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MICROBIOLOGICAL ASSAY OF COLISTIN SULFATE ANTIBIOTIC IN PHARMACEUTICAL FORMULATIONS

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A simple, sensitive and specific agar cup diffusion bioassay for the antibacterial Colistin sulfate was developed. Using a strain of Escherichia coli ATCC 8739 as the test organism, Colistin sulfate at concentrations ranging from 100 to 1600 µg/ml could be measured in pharmaceuticals. A prospective validation of the method showed that the method was linear (r^2= 0.999), precise (RSD< 2.8%) and accurate (percent recovery ranges between 98-102%). The method shows that results confirm its precision, not differing significantly from the other method described in the literature. We conclude that microbiological assay is satisfactory using Escherichia coli ATCC 8739 for quantitation of in-vitro antibacterial activity of Colistin sulfate.

INTRODUCTION

Colistin sulfate is the sulfate salt of an antibacterial substance produced by the growth of *Bacillus polymyxa* var. *colistinus*[1]. Colistin is an antibiotic of the polymyxin group and is identical to polymyxin E[2]. It consists of acyclic heptapeptide and a side-chain of three amino acids acylated at the N-terminus by a fatty acid (Fig. 1). It is a complex mixture of at least 30 different components. The two main components are Colistin A (polymyxin E1) and Colistin B (polymyxin E2), which differ only in the fatty acid side chain[3].

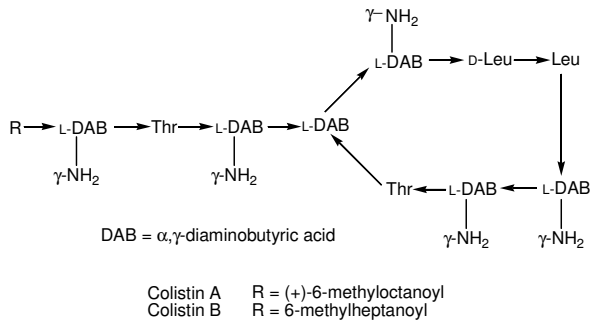

Fig. 1: Structural formula of Colistin A-B *(quoted after[4]).*

Colistin, with its similar structure to polymyxin B, is believed to have an identical mechanism of action[5]. Polymyxin B interacts electrostatically with the outer membrane of Gram negative bacteria and competitively displaces divalent cations (calcium and magnesium) from the negatively charged phosphate groups of membrane lipids[6]. Insertion of polymyxins disrupts the outer membrane and lipopolysaccharide is released[7].

Colistin exhibits a narrow antibacterial spectrum, mostly against common Gram-negative clinical isolates. Colistin is active against the common species of the *Enterobacteriaceae* and *Aeromonas*, but not Vibrio species[8]. Of the common or important non-fermentative Gram-negative bacteria, *P.aeruginosa* and *Acinetobacter* species are naturally susceptible[9-11]. Of particular importance is its activity towards multi-resistant *P. aeruginosa*[12]. *E. coli, Enterobacter, Salmonella, Shigella* and *Klebsiella* are also

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susceptible. Colistin is also active against *Haemophilus influenza*[9&13] *Bordetella pertussis* and *Legionella pneumophila*[14].

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms. A reduction in antimicrobial activity also will reveal slight changes not demonstrable by chemical methods. Accordingly, microbial or biological assays remain generally the standard for resolving doubt with respect to possible loss of activity[1].

Microbiological assay (MBA) can be defined as the estimation of potency of a growth-promoting substance (GPS) or growth-inhibiting substance (GIS) by comparing its quantitative effect on the growth of a specific microorganism with that of a reference standard of defined potency[15].

Biological methods are advantageous because the parameters that are measured with these techniques and the properties for the drug used are the same. Thus, impurities and the related substances do not interfere, maintaining the precision of the analytical method[16].

To assess the potency of Colistin sulfate antibiotic in pharmaceutical formulations, valid microbiological assay methods should be developed using sensitive microorganisms.

MATERIAL AND METHODS

Preparation of Colistin sulfate reference standard solutions

The assay design used is a 1-level assay with standard curve[1] in which dilutions representing 5 test levels of the standard and a single test level of the unknown corresponding to the median test dilution of the standard were prepared.

