Journal of Al Azhar University-Gaza *(Natural Sciences)*,**2019, 21** : **69-86**

Spectrophotometric Method for Determination of Gentamicin in Bulk and Pharmaceutical Dosage Forms using Salicylaldehyde

تطوير واختبار مدي كفاءة طريقة التحليل الطيفي لتحديد كمية الجنتاميسين في المادة الخام والأشكال الصيدلانية باستخدام الساليسيلألدهيد

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Received 25/9/2019 *Accepted* 20/11/2019

Abstract:

A sensitive, simple and cost effective spectrophotometric method was developed and validated for determination of gentamicin (GEN) in bulk and pharmaceutical preparations. The method is based on schiff base condensation reaction of the primary amino group of gentamicin with salicylaldehyde reagent (SA) in presence of acetate buffer solution (pH 5.5) at 45 °C for 15 minutes. The obtained yellow colored derivative in methanolic medium showed absorption maxima at 416 nm. The validity of the described method was assessed according to the International Conference on Harmonization (ICH) guidelines. The regression line equation was Y = 0.0053X + 0.0527 for the concentration range 14-170 µg/mL, and a regression coefficient 0.994 (n = 5). The limit of detection (LOD) and limit of quantification (LOQ) were found to be 2.17 and 6.6 µg/mL, respectively. The percent of recovery performed at three different concentrations using standard addition method was in the range 99.77–101.78 ± 0.15-1.55. The precision of the method was satisfactory, the values of relative standard deviation (RSD) were less than 1.22%. No interference liabilities were observed from excipients of GEN dosage forms. The method was successfully applied for determination of GEN; the label claim percentages were 99.24 ± 1.48 and 100.53 ± 2.64 for injections and eye drops, respectively. The results were compared favorably with a validated reference method. The developed method is practical and valuable in terms of its application in quality control laboratories.

Keywords: Spectrophotometric, gentamicin, salicylaldehyde, validation.

الملخص:

يتضمن البحث تطوير ومعايرة طريقة تحليل طيفي تتميز بأنها حساسة، بسيطة واقتصادية لتحديد كمية الجنتاميسين(GEN) سواء كان في حالته النقية أو في المستحضرات الصيدلانية. اعتمدت الطريقة على تكوين مركب ايميني من خلال تفاعل مجموعة الأمين الأولية الموجودة في الدواء مع مجموعة الكاربونيل النشطة الموجودة في مادة الساليسيلألدهيد(SA) في وجود محلول الاسيتات عند الرقم الهيدروجيني 5.5 ودرجة حرارة 45 درجة مئوية لمدة 15 دقيقة. المركب الاصفر المتكون من هذا التفاعلفي وجود الميثانول كمذيب اظهر أعلى شدة امتصاص عند الموجة 416 نانومتر. تم معايرة وتقييم طريقة التحليل حسب مبادئ (ICH). المعادلة الخطية كانت ص = 0.0053 س + 0.0527 والذي ينطبق على مجال التركيز 14−170 ميكروجرام/مليلتر ومعامل ارتباط 0.994 للعدد 5. كانت قيمة كل من (LOD) و (LOQ) 2.17 و6.6 ميكروجرام/مليلتر. تم فحص الدقة و تراوحت نسبة الاسترجاع المئوية للجنتاميسين والتي تم فحصها باستخدام ثلاث مستويات مختلفة عن طريق إضافة المادة القياسية من99.77−101.78 وبانحراف عياري 0.15− 1.55. تم إثبات قابلية الطريقة للتكرار حيث لم يتجاوز الانحراف المعياري النسبي 1.22% ولم يسجل تداخل من المواد المضافة للأشكال الصيدلانية بالطريقة المطورة. تم تطبيق الطريقة بكفاءة في تحديد كمية الجنتاميسين والتي كانت بنسبة مئوية 99.24±1.48 في الحقن و100.53±2.64 في قطرة العين وتم مقارنة النتائج بطريقة مرجعية. وبناءا علي ذلك فان استخدام طريقة تحليل الجنتاميسين المطورة يعد مجديا لضبط الجودة في مراكز تحليل الأدوية.

