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RESEARCH ARTICLE

Consistency evaluation between matrix components ratio and microbiological potency of tylosin major components

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Abstract

Background The aim of our research work was to investigate the relative potencies of matrix components of tylosin, a multi-component antibiotic, and establishing a quantitative relationship between content and potency of each component.

Methods The potencies of tylosin matrix components were determined by using three bioassay methods. The content of tylosin components (tylosin A, B, C, and D) in different tylosin samples were determined by using high pressure liquid chromatography (HPLC) technique and their theoretical potencies were calculated. Equivalency of theoretical and microbiological potencies for each sample was evaluated using statistical analysis.

Results The highest amount of tylosin B content was found in tylosin phosphate and tartrate (up to 19%). Tylosin D content in all tylosin samples varied in the range of 0.03 to 18.73%. Tylosin A, B, and C showed similar sensitivity to the *Kocuria rhizophila*, the test organism in agar-diffusion method, while the potency of tylosin D was 39% of A. In the turbidimetric methods by *Staphylococcus aureus*, tylosin D and B responses to A component were ranged from 22.5 to 22.8% and 77.3 to 79.3%, respectively, while potencies of tylosin C and A were almost equal. The biopotency conversion factors were not resulted to a single factor, due to the different antibacterial activity of tylosin components.

Conclusion Our findings indicated that defining individual limit for the low active matrix components and for the total of other components with similar high activity could improve the accuracy of potency results.

Keywords Tylosin · Multi-components antibiotic · HPLC · Microbiological potency · Component ratio

Background

Tylosin, as a multi-component antibiotic, is a complex mixture of closely related components produced by a strain of *Streptomyces fradiae*. Through binding to 50S ribosome and inhibiting bacterial protein synthesis, tylosin exerts antibacterial activity on most Gram-positive bacteria and *mycoplasma* species. It is used in veterinary medicine for disease treatment and the promotion of growth in swine, cattle, and poultry [1, 2]. Tylosin A, B (desmycosin), C (macrocin), and D (relomycin) were found to be common components of tylosin. It is claimed that the antimicrobial activity of these components against microbial species is different [1]. Small amounts of other components, such as lactenocin, demecinosyl-tylosin (DMT), and O-mycaminosyl-tylonolide (OMT), may also be present in commercial samples [3]. The British Pharmacopoeia (BP) and the United States Pharmacopeia (USP) have provided the specifications of tylosin base, tartrate and phosphate, listing a minimum tylosin A content of 80% and a minimum of 95% for the combined sum of tylosins A, B, C, and D. For tylosin injection, minimum of 80 and 75% of tylosin A and 90 and 85% for the combination of all four tylosin components are considered according to USP and BP, respectively. None of the monographs considered individual limits for tylosin B, C, and D [4, 5]. The proportion of tylosin components may vary from lot-to-lot production due to the use of different strains of

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Streptomyces fradiae and the modification made to the fermentation condition and downstream process. This may affect antibiotic clinical efficacy. A recent review of the literature on multi-components antibiotics indicated that the subcomponents of colistin and teicoplanin antibiotics differ in their fatty acid, amino acid composition, the lipophilic and protein binding characteristics. These differences may affect the pharmacokinetics and their final clinical efficacy [6].

It was found that the potency value obtained by the bioassay methods depends on the matrix components ratio of the reference standard and sample as well as on the condition of bioassay (e.g., the test organism, media, and buffers). These factors may cause assay problems of day-to-day, lab-to-lab, and method-to-method variations and affect the accuracy of microbiological assays [7, 8]. The antimicrobial activity of the various components in the multi-components antibiotics may differ due to their structural differences. One of the main accuracy requirements of microbiological assay on antibiotics is consistency of reference standard and test sample. On this basis when measuring the potencies of multi-components antibiotics, it is necessary to use standard and test samples with similar compositions to guarantee the accuracy of results. If significant differences between reference standard and test sample composition are observed, then the matrix components of multi-components antibiotic should have similar sensitivities against test organism in the bioassay methods [8, 9]. Antibiotic potency measurement by using microbiological assay has been adopted by official methods to express antibiotic content for several antibiotics. However, to simplify quality-control procedures, most of the pharmacopoeias have replaced microbiological assays with physicochemical methods to determine the antibiotic content for single-component antibiotics [10]. Due to the unknown relationship between the potency and purity of the multi-components antibiotics, microbiological assays are still used for the potency determination and their quality control protocols include high pressure liquid chromatography (HPLC) purity and microbiological potency determination [11]. Chang et al. established a quantitative relationship between the antibiotic purity (by HPLC) and potency (using microbiological assay) to determine the potency of the main active components of vancomycin, norvancomycin, amphotericin B, and gentamycin by HPLC method [10–12]. Few studies have been published on the multi-components antibiotics bioassays, but due to their quality control problems, it remains an important and serious issue in this area which needs special attention.