The five test levels of Colistin sulfate reference substance (RS) (Sigma-Aldrich, U.K.) were prepared in phosphate buffer pH 6(10%) with the following concentrations: L_1 (100 µg/ml), L_2 (200 µg/ml), M (400 µg/ml),H_1 (800 µg/ml) and H_2 (1600 µg/ml).

Another two different concentrations representing 90% (360 µg/ml) and 110% (440 µg/ml) of M standard dose were prepared using Colistin sulfate (RS) material and used for studying the validation parameters like the accuracy and the intermediate precision.

Plate assay

- *Escherichia coli* ATCC 8739 (Microbiologics, Inc., U.S.A) was suspended in sterile purified water and the inoculum suspension was standardized to the density of McFarland 0.5 standard.
- Two hundred fifty milliliters of molten nutrient agar (Himedia, India) was cooled to about 45°C and seeded with two milliliters of organism suspension and mixed well to obtain homogenous dispersion.
- Twenty milliliters of the seeded agar was then added to a 90 mm Petri dish to obtain a thickness of agar layer of approximately 4mm &spreaded evenly by rotating the plates gently.
- The plates were placed on flat surface until the agar had solidified. Then 6 holes in each plate were punched out with cork borer with suction device to obtain holes of 9mm diameter.
- Two plates for each dose level (L_1, L_2, H_1 and H_2) of the standard were prepared.
- Each plate accommodated only two test solutions that were applied in triplicate. Position R was for the reference solution, which is the mid dose (M) of the five standard doses. The same test solution was applied in position R for every plate in the assay. Position S was for a single dose of the unknown or for any one of dose levels L_1, L_2, H_1, or H_2 of the reference standard[15].
- The pattern for the distribution of test solutions in small (90-mm) plate is shown in figure 2.

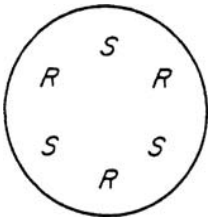

Fig. 2: Pattern for the distribution of test solutions in small plate *(quoted after*[15]*)*.

- Each hole in the plate was loaded with 0.1 ml of solution. Then the plates were incubated at 32-35ºC for 18-24 hrs.
- After incubation, the diameters of zones of organism growth inhibition in the different concentrations were measured and the mean zone diameter (mZD) for each concentration was calculated.

Validation of method

The linearity was evaluated by plotting the (mZD) as a function of the corresponding log concentration of different dilutions and estimating the co-efficient of determination and slope of the regression line[17].

The accuracy was tested by preparing two different concentrations representing 90% and 110% of (M) standard dose using Colistin sulfate (RS) and applying the procedures mentioned in plate assay using two plates for each concentration (L_1, L_2, H_1, H_2, 90% and 110%) then calculating the recovered potency of both concentrations from which the percent recovery of both is calculated which should be in the range of 98-102%[18].

The precision may be considered at two levels, repeatability and intermediate precision. Repeatability was evaluated by calculating the relative standard deviation (RSD) of the zone diameter readings for each concentration within the same assay and the intermediate precision was evaluated by calculating the RSD for the assay results for the prepared 90% and 110% concentration performed on three consecutive days. The value of RSD in these tests should be not more than 2.8%[18]. Relative standard deviation is the standard deviation as a fraction of the mean, i.e. S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It is more reliable expression of precision. % Relative Standard Deviation (RSD)= S*100/x[19].

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity[17&19]. For assay tests, the ICH (2005) requires the minimum specified range to be 80 to 120 percent of the test concentration. The studied range using *E. coli* ATCC 8739 was from 25 (L_1) to 400 (H_2) percent of the test concentration.

Colistin sulfate sample analysis

Five syrup samples were collected from the Egyptian market and analyzed with the previously validated methods for their potency which should be not less than 90.0 percent and not more than 120.0 percent of the labeled amount of Colistin[1].

The potency of Colistin sulfate in pharmaceutical products is expressed as the number of international units (IU). So, sample solution of unknown presumed to be of equal activity with median dose of standard (M) was prepared taken into consideration that one unit of Colistin sulfate is equivalent to 0.04 µg of Colistin A as a conversion factor according to[20].

