Determination of Gentamicin Released from Orthopedic Carrier System by a Novel HPLC Method

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Abstract

A new, fast high-performance liquid chromatograpic-UV method has been developed and validated for quantitative analysis of gentamicin carrier samples drawn in drug release studies. The samples of the calibration curve were prepared in physiological salt solution, which were applied as dissolution medium for the drugs. The calibration curve is in the range of 1–100 µg/mL. The mobile phase consisted of methanol-water-acetate buffer (0.02 M ammonium acetate solution, adjusted with cc. ammonia to pH = 9), a reverse phase, Zorbax Rx-C-18 2.1 × 150 mm, 5 µm column has been used. Validation procedure of this newly developed method is carried out. Linearity, specificity, intra- and inter-day precision and accuracy, robustness, limits of detection, and quantitation are investigated.

Introduction

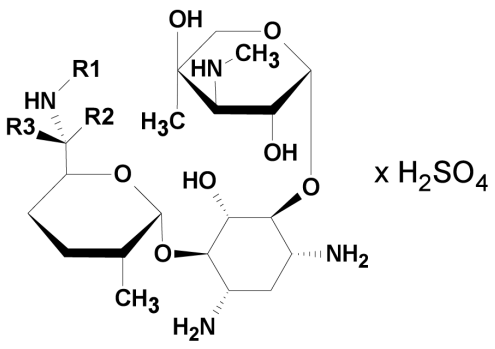
Gentamicin (GEN) is a broad spectrum, aminoglycoside-type antibiotic that is isolated from *Micromonispora purpurea* (Figure 1). GEN kills bacteria by damaging the plasma membrane and binding to the 16S ribosomal RNA, leading to the inhibition of microbial protein synthesis. It is effective against a wide spectrum of gram-negative and gram-positive bacteria. In case of systemic administration, ototoxic, and nephrotoxic side effects can happen (1).

Several methods have been developed to determine GEN content based on microassay (2), immunoassay (3,4), gas chromatographic method coupled with mass spectrometry (5), liquid chromatography (LC) (6,7,8), and capillary electrophoresis (CE) (9,10). The disadvantage of most of these methods is that they are time-consuming.

Yusuf et al. investigated a simplified high-performance (HP) LC method for the determination of GEN components in plasma microsamples using derivatizing agent. The runtime was 33 min, the flow rate was 2.5 mL/min. This method is able to use small sample size (50 µL), as well as maintain a high degree of accuracy (6).

The research group of Megoulas developed an LC method with evaporative light scattering detector for the determination of GEN in raw materials and pharmaceuticals. Their method was applied for different pharmaceutical formulations (injection, drops, and cream). No pretreatment was used except creams, for which liquid–liquid extraction was required. The different limit of detection (LOD) values happened to be 1.2–2.4 µg/mL for the GEN components (7).

A rapid (runtime 10 min) and simple method for the separation and quantitation of gentamicin sulphate by HPLC coupled with evaporative light scattering detection has been developed by Clarot et. al. Their method does not need any derivatization. This method was used to evaluate the composition of gentamicin sulphate of commercial samples from different sources. Mass spectrometric studies comfirmed that no impurities co-elute with gentamicin components. The determined LOD relative to the C_{1a} peak is 0.16% (m/m) (8).



Gentamicin	R1	R2	R3
C1	CH_3	CH_3	H
C1a	H	H	H
C2	H	CH_3	H
C2a	H	H	CH_3
C2b	CH_3	H	H

Figure 1. The structural formula of gentamicin.

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The method of Yusuf et al. has good applicability for blood samples after derivatizing, but has 33 min of retention time. The other two methods mentioned previously do not a need derivatization agent during analysis. The method of Megoulas is applicable for the analysis of the four components of GEN in different pharmaceutical formulations and raw materials. Clarot et al. described another HPLC method with evaporative light scattering detection, for the separation of GEN components and minor components. The last two methods have a retention time approximately 10 min.