الكلمات المفتاحية: تحليل طيفي، جنتاميسين، ساليسيلألدهيد، اختبار الكفاءة.

Introduction

Gentamicin (GEN) is a mixture of sulfates of antimicrobial substances produced by *Micromonospora purpurea* and belongs to aminoglycoside antibiotics (Figure 1). It is a potent broad-spectrum antibiotic, used for the treatment of many types of bacterial infections, particularly those caused by aerobic gram-negative bacteria (Martindale 36, 2009). It has a bactericidal effect through inhibition of protein synthesis (MacDougall and Chambers, 2011).

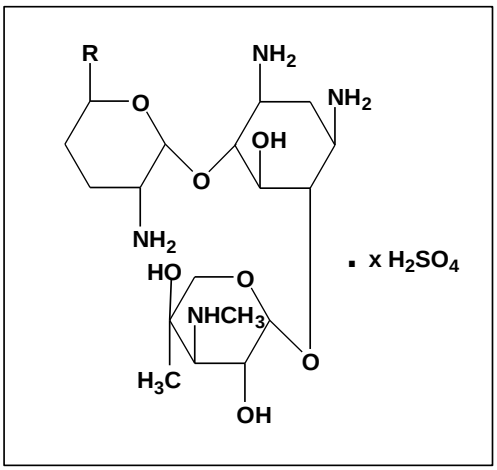

Figure 1: Chemical structure of gentamicin sulfate.

Literature review revealed various analytical methods for gentamicin analysis which were high performance liquid chromatography (HPLC) (Clarot *et al.*, 2004, Joseph and Rustum, 2010; Stypulkowska *et al.*, 2010, Chopra *et al.*, 2013; Manyanga *et al.*, 2008; Kim *et al.*, 2003; Claes *et al.*, 1984; Freemana *et al.*, 1979, Kowalczuk *et al.*, 2010, Kuehl *et al.*, 2012; Laki *et al.*, 2011) , capillary electrophoresis (Curiel *et al.*, 2007; Kaale *et al.*, 2000, Kaale *et al.*, 2001; Santos *et al.*, 2010), UV-Vis spectrophotometry (El-Didamony *et al.*, 2006; Frutos *et al.*, 2000; Krzek *et al.*, 2009; Mukhamedzianov and Likhoded, 1991; Omar *et al.*, 2013b) and spectrofluorimetry (Omar *et al.*, 2013a) based assays for GEN analysis in bulk, pharmaceutical formulations and/or biological samples, either alone or in combination. The official methods for the identification and quantitation analysis of GEN are

prescribed in BP and USP (BP, 2015; USP 32, 2009). USP described for quantitation of GEN a HPLC assay based on prior GEN derivatization with o-phthalaldehyde and detection at 330 nm (USP 32, 2009). A LC method with pulsed electrochemical detection is described in BP (BP, 2015).

UV-Vis spectrophotometry is advantageous for analysis due to robustness, simplicity, good accuracy and precision and cost effectiveness. When drugs like GEN lack adequate chromophores, which can permit analysis at wavelength regions beyond the nonspecific UV-regions of the electromagnetic spectrum, chemical derivatization is necessary. Derivatization reactions are carried out to convert such drugs to readily determinable compounds whose properties and concentration can be related to the original compound (Adegoke, 2012; El-Didamony *et al.*, 2006; Mukhamedzianov and Likhoded, 1991; Omar *et al.*, 2013a). Salicylaldehyde (SA) is a derivatizing agent that through carbonyl group can condense with a primary amine group of GEN to produce an imine chromogen (Figure 2). SA is one of such aldehyde, which can be applied efficiently in analysis (Hassouna *et al.*, 2013; Almani *et al.*, 2013; Humedy, 2015; Wang *et al.*, 2016, Almasri *et al.*, 2019).