In the assay procedures, two plates for each dose level (L_1, L_2, H_1 and H_2) of the standard and two plates for each unknown sample were prepared as was mentioned in the plate assay.

Calculation of potency of unknown

In the assay of a sample of unknown potency using a 1-level assay with standard curveas assay design, calibration curve is constructed in which the (mZD) is plotted as a function of log corresponding concentration. The relationship is linear (y= mx+b) with a high coefficient of determination approximately equals ($r^2 \approx 0.999$). The quantities (m) and (b) are called the least-squares coefficients[21]. The coefficient (m) is the slope of the least-squares line, and the coefficient (b) is the y-intercept.

As the sample solution of unknown was prepared presuming to be of equal activity with that of median dose of standard (M), the (mZD) of unknown should not differ significantly from that of (M) dose of standard and the potency can be calculated as followed:

Log potency ratio (R)=
(mZD of unknown - mZD of M standard dose)/ slope **(1)**

Where slope is the coefficient (m) in the regression line equation (y= mx+b) or may be obtained graphically by the following equation

$$Slope\,(m) = \frac{mZD\,of\ H_2 - mZD\,of\ L_1}{Log\ H_2 - Log\ L_1} \quad (2)$$

From eq. (1)

% of unknown= antilog (R) x 100 (3)

RESULTS AND DISCUSSION

Results

- The proposed method of assay showed good repeatability as the RSD for the zone diameter readings of each concentration is less than 2.8% as shown in tables 1-3.

Table 1: First day assay data using *E. coli* ATCC 8739 as test organism.

Conc.[a]	H_2 (1600 µg/ml)	M (400 µg/ml)	H_1 (800 µg/ml)	M (400 µg/ml)	L_2 (200 µg/ml)	M (400 µg/ml)	L_1 (100 µg/ml)	M (400 µg/ml)	90% (360 µg/ml)	M (400 µg/ml)	110% (440 µg/ml)	M (400 µg/ml)
1st zone [b]	26.1	23.1	24.5	23.3	21.9	22.85	19.95	22.75	22.25	22.55	22.35	22.45
2nd zone [b]	24.85	22.7	24.4	22.75	21.55	23.2	20.65	23.25	22.7	22.15	22.75	23.2
3rd zone [b]	25.6	23.25	23.7	22.85	20.8	22.7	20.65	22.8	21.55	22.6	23.5	22.55
4th zone [b]	25.55	23.1	24.15	23	21.45	23	20.95	23	22.25	22.55	22.5	22.75
5th zone [b]	26	23.45	24.4	22.7	21.5	22.65	19.7	23.15	22	22.9	23.5	22.9
6th zone [b]	25.45	22.55	23.65	22.5	21.3	22.65	19.85	22.5	22.35	21.6	22.3	21.7
mZD	25.591667	23.025	24.13333333	22.85	21.41667	22.84166667	20.29167	22.90833	22.1833333	22.39166667	22.8166667	22.59167
SD	0.4465609	0.33874769	0.373720038	0.275680975	0.361478	0.222298598	0.520016	0.278239	0.38427421	0.4554302	0.55196618	0.511289
RSD	1.7449467	1.47121691	1.548563693	1.206481291	1.687837	0.973215314	2.562707	1.214573	1.7322654	2.033927203	2.41913594	2.263176

(H_2) high dose 2, (H_1) high dose 1, (M) median dose, (L_2) low dose 2, (L_1) low dose 1, (mZD) mean zone diameter, (SD) standard deviation, (RSD) relative standard deviation, [a] concentration, [b] zone diameter in millimeter.

Table 2: Second day assay data using *E. coli* ATCC 8739 as test organism.