Manyanga et al. investigated an LC method with pulsed electrochemical detection. The base of their researches was the method described in the European Pharmacopoeia, which was improved by changing separation parameters. The developed method is repeatable, linear and robust. Also the different GEN components can be separated (11,12).

Several papers deal with UV detection in case of GEN (1,13–16). Curiel et al. developed a CE under reversed polarity to achieve a complete baseline separation between the components with direct UV detection (195 nm) for underivatized GEN (10). Chai et al. measured GEN adsorbed to hydroxyapatite with spectrophotometric method (17). Wang et al also described a method which was used for analyzing GEN carried calcium sulphate implants with direct UV detection (18). Doadrio et al. investigated mesoporous SBA-15 carrier system containing GEN. In their case GEN was analyzed with reversed-phase (RP-HPLC) (19).

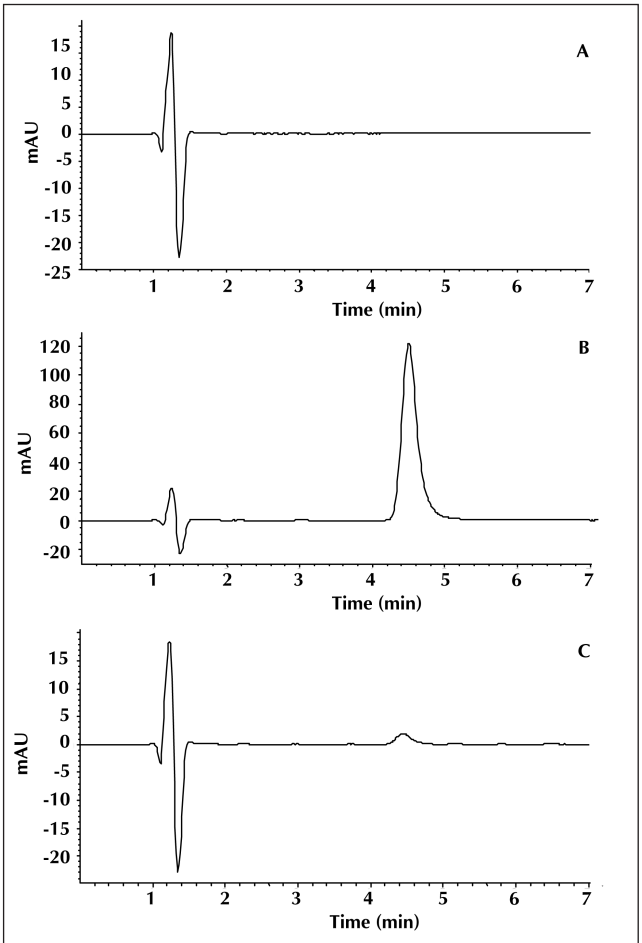

Figure 2. Chromatogram of blank (A), calibration (60 µg/mL) (B), and sample injection (C).

The objective of the research was to develop a simple analytical method that is applicable in the investigation of GEN containing drug carrier systems for orthopedic use that release the antibiotics for prolonged time, at least for several weeks. These novel drug delivery systems should be tested first under in vitro conditions in order to evaluate the release profile of the active ingredient. For the design and testing of the GEN carrier system, it is necessary to apply a simple and rapid analytical method for the qualitative and quantitative analysis. In this paper, a new and rapid method is presented, which determines total amount of GEN (without separation of different components) from samples drawn from in vitro drug release tests in physiological salt solution without any pretreatment. Accordingly, in the present work a novel HPLC–UV method was developed and validated.

Experimental

Materials

Gentamicin sulphate (meets EP and USP testing specifications) and gradient grade solvents (water for HPLC, methanol, ammonium acetate buffer) used for the chromatographic measures were purchased from Sigma Aldrich Ltd., (Budapest, Hungary).

White wax and white petrolatum, used for the preparation of GEN carrier systems, were obtained from Hungaropharma Co. (Budapest, Hungary) meeting the quality requirements of the European Pharmacopoeia.