In this study we aimed to develop and validate a method for GEN analysis after chemical derivatization with SA using a simple, rapid, and one step procedure, and avoiding vigorous conditions. The proposed method was applied to GEN dosage forms and the results were compared with a reference method (El-Didamony *et al.*, 2006).

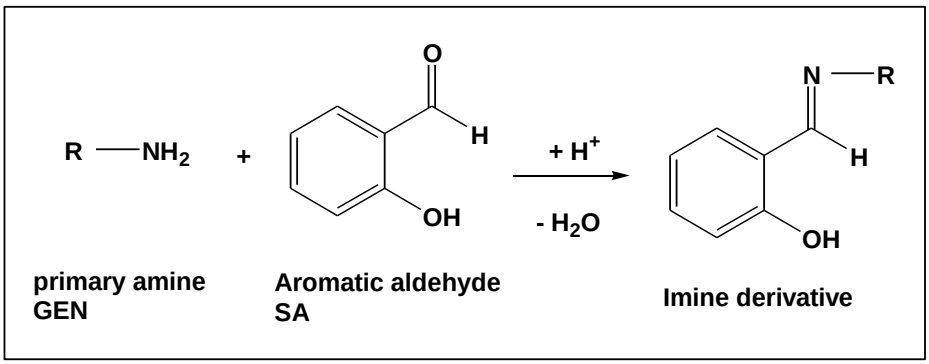

Figure 2: Condensation reaction of primary amine with aldehyde. GEN: Gentamicin, SA: Salicylaldehyde.

Materials and methods

Instruments

Double beam scanning UV-Vis Spectrophotometer (Shimadzu 1601, Japan) with 1 cm matched quartz cell connected to a computer with UV-Probe software. Thermostatically controlled water bath (SB-24, Tokyo Aikakikai, Japan) and pH meter (Shimadzu, Japan) were used.

Chemicals and reagents

Gentamicin sulfate standard (Certified Reference Material, CRM) was kindly donated by Middle East Pharmaceutical and Cosmetics (Megapharm) company limited (Gaza, Palestine). Commercially available pharmaceutical dosage forms, gentamicin ampoules (40 mg/mL) and gentamicin eye drops (3 mg/mL), were purchased from the local pharmacies. Methanol p.a was purchased from Merk (Germany). Salicylaldehyde, glacial acetic acid and sodium acetate supplied by (Sigma-Aldrich, Germany). Pharmaceutical grade excipients: like methyl paraben and propyl paraben were kindly supplied by domestic pharmaceutical companies.

SA solution 0.3% (v/v): 0.30 mL of SA were transferred into 100 mL volumetric flask, mixed thoroughly with warm methanol and diluted with methanol. The solution was freshly prepared daily and protected from light.

Sodium acetate solution 0.6% (w/v): 0.60 g of sodium acetate were accurately weighed, put in 100 mL volumetric flask and dissolved in distilled water.

Acetate buffer solutions: It was prepared by mixing portions of sodium acetate and glacial acetic acid 2M in 1 L volumetric flask according to USP (USP 32, 2009).

GEN standard stock solution (1 mg/mL)**:** It was prepared by dissolving 25.0 mg of anhydrous gentamicin sulphate standard in 25 mL volumetric flask using distilled water.

Selection of analytical wavelength for chromogen

0.45 mL and 1.7 mL of GEN stock solution were transferred into 10 mL volumetric flasks, followed by 2.0 mL of SA solution, and 1.0 mL acetate solution buffer pH 5.5. The content was shaken thoroughly and heated on a water bath at 45°C for 15 minutes. The flasks were left to cool for 10 minutes, then volume was completed with methanol to have 45 and 170 µg/mL GEN, respectively. The absorption spectra and absorption maxima of the resulting solutions (chromogen) were determined against a blank prepared in the same manner without the examined drug**.**

General procedure

0.8 mL of GEN stock solution (1 mg/mL) was transferred into 10 mL volumetric flask, followed by 2.0 mL of SA reagent and 1.0 mL of acetate solution buffer pH 5.5. The content of the flask was shaken thoroughly and heated on a water bath at 45 °C for 15 minutes. The flask left to cool for 10 minutes and the volume was completed with methanol. The absorbance of the resulting solution was measured at 416 nm against a blank.

