMINED BY HPLC, EMIT®, AND MB

HPLC	EMIT®	MB
0.3	0.1	<1
0.8	0.6	<1
1.3	1.2	1.6
0.8	0.6	<1
4.9	4.6	4.5
1.3	1.1	<1
1.2	1.0	<1
3.3	3.3	2.9
1.3	1.4	<1
3.6	3.1	3.8
4.3	4.2	5.6
2.0	2.2	4.5
0.8	1.2	1.0
2.0	1.7	2.2
7.0	6.3	11.0
2.4	2.3	
4.8	4.4	
7.0	6.3	
9.1	7.5	
9.1	8.1	
10.7	9.4	
5.7	5.5	
13.8	11.7	
7.4	6.9	

The sensitivity was defined as the lowest concentration giving a peak at least ten times higher than the noise on the base line. In this way the lowest concentration giving safe quantitation was 0.2 mg/l.

The accuracy and reproducibility for clinical use was found to be within acceptable limits (Table II).

Tests of specificity, i.e. adding different drugs to the samples, were not carried out but we never detected any interfering peaks.

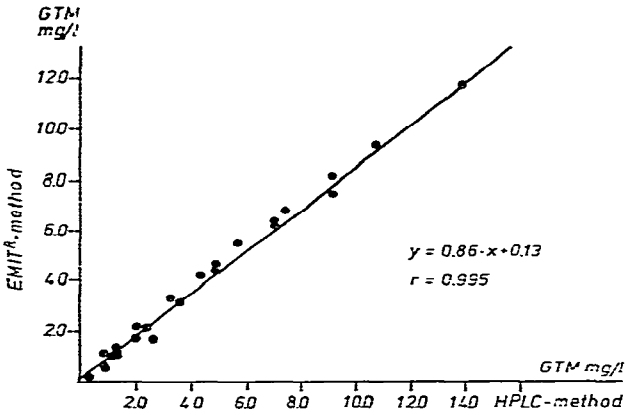

Fig. 2. Relationship between the EMIT® and the HPLC procedures. For both methods 24 serum samples were measured.

The composition of the mobile phase makes it possible to inject a new sample every 6 min, giving a (theoretical) capacity equal to 10 samples per h.

Inter-methods correlations were carried out by analysing randomly selected serum samples from patients treated with ordinary dosages of GTM. The samples were analyzed using the HPLC, MB and the EMIT® methods. The results obtained are given in Table III. As seen the methods gave almost identical results for concentrations up to 13 mg/l. Fig. 2 demonstrates corresponding values from the HPLC and the EMIT® methods. A correlation coefficient of 0.995 was obtained. The equation for the straight line, $y = 0.86x + 0.13$, further confirms the uniformity between the methods.

DISCUSSION

Direct UV detection of GTM is impossible because of the lack of an UV chromophore. Chemical derivatization with e.g. BSC is necessary to enhance UV absorbance. The isolation of GTM from other serum constituents is difficult due to the excellent solubility of GTM in water or phosphate buffer. Direct derivatization of GTM in human serum samples is obstructed by albumin, therefore precipitation of this compound is necessary before the reaction. Acetonitrile is found to be an excellent reagent for precipitation of proteins, due to the formation of a solid mass that adheres to the wall of the tube [8]. Addition of methylene chloride removes most of the acetonitrile into the lower organic phase, resulting in an increased concentration of GTM in the upper layer.

Many procedures for the determination of GTM using liquid chromatography have been reported [8—13], and the method described in this paper can not be claimed to be superior to these assays, as the demanded sample volume, the detection limit, the specificity and the accuracy of all these methods are almost similar. However, in one point our method differs from the other liquid chromatographic methods, as no separation of the three constituents of GTM (C_1, C_{1a}, C_2) is obtained (Fig. 1). No attempts have been made to achieve specific estimations of each of the three compounds, as the main purpose with this assay was to design a routine method.

The derivatization with BSC is a critical point in the procedure, as too long a reaction time and too high a temperature apparently destroy the formed compound. The reaction time and temperature have to be maintained within narrow limits. The addition of sodium hydroxide immediately before the reaction with BSC is important to ensure a pH between 7 and 9 after the derivatization. Experiments have demonstrated the importance of a certain ratio between the amounts of sodium hydroxide and the BSC.

The results given in Fig. 2 show that the EMIT® and the HPLC methods give almost identical results when applied to the same serum samples drawn from patients in ordinary therapeutic treatment. This indicates an acceptable specificity achieved by both methods, when identical results are obtained using totally different analytical techniques.

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