Analytical measurement

The analysis was carried out with a HP 1050 High Performance Liquid Chromatographic instrument (Agilent Technologies Inc., Palo Alto, CA) with HP Chemstation software Rev. A10.02 (Agilent, Waldbronn, Germany). The system consisted of a binary pump, on-line degasser, autosampler, column heater, and a diode array detector (DAD). A Zorbax Rx-C-18 (2.1 × 150 mm, 5 µm) column (Agilent, Waldbronn, Germany) was used for the separation. Column temperature was 30°C, the volume of injection was 20 µL and the flow rate was 0.3 mL/min. In the isocratic separation, the mobile phase consisted of methanol–water–ammonium acetate buffer (0.02M, adjusted with concentrate ammonia to pH = 9): 35:60:5 (v/v/v). The chromatograms were recorded at 280 nm according to the absorption maximum with DAD.

Preparation of standard stock solution and quality control solutions

For the preparation of the 100 µg/mL solution (standard stock solution), 0.0100 g of GEN was dissolved in physiological salt solution (0.9% NaCl). Calibration solutions (1, 2, 5, 10, 50, 80, and 100 µg/mL) were diluted from the standard stock solution, in the concentration range of 1–100 µg/mL.

The concentration of quality control samples were 5, 20, and 80 µg/mL prepared by the same manner described previously (diluting from daily stock solution).

Formulation of drug delivery system

GEN was suspended with the portions of the melt mixture of white wax and white petrolatum in different molar ratio (Composition I, II, and III), then moulded into plastic blisters of 1.5 cm diameter and 0.5 cm height, then left to form tablets after cooling (20).

In vitro drug release test

The in vitro drug delivery test of samples was done for 2 weeks. The dissolution medium was 100 mL physiological salt solution tempered at 37°C.

The pharmaceutical formulation, containing 4 mg of GEN, was placed into the dissolution medium without stirring. Drawing of 5 mL samples was performed in every 24 h. The dissolution medium was replaced by fresh medium every day. Purification of the samples was not necessary.

Table I. Statistical Evaluation of Linearity

Concentration	1–100 µg/mL
Calibration fitting	$y = a + b \times x$
r^2	0.99908
$F_{Lack\text{-}of\text{-}fit}$	26.3602
$p_{Lack\text{-}of\text{-}fit}$*	0.0000854
$a \pm s_a$†	49.6498 ± 8.2306
$b \pm s_b$†	22.7302 ± 0.1578
Standard error of residuals	10.9999

*Significance level 95 % † s = standard error

Results and Discussion

Method development

A Zorbax Rx-C-18 column, maintained at optimal temperature (30°C), was used for method development. The mobile phase, water–methanol–acetate buffer (0.02 M, pH = 9, adjusted with cc. Ammonia), at a flow rate of 0.3 mL/min was selected after several preliminary investigatory chromatographic runs using different eluent compositions. The injection volume was increased from 10 µL to 20 µL, and the flow rate was changed to 0.5–0.3 to enhance method sensitivity without sacrificing chromatographic performance. The investigations were done on the basis of suitability for drug content estimation and cost, because rapid and economic analysis is becoming increasingly important in pharmaceutical analysis.

The new analytical HPLC–UV method was simple, uses isocratic mobile phase and has a short retention time (Figure 2). During in vitro dissolution tests there was no need for a special purification step due to the lack of biological samples. The present analytical method can be used during pharmaceutical development of drug delivery systems containing GEN. The developed simple HPLC–UV method facilitated the research work, due to the short retention time allowing a large number of measurements within a short time.

Validation Procedure

Validation procedure of the method was carried out (21). Results are demonstrated in Tables I, II, and III.