Optimization of reaction conditions

Different reaction parameters were studied: Solvent, concentration and volume of SA, sodium acetate solution, acetate buffer, pH, temperature, reaction time, and order of addition.

Stability of chromogen

The effect of time on the stability of chromogen was studied by following the absorption of the resulting GEN derivative at different time intervals up to 1 h after dilution at 416 nm.

Method validation

The developed method was validated according to the ICH guidelines (ICH, 2005). They included linearity, range, accuracy, precision, specificity, LOD and LOQ.

Linearity and range:

Different aliquots (0.14 – 1.7 mL) were transferred from stock solution into 10 mL volumetric flasks. The analysis followed the general procedure mentioned above. Three measurements were made

per concentration. The concentration range was 14-170 µg/mL. The absorbance was plotted against concentration. The regression line and

correlation coefficient were determined and was repeated over five consecutive days.

Specificity:

0.2 g of common excipients, which may present in GEN formulations like methyl paraben and propyl paraben were added to 0.1 g GEN standard in 100 ml volumetric flask, mixed and diluted with distilled water. 0.6 and 1.2 mL of the resulting solution were transferred into 10 mL volumetric flask and analysis was then completed as described above. Percent recovery was calculated.

Accuracy:

accuracy of the method was ascertained by performing recovery studies using standard addition method, in which standard drug aliquots were added at three different levels (30 µg/mL, 60 µg/mL and 90 µg/mL) to a pre-analyzed sample of GEN ampoules equivalent to 60 µg/mL. These three concentrations were measured and each of them was repeated three times to calculate accuracy.

Precision:

Intraday precision was assessed by analysis of three different concentrations (30, 60 and 120 µg/mL) of the drug at five different time periods of the same day. On the other hand, inter-day precision was studied by repeating the procedure for three different concentrations (30, 60 and 120 µg/mL) of the drug over six consecutive days. The percent relative standard deviation (RSD) was calculated.

LOD and LOQ:

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using formulas LOD= 3.3 σ/S, and LOQ= 10 σ/S; where, σ

is the standard deviation of residuals of the regression line, S is the slope of the regression line (ICH, 2005).

Analysis of commercial gentamicin pharmaceutical products

Ampoules:
The content of five GEN ampoules (40 mg/mL) was mixed and an accurately measured volume equivalent to 25 mg GEN was transferred into 25 mL volumetric flask and diluted with distilled water. From resulting solution 0.5, and 1.0 mL aliquots were transferred into two 10 mL volumetric flasks then the procedure was followed as described under general procedure. Triplicate measurements were made for each concentration, and the percentage of recovery was calculated.

Eye drops:
The content of three bottles of GEN eye drops (3 mg/mL) was mixed and an accurately measured volume equivalent to 25 mg GEN was transferred into 25 mL volumetric flask and diluted with distilled water. From resulting solution 0.5, and 1.0 mL aliquots were transferred into two 10 mL volumetric flasks then the procedure was followed as described as described under general procedure. Triplicate measurements were made for each concentration, and the percentage of recovery was calculated from the calibration curve. The obtained results were compared with a reference method.

Results and discussion

A common problem of amine is the low ultraviolet absorptivity of the functional group as the case of GEN. Chemical derivatization is the most widely employed solution that allows the UV-Vis detection (Krzek et al., 2009). Imine derivative is a red shifted chromophore. Imines (Schiff bases) are generated by the reaction of aldehydes or ketones with primary amines by acid catalyzed nucleophilic addition of the amine to carbonyl group (Figure 2). In this study salicylaldehyde as a derivatizing agent was applied to enhance UV-Vis detection of GEN.