Within the framework of these criteria, the aim of present study was to compare the responses of four tylosin components by three microbiological assay methods. In addition, the chemical composition of different tylosin samples (tylosin base, tartrate, phosphate, and injection) from different manufacturers were analyzed by HPLC method. Then, the potency and purity of total tylosin was investigated by establishing a quantitative relationship between the contents obtained by HPLC method and the responses of tylosin A, B, C, and D in each microbiological method.

Methods

Materials

Tylosin components (tylosin A, B, C) were obtained from Toku-E Company (Bellingham, WA, USA). Purity was reported by the manufacturer as >99% for tylosin A, ≥95% for B, >85% for C. The European Pharmacopoeia Chemical Reference Substance of tylosin (EP-CRS, Cat. No. T2880000, batch 2, 1025 IU/mg) and tylosin D (Cat. No. T2880100, batch 2) provided from European Directorate for Quality Medicines & HealthCare (EDQM) were purchased from Sigma-Aldrich (USA). Different samples of tylosin base, tylosin tartrate, tylosin phosphate, and tylosin injection were provided by different manufacturers.

Acetonitrile, methanol, and phosphoric acid were obtained from Sigma-Aldrich (USA). All chemicals were of HPLC or analytical grade. Potassium phosphate dibasic and potassium phosphate monobasic were supplied from Merck (Darmstadt, Germany). De-ionised water was used throughout the study. Antibiotic assay media A (pH 7.9–8.1), C (pH 6.9–7.1), No. 3 (pH 6.95–7.05), and No. 39 (pH 7.8–8.0) were purchased from Hi-Media Laboratories Pvt. Ltd. Tryptic Soy Agar (TSA) was purchased from Merck (Darmstadt, Germany).

Micro-organisms and cultivation conditions

The test strains were obtained from the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology, Tehran, Iran. The *Kocuria rhizophila* ATCC 9341, and *Staphylococcus aureus* ATCC 9144 were used for agar diffusion and turbidimetric assays, respectively. The *K. rhizophila* was grown in TSA at 32–35 °C. The *S. aureus* was cultured in antibiotic medium No. 3 at 37 °C.

HPLC analysis of tylosin samples and reference substance

The purity of tylosin samples and reference substance was determined by using the described method in the 2014 edition of USP [4]. Briefly, the Knauer HPLC system (Berlin, Germany) equipped with a model k-1001 LC pump, model k-2600 UV detector set at 280 nm and a model 2003 degasser was used. The analyses were performed on Nucleosil ODS (4.6 mm × 250 mm, 5 μm particle size) in the analytical column. Column temperature was maintained at 25 °C. Injection

volume was 20 μl. The mobile phase was acetonitrile-sodium perchlorate (pH 2.5; 2 M for tylosin base and tylosin phosphate, 200 g/l for tylosin tartrate, and 184 g/l for tylosin injection) (40:60, *v*/v). The flow rate was 0.7 ml/min except for tylosin tartrate which was 1 ml/min with isocratic elution. To verify the used analytical method, system suitability parameters were determined.

Determination of minimum inhibitory concentrations (MICs)

The MICs of four tylosin components against the *K. rhizophila*, and *S. aureus* and several other Gram-positive strains were determined by the microdilution method using 96 U-shaped wells plates [13]. Briefly, stock solution of tylosin components (50 μg/ml in 2.5% *v*/v methanol in 0.1 M phosphate buffer, pH 7) were serially two-fold diluted ranging from 50 to 0.098 μg/ml in Mueller–Hinton broth (MHB). Bacterial suspensions with the optical density (OD) of about 0.25–0.3 at 600 nm were prepared in sterile saline from over-night cultures of *K. rhizophila*, and *S. aureus* on MHB. Their cell concentrations, that were determined previously by serial dilution and pour plate method, were about 1×10^8 CFU/ml. Then, 1 ml of each bacterial suspension was added to 9 ml of MHB to reach the final concentration of about 1×10^7 CFU/ml. In each well of microtiter plate, 100 μl of the tylosin solution was co-incubated with 100 μl of the bacterial suspension (1×10^7 CFU/ml in MHB) at 37 °C for 24 h. Control wells containing 100 μl of 2.5% *v*/v methanol in 0.1 M phosphate buffer instead of antibiotic solution were also mixed with bacterial suspension and incubated. The MIC was defined as the lowest concentration of each component that the test strain does not showed visible growth in comparison with the control well containing only the test strain suspension.