Conc.[a]	H_2 (1600 µg/ml)	M (400 µg/ml)	H_1 (800 µg/ml)	M (400 µg/ml)	L_2 (200 µg/ml)	M (400 µg/ml)	L_1 (100 µg/ml)	M (400 µg/ml)	90% (360 µg/ml)	M (400 µg/ml)	110% (440 µg/ml)	M (400 µg/ml)
1st zone[b]	25.55	23.15	24.7	23	21.75	23.4	21	23.45	23.9	23.75	23.45	23.5
2nd zone[b]	26.65	24	24.65	24.1	22.8	23.4	20.9	24.1	24.3	23.8	24	24.1
3rd zone[b]	26.1	22.7	25.85	24	22.4	23.15	22.1	23.9	23.35	23.95	24.8	23.4
4th zone[b]	26.9	24.3	25.05	24.35	21.75	22.45	21	23.25	23	23.7	23.75	23.2
5th zone[b]	26.4	23.3	25.85	24.1	22.5	23.25	21.2	23.95	23.4	23.6	23.5	23.9
6th zone[b]	26.65	23.65	24.9	23.5	22	23.8	20.9	23.95	22.65	22.8	23.45	23.75
mZD	26.375	23.5166667	25.16666667	23.84166667	22.2	23.24166667	21.18333	23.76667	23.4333333	23.6	23.825	23.64167
SD	0.4865696	0.58537737	0.5483308	0.498414152	0.432435	0.446560933	0.462241	0.335659	0.59637796	0.408656335	0.524166	0.335286
RSD	1.8448137	2.48920215	2.178797882	2.090517239	1.947905	1.921380851	2.182097	1.412308	2.54499839	1.731594639	2.20006717	1.418199

(H_2) high dose 2, (H_1) high dose 1, (M) median dose, (L_2) low dose 2, (L_1) low dose 1, (mZD) mean zone diameter, (SD) standard deviation, (RSD) relative standard deviation, [a]concentration, [b]zone diameter in millimeter.

Table 3: Third day assay data using *E. coli* ATCC 8739 as test organism.

Conc.[a]	H_2 (1600 µg/ml)	M (400 µg/ml)	H_1 (800 µg/ml)	M (400 µg/ml)	L_2 (200 µg/ml)	M (400 µg/ml)	L_1 (100 µg/ml)	M (400 µg/ml)	90% (360 µg/ml)	M (400 µg/ml)	110% (440 µg/ml)	M (400 µg/ml)
1st zone [b]	27.85	25.55	26.45	24.75	23.1	24.35	22	24.5	24.85	24.85	24.6	24.6
2nd zone [b]	28.1	24.9	26.1	25.2	23.65	25.7	22.2	25.25	24.5	25.35	24.7	24.4
3rd zone [b]	27.25	24.45	26.8	25.85	24.5	25	22.65	24.1	25.9	26.15	24.55	24.65
4th zone [b]	26.9	24.2	26	24.6	23.3	25.75	22.5	25.4	25.5	25.4	25.2	25.75
5th zone [b]	26.5	24.35	26.7	25.7	24.1	25.1	22.1	25.9	24.45	24.2	25.2	24.1
6th zone [b]	27.85	25.25	26.75	25.6	23	24.6	22.5	24.9	24.35	24.75	25.6	25.1
mZD	27.4083333	24.783333	26.46666667	25.28333333	23.60833	25.08333333	22.325	25.00833	24.925	25.116667	24.975	24.76667
SD	0.62882165	0.5400617	0.345928702	0.520256347	0.593647	0.566274374	0.260288	0.648395	0.63619965	0.6705719	0.42160408	0.582809
RSD	2.29427175	2.1791327	1.307035397	2.057704735	2.514565	2.257572255	1.165905	2.592718	2.55245598	2.6698284	1.688104423	2.3532

(H_2) high dose 2, (H_1) high dose 1, (M) median dose, (L_2) low dose 2, (L_1) low dose 1, (mZD) mean zone diameter, (SD) standard deviation, (RSD) relative standard deviation, [a] concentration, [b] zone diameter in millimeter.

- The method showed good accuracy as the mean percent recovery for the prepared two prepared 90% and 110% concentration lies within the range 98-102% as shown in table 4
- The method also showed good intermediate precision as the RSD for the assay results obtained on three successive days for the prepared 90% and 110% concentrations is less than 2.8% as shown in table 5.
- The proposed method of assay showed good linearity within the selected range as the r^2approximately equals 0.999 as shown in figures 3-5 and table 6.
- The assay plates representing the development and validation of the assay method are shown in figure 6.
- The assay results of syrup samples are shown in table 7. All samples comply the specification limit (90–120% of the labeled amount) mentioned in US Pharmacopoeia[1].