Selection of analytical wavelength for chromogen

The derivatization reaction of GEN produces a yellow-colored product, which dissolved in methanol. The spectra are shown in Figure 3 and 4. The absorbance of the derivative increased directly

with GEN concentration and exhibiting a λ_{max} at 416 nm. The sensitivity was enhanced by the formation of a colored shiff base derivative.

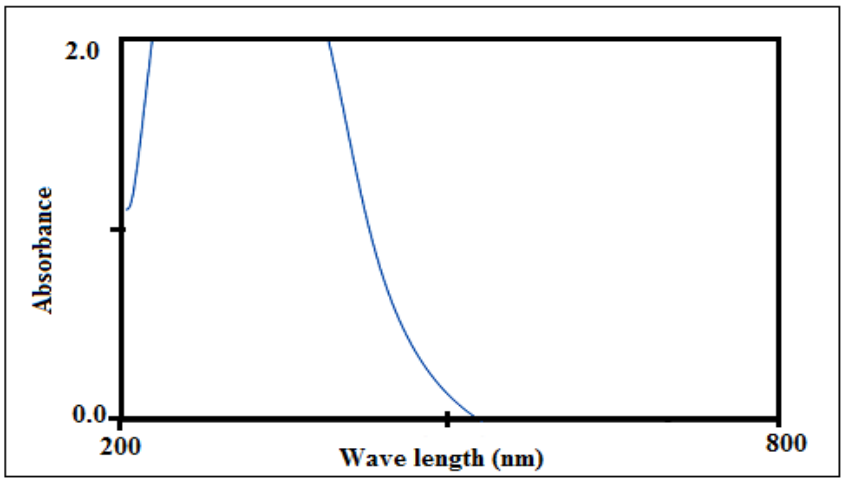

Figure 3: Absorption spectrum of blank solution of SA and sodium acetate against methanol.

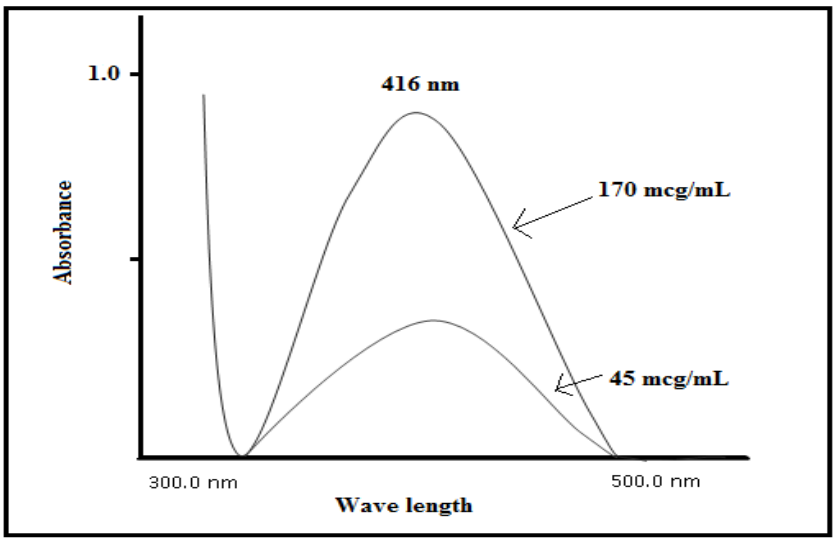

Figure 4: Absorption spectrums of GEN-Derivative against blank. (GEN concentration: 45 µg/mL and 170 µg/mL).

Optimization of reaction conditions

Different reaction variables were examined to optimize the derivatization reaction. The results of are summarized in Table 1.

The pH is critical for imine-producing reactions. In fact the fastest imine formation is at pH 4-6. At higher pH there is insufficient acid present to allow dehydration and at lower pH the amine will be protonated rendering it unable to do a nucleophilic attack at the carbonyl carbon (Sorrell, 2006). The optimum pH was 5.5. The solubility of the yellow imine product required an organic solvent, which was methanol.

Table 1: Summary of optimum conditions for reaction of GEN with SA.