Table II. Summary of Inter- and Intra-day Accuracy and Precision

Aspects	Intra-day (n = 25/conc.)			Inter-day (n = 54 /conc.)		
Nominal conc.	Measured conc. (µg/mL)	RSD (%)	Accuracy (%)	Measured (µg/mL)	RSD (%)	Accuracy (%)
5 µg/mL	4.9606 ± 0.0933	1.8660	99.212	5.0610 ± 0.0979	1.9580	101.220
20 µg/mL	20.4590 ± 0.1975	0.9875	102.295	20.2451 ± 0.2022	1.0110	101.255
80 µg/mL	79.3246 ± 1.5954	1.9943	99.156	78.6213 ± 1.2389	1.5486	98.276

Table III. Summary of System Suitability Test of HPLC Method*

	Starting conditions	Flow rate (mL/min)		Methanol		Temperature		Wavelength	
		0.27	0.33	–1%	+1%	–5°C	+5°C	–5 nm	+5 nm
Retention time (min)									
5 µg/mL	4.449	4.817	3.930	4.809	4.152	4.628	4.405	4.602	4.762
20 µg/mL	4.451	4.828	3.936	4.916	4.155	4.693	4.415	4.605	4.712
80 µg/mL	4.479	4.849	3.940	5.036	4.189	4.740	4.440	4.762	4.652
Asymmetry factor									
5 µg/mL	0.918	0.856	0.847	0.835	0.827	0.913	0.899	0.911	0.876
20 µg/mL	1.036	0.874	0.868	0.889	0.903	1.019	0.990	1.028	1.021
80 µg/mL	0.984	0.933	0.942	0.906	0.942	0.995	1.044	1.005	1.026
Capacity factor (k')									
5 µg/mL	3.096	3.369	2.565	3.361	2.138	3.198	2.996	2.478	3.321
20 µg/mL	3.039	3.379	2.570	3.459	2.769	3.257	3.005	3.177	3.274
80 µg/mL	3.063	3.398	2.574	3.568	2.799	3.299	3.027	3.319	3.220

* RSD of mean area < 2%

Specificity

In order to exclude the presence of any interfering components in the retention area, specificity and selectivity of the method were tested. Physiological salt solution was injected 6 times. Chromatograms of blank, calibration, and sample injections are shown in Figure 2. No disturbing component was detected in the retention area, and the peak purity chromatogram is shown in Figure 3.

Linearity

The linearity of the method was studied by analyzing standard solutions at seven different concentrations ranging from 1 µg/mL to 100 µg/mL with triplicate determination at each level. The calibration plot was constructed by plotting mean response against corresponding concentration injected, using least squares method. Statistical evaluation of linearity has been carried out by using Table Curve 2D Version 5.01 (Systat Software INC., Chicago, IL). Results including also lack-of-fit test are shown in Table I.

Inter- and intra-day precision and accuracy

To examine inter- and intra-day confidence, quality control samples (three concentration level, n = 3) were studied for six consecutive days. On the basis of the daily calibration curve, the concentration, accuracy, and precision of the quality control samples

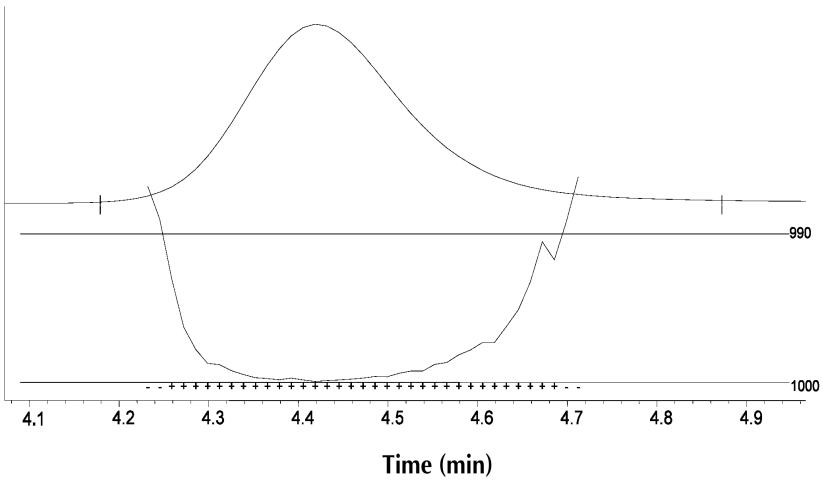

Figure 3. Peak purity chromatogram of standard stock solution.