Table 4: Experimental values obtained in the recovery test using *E. coli* ATTCC 8739.

Concentration	1st day (percent recovery)	2nd day (percent recovery)	3rd day (percent recovery)	Mean (percent recovery)	SD[a]	RSD[b]
Sample 1 (90%)	99.692543	101.90041	100.150246	100.581066	1.16527842	1.158546
Sample 2 (110%)	102.20443	99.987802	101.773567	101.321933	1.175304163	1.1599701

[a]standard deviation, [b] relative standard deviation.

Table 5: Assay results for the prepared 90% and 110% in three successive days showing good precision.

Concentration	1st day (recovered potency)	2nd day (recovered potency)	3rd day (recovered potency)	Mean (recovered potency)	SD[a]	RSD[b]
Sample 1 (90%)	89.72328895	91.71037052	90.13522178	90.52296041	1.048751271	1.158547253
Sample 2 (110%)	112.4248716	109.9865823	111.9509241	111.454126	1.292834072	1.159969683

[a]standard deviation, [b] relative standard deviation.

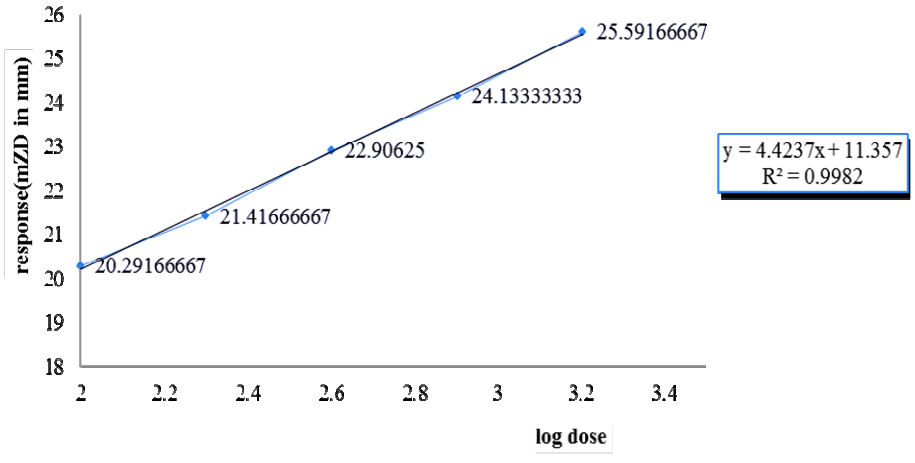

Fig. 3: Calibration curve for Colistin sulfate showing linearity within the selected range (L_1 to H_2) using *E. coli* ATCC 8739 (1st day assay study).

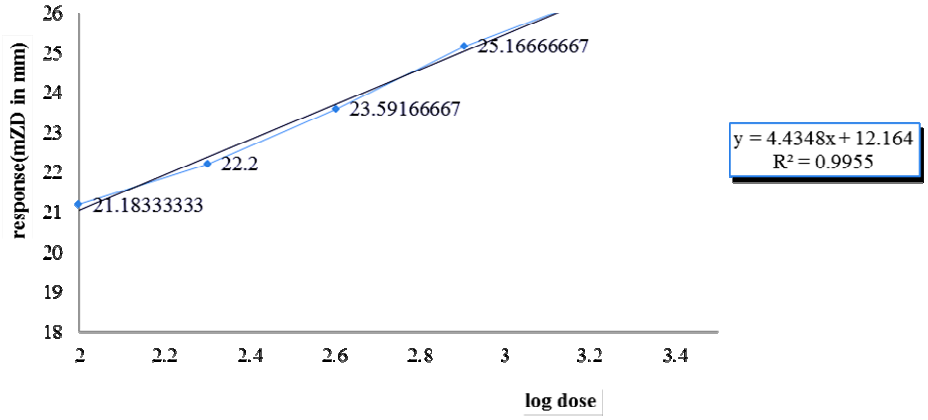

Fig. 4: Calibration curve for Colistin sulfate showing linearity within the selected range (L_1 to H_2) using *E. coli* ATCC 8739 (2nd day assay study).