Variable	**Studied range**	**Result**
Solvent	Methanol or Water	Methanol
Volume of SA solution 0.3% (v/v)	1.0 - 2.5 (mL)	2.0 (mL)
Volume of sodium acetate solution 0.6% (w/v)	0.5 – 2.0 (mL)	1.0 (mL)
pH	4 – 7.5	5.5
Temperature	25 -70 (°C)	45 (°C)
Reaction time	5 – 30 (min)	15 (min)
Order of addition	Different	No effect

Heating was required to enhance the reaction. Higher temperature has not effectively improved formation of the product. The reaction proceeded at 45 °C. Stability of imine is pH, temperature, and humidity dependent (Clayden *et al.*, 2012).

Stability of chromogen

It was found that the use of methanol as a solvent yielded good results in terms of stability of GEN derivative which remained at least for 1 h after dilution as shown in Figure 5. This indicated the ability to perform many samples.

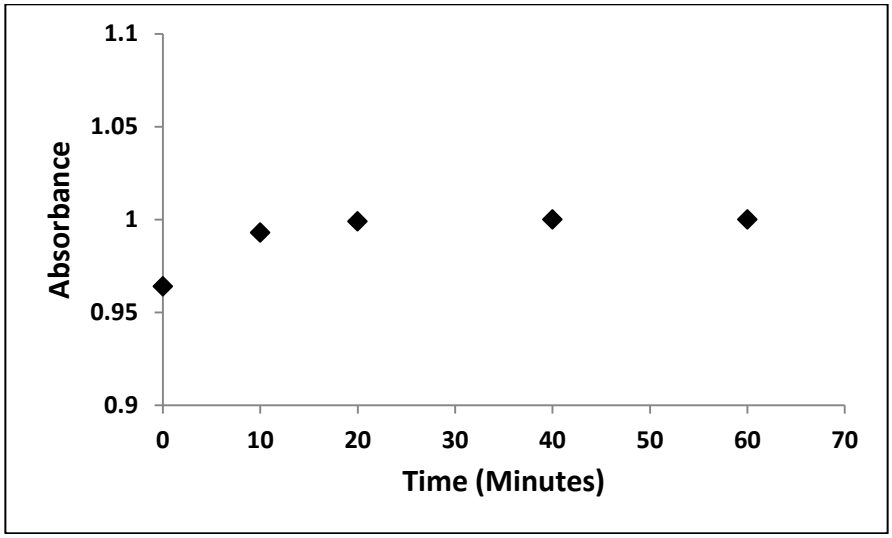

Figure 5: Stability of GEN-Derivative.

Method validation

Linearity

The regression line equation was determined by plotting the concentration versus absorbance. The linearity range was 14-170 µg/mL. The regression line equation is given in Figure 6, which showed a regression coefficient $R^2 = 0.994$.

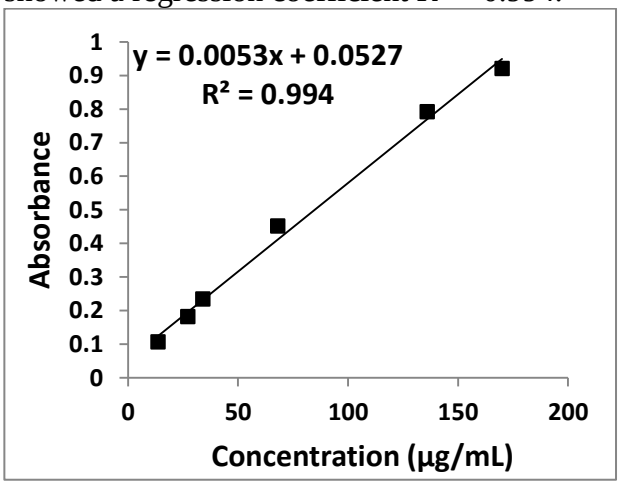

Figure 6: Calibration curve of GEN analysis (n = 5).