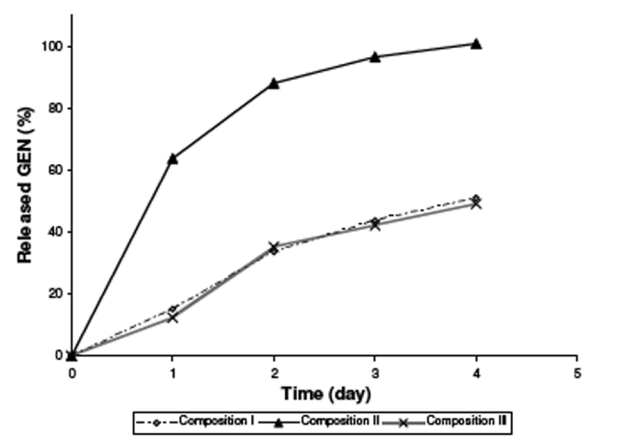

Figure 4. The in vitro releasing profile of drug carrier systems containing gentamicin (RSD < 2%, *n* = 5).

were calculated (RSD < 2%). Results are shown in Table II. The accuracy of the method was determined by the measurement of recovery percentage. The results were between 98% and 103%, which proves the accuracy of method.

Robustness study

The robustness of the method was investigated by making small changes in the chromatographic and detection conditions (flow rate, amount of methanol in the mobile phase, temperature, and wavelength) at three concentration levels (22). The changes were monitored when only one condition was varied. The results (shown in Table III.) obtained by comparing mean areas with low value of RSD (RSD < 2%) indicate that none of the changes had a significant effect on the analysis.

LOD and limit of quantitation

The LOD is defined as the lowest concentration of an analyte that can be detected but not necessarily quantified. The limit of quantitation is defined as the lowest concentration of an analyte that can be quantified with acceptable precision and accuracy. The limits of detection and quantification were determined by the method based on the standard deviation (σ) of responses for triplicate blank injections and the slope (S) of calibration curve, using the formulae LOD = $3.3/\sigma S^{-1}$ and in case of the limit of quantitation (LOQ) of this method, the lowest point of calibration curve was selected. The LOD was found to be 0.1658 µg/mL and 1.0 µg/mL as LOQ, respectively. Comparing these results to the LOD values to previously described methods, the sensitivity of this developed novel method is higher (7).

Solution stability

To demonstrate the stability of standard solutions, they were analyzed over a period of 48 h at room temperature. The results confirmed that the retention time and peak area of GEN remained unchanged (RSD < 1%).

In vitro dissolution studies

According to the aim of the formulation work, a drug delivery system was developed with prolonged release (for several weeks). During the formulation work, various GEN concentrations were incorporated into different hydrophobic matrices for GEN drug delivery. The in vitro drug dissolution from the samples was studied over a period of 4 days (RSD < 1%).

Composition I, II, and III contained 4 mg GEN/tablet. The release profiles are demonstrated in Figure 4. In case of Composition I, after four days of test, 51% (2.04 mg) was released. Composition II liberated 99% of GEN (3.96 mg) in four days, in case of Composition III this amount was 49% (1.96 mg). These results show, that Composition II was not able to release the active ingredient for extremely prolonged release, but the other two compositions are seem to be promising for further development.

Conclusion

The aim was to develop a new, robust and reproducible analytical HPLC–UV method supporting the formulation of drug delivery systems. The isocratic HPLC method was suitable for the quantitative and qualitative determination of GEN in physiological salt solution during in vitro drug release tests. The short retention time allowed fast analysis of large number of samples.

The proposed method was applied successfully for the determination of GEN in pharmaceutical formulations without any pretreatment and with sufficient recoveries.

Acknowledgement

The authors thank the Hungarian Scientific Research Fund for the financial support (Grant No.: T049480).

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Manuscript received June 9, 2009;
revision received September 7, 2009.