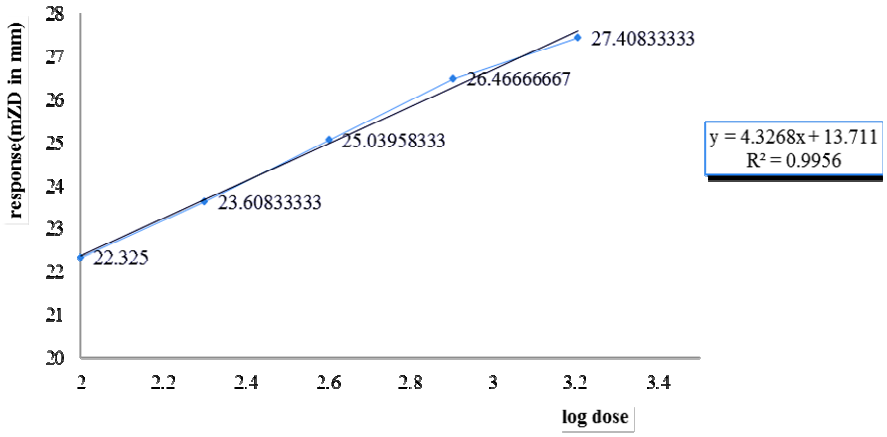

Fig. 5: Calibration curve for Colistin sulfate showing linearity within the selected range (L_1 to H_2) using *E. coli* ATCC 8739 (3rd day assay study).

Table 6: Evaluation of the linearity of assay method using *E. coli* ATTCC 8739**.**

Test	R^2 value		Hypothesis[a]	Experimental t	Theoretical t	decision
correlation	1st day	0.9982	H_0: $R^2 = 0.999$	2.9414	4.303	Accept H_0
	2nd day	0.9955	H_1: $R^2 \neq 0.999$			
	3rd day	0.9956				

[a]at 0.05 level of significance.

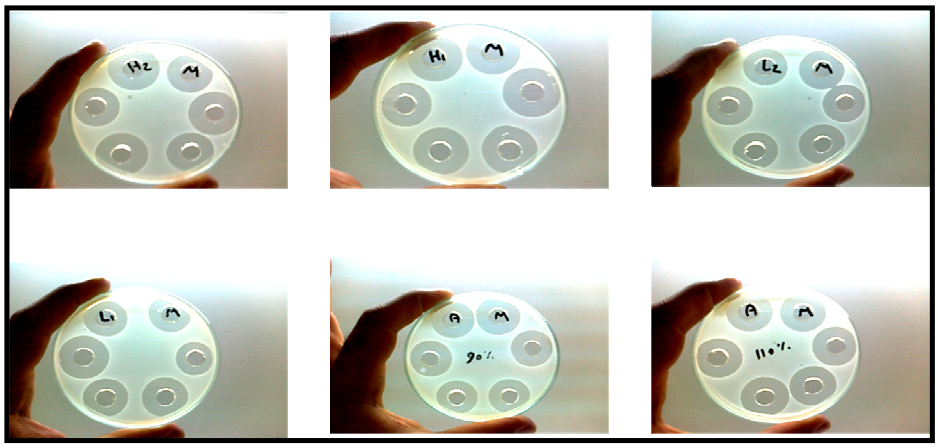

Fig. 6: Validation of microbiological assay method of Colistin sulfate using *E. coli* ATTCC 8739.

Table 7: Calculation of potency of five syrup samples.

	Sample A1	Sample A2	Sample A3	Sample A4	Sample 5
log potency ratio[a]	-0.009698	0.0193961	-0.0035004	-0.0070007	-0.0019396
recovered potency[b]	97.791692%	104.56735%	99.197249%	98.400942%	99.554384%

[a]calculated using equation (1).
[b]calculated using equation (3).