The developed method has enhanced sensitivity. LOD of developed method was 2.17 µg/mL, which indicated a better sensitivity than assay based on oxidation of GEN using permanganate (Fraihat, 2015). Analysis of GEN by developed method has expanded range in comparison to that recorded in the literature, 4 - 9 µg/mL (El-Didamony *et al.*, 2006), 10 - 100 µg/mL (Omar *et al.*, 2013b) and 20-125 µg/mL (Fraihat, 2015). A colorimetric assay using ninhydrin for GEN analysis showed a range of 30-120 µg/mL (Frutos *et al.*, 2000).

Specificity

The interference liabilities of excipients was evaluated by measuring the percentage of recovery at concentration levels 60 and 120 (µg/mL) GEN. No interference was found in the analysis of GEN by the developed method as shown in Table 2.

Table 2: Results of specificity study for GEN analysis.

Concentration (µg/mL)	**% Recovery [a] (Mean ± SD)**
60	101.67 ± 0.82
120	100.96 ± 0.22

[a]: Values are mean of three determinations.

Accuracy

Accuracy of the method was ascertained by performing recovery studies using standard addition method. The obtained results are given in Table 3. The percent recovery values were 101.78 - 99.77 ± 0.15-1.55 with SD ≤ 1.55, which indicate high accuracy of the developed method.

Table 3: Results of recovery study for GEN analysis.

Base level (µg/mL)	Spiked amount (µg/mL)	% Recovery [a] (Mean ± SD)
60	30	101.78 ± 1.15
60	60	101.10 ± 1.55
60	90	99.77 ± 0.15

[a]: Values are mean of three determinations.

Precision

Intra- and inter-day precision were assessed and the results are summarized in Table 4. The percent of relative standard deviation RSD values were less than 2%, indicating high repeatability of the developed method.

Table 4: Intra-and inter- day precision data for GEN analysis.

GEN conc. (µg/mL)	Intra-day (n = 5)		Inter-day (n = 6)	
	Mean	% RSD	Mean	% RSD
30	30.68	1.05	30.71	1.22
60	60.61	0.57	60.72	0.79
120	120.42	0.66	120.99	1.02

RSD: Relative standard deviation.

Analysis of commercial gentamicin pharmaceutical products

The developed spectrophotometric method has been successfully applied to the determination of GEN in ampoules and eye drops. The results are given in Table 5. The percentage of recovery were 99.24 ± 1.48 for ampoules and 100.53 ± 1.64 for eye drops, which are very

close to the label claim, and lie within the acceptable pharmacopoeial requirement (97%-110% for ampoules and 90%-120% for eye drops (BP, 2015). The estimated results were compared with validated published method (El-Didamony *et al.*, 2006) using t-test. No significant difference was found at 95% confidence level (p value < 0.05) as shown in table 5.

Table 5: Determination of GEN in dosage forms by the developed method and comparison with validated published method.

Dosage form	% Recovery[a] (Mean ± SD)	
	Developed method	**Published method**
GEN ampoules (40 mg/mL)	99.24 ± 1.48 t = 1.24[d]	100.04 ± 0.57[b]
GEN eye drops (3 mg/mL)	100.53 ± 1.64 t = 0.19[d]	100.14 ± 0.41[c]

[a]: Values are mean of three determinations, [b]: Results for GEN determination in ampoule by published method (El-Didamony*et al.*, 2006), [c]: Results for GEN determination in eye drops by published method (El-Didamony*et al.*, 2006), [d]: Tabulated t-value = 2.23 at 95% confidence level and 10 degrees of freedom.

Conclusion

The present work described the successful derivatization of gentamicin using a cheap and available derivatizing agent salicylaldehyde. The procedure was simple and the derivatization product showed high stability and increased the sensitivity and selectivity of GEN spectroscopic analysis. The developed method did not require prior extraction of drug from dosage forms. Validation

showed precise and accurate method that was applied successfully for GEN analysis in dosage forms using a cost effective procedure and UV-Vis spectrophotometer.

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