Discussion

Considering that the potency of an antibiotic may be demonstrated under suitable conditions by comparing the inhibition of growth of susceptible microorganisms induced by known concentrations of the antibiotic to be tested and the reference standard[1&22], a microbiological assay was proposed as a suitable method for determination of Colistin sulfate in pharmaceutical dosage forms.

Biological methods are advantageous because the parameters that are measured with these techniques and the properties for the drug used are the same. Thus, impurities and the related substances do not interfere, maintaining the precision of the analytical method[16]. Therefore, microbial or biological assays remain, in general as the standard for resolving doubt with respect to possible loss of activity[1].

The proposed method of assay showed good linearity within the range of 100 (L_1)-1600µg/ml (H_2) with a coefficient of determination (R^2) reached 0.999 as evaluated by testing the hypothesis of R^2 values obtained from the assay procedures carried out three times in three successive days. This correlation is better than that obtained by Diaz *et al.*[23] (R^2= 0.9907) in the assay of vancomycin using *Bacillus Subtilis* ATCC 6633. Again this correlation is more or less matched with that obtained by M. J. Souza *et. al.*[24] in the assay of enrofloxacin injection showing a good correlation (R^2= 0.99996) but using only three standard doses within a narrower range (3.2-12.8 µg/ml).

The proposed method also showed good accuracy as the percent recovery for the prepared authentic concentrations (90% and 110% of M dose) was ranging between 98-102%). It also showed good repeatability as the R.S.D of the zone diameter readings for different concentrations L_1, L_2, M, H_1 and $H_2$was ranging from 0.97 to 2.6%. These values are better than that obtained by Staub *et al.*[25] in the assay of ketoconazole in shampoo showing RSD value reached 4.04% for the low dose, 2.16% for the medium dose and 2.34% for the higher dose.

The method also showed good intermediate precision as the R.S.D for the

assay results obtained on three successive days for the prepared authentic 90% and 110% concentrations was 1.158 and 1.159 respectively (less than 2.8%). The sensitivity of method as expressed by the slope of the regression line (m≈ 4.4) was higher than that obtained by Diaz *et al.*[23] (m≈ 2.6) in the assay of vancomycin using *Bacillus Subtilis* ATCC 6633.

The proposed method of assay in this study using *E. coli* ATCC 8739 was linear, accurate and precise and can thus be used for the measurements of Colistin sulfate in pharmaceutical formulations.

The potency of the five syrup samples lies within the specified range (90-120% of labeled amount) stated in[1]. This good results may be attributed to true definition of the international unit (IU) of Colistin sulfate mentioned by the manufacturer in preparing these samples (3000,000 IU= 100 mg Colistin base or 120 mg Colistin sulfate) which complies that defined in The Japanese Pharmacopoeia[20].

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نشرة العلوم الصيدلية
جامعة أسيوط

القياسات الميكروبيولوجية للمضاد الحيوي سلفات الكوليستين في المستحضرات الصيدلية

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تهدف هذه الدراسة إلى تطوير طريقة حيوية بسيطة وحساسة ودقيقة وذلك لقياس عقار كوليستين سلفات في المستحضرات الصيدلانية.بإستخدام إيكولاي آي تي سي ٨٧٣٩ ككائن إختبار، فقد تبين أنه من الممكن قياس هذا العقار في نطاق تركيزات تتراوح من ١٠٠ إلى ١٦٠٠ ميكروغرام/مل. وبالتحقق من صحة الطريقة المقترحة فقد أظهر إختبار فحص الخطي علاقة جيدة ضمن هذا النطاق (١٠٠-١٦٠٠ ميكروغرام/مل) مع معامل تحديد بلغ ٠,٩٩٩. كما أظهرت الطريقة المقترحة دقة جيدة حيث كانت نتيجة الإسترداد بالمائة في نطاق يتراوح من ٩٨٪ و ١٠٢٪. كما أظهرت أيضا تكرارية ودقة وسيطة جيدة حيث لم تتجاوز قيمة معامل الإنحراف المعياري النسبي ٢,٨٪.

ومن ذلك نخلص أن الطريقة الحيوية المقترحة بإستخدام إيكولاي آي تي سي ٨٧٣٩ هي طريقة مقبولة وذلك لقياس قوة النشاط المضاد للبكتريا لهذا العقار.