The MIC of tylosin A and D against a series of Gram-positive bacteria including *Staphylococcus aureus* ATCC 9144, *S. aureus* ATCC 29737, *S. haemolyticus* ATCC29970, *Kocuria rhizophila* ATCC 9341, *S. aureus* ATCC 1112, *S. warneri* ATCC 27836, *S. xylosus* ATCC 29971, *S. epidermidis* ATCC 12228, *S. saprophyticus* ATCC 15305, *Bacillus subtilis* ATCC 6633 were also determined by microdilution method as described above.

Potency determination by microbiological assay methods

Three commonly microbiological assay methods (the BP agar diffusion method, the BP turbidimetric method, and the USP turbidimetric method) were used in this study as described in the general chapter of "antibiotics-microbial assays" in USP 37 and according to the general guidance in the appendix XIV of Volume V, the 2015 edition of the BP [14, 15]. The potencies were calculated according to 3 × 3 and 4 × 4 experimental design with the BP agar-diffusion and the BP turbidimetric methods, respectively, and 5× 1 experimental design with the USP turbidimetric method. For the BP agar-diffusion method, three concentrations ranging from 2 to 8 IU/ml with dose ratio of 2:1 and for the BP turbidimetric method, four concentrations ranging from 1 to 8 IU/ml with dose ratio of 2:1 were prepared. The USP turbidimetric method was performed with five concentrations ranging from 2.56 to 6.25 μg/ml with dose ratio of 5:4.

In the diffusion method, 2.5% *v*/v solution of methanol in 0.1 M phosphate buffer solution pH 7.0 was used as the solvent to prepare stock standard and test solutions of 1 mg/ml. Dilutions were made from the stock solutions and with the mixture of methanol and 0.1 M phosphate buffer solution pH 8.0 (2:3) as final diluent. The plates were prepared by adding 25 ml of antibiotic medium A inoculated with suspension of *K. rhizophila* ATCC 9341 with 25 ± 2% transmittance at 580 nm in 0.9% sodium chloride sterile solution. After solidification, wells were punched on each plate. 100 μl of each standard and test solutions were distributed into each well. The plates were incubated at 33 °C for 24 h and then the inhibition zone diameters were measured.

For the turbidimetric methods, cultures of *S. aureus* ATCC 9144 were cultivated on antibiotic medium No. 3 at 37 ± 2 °C for 18 h. After preparing the microbial suspension with desired optical density of 1 ± 0.1 at 580 nm, portions of 2.5 ml of this suspension were added to 100 ml of antibiotic medium No. 39 and antibiotic assay medium C according to the USP and BP turbidimetric methods, respectively. Dilutions were made from the standard and test stock solutions of 1 mg/ml with the mixture of methanol and 0.1 M phosphate buffer solution pH 8.0 (1:1) and 0.1 M phosphate buffer solution pH 7.0 as final diluents according to the USP and BP turbidimetric methods, respectively. Then 100 μl aliquot of each antibiotic solution along with 9 ml of inoculated medium were mixed into the test tube. After 4 h incubation at 37 ± 0.1 °C, optical density of each test tube was measured at 580 nm.

Quantitative relationship between content by HPLC and potency of Tylosin A, B, C, and D

To determine the theoretical potency, we selected randomly four samples from tylosin base and tylosin phosphate containing different ratios of matrix components (Table 10). Also, five mixed samples from four tylosin components were prepared in the laboratory and their theoretical and microbiological potencies were determined (Table 11). Microbiological potency of each selected sample was also determined by three bioassay methods as described above. The theoretical potency for each tylosin sample was obtained from the sum of

Table 1 Chromatographic characteristics of system suitability tests for tylosin base and phosphate analytical method

Parameter	Value			
	T[a]A	TB	TC	TD
Peak area %RSD[b]	1.11	1.99	2. 0	1.41
Retention time %RSD	0.12	0.21	0.49	0.20
Tailing factor	1.45	1.31	0.87	0.94
Resolution between TA-TD, TC-TB	2.11		2.15	

[a] T, tylosin

[b] RSD, relative standard deviation

Table 3 Chromatographic characteristics of system suitability tests for tylosin tartrate analytical method

Parameter	Value			
	T[a]A	TB	TC	TD
Peak area %RSD[b]	1.7	1.04	1.9	2
Retention time %RSD	0.3	0.9	0.75	0.6
Tailing factor	1.3	0.84	0.67	0. 95
Resolution between TA-TD, TC-TB	2.1		2.7	

[a] T, tylosin

[b] RSD, relative standard deviation

theoretical potency of each component according to the following formula:

$$\text{Theoretical potency (unit or } \mu g/mg) = P_A{}^*(\text{TA purity}) + P_B{}^*(\text{TB purity}) + P_C{}^*(\text{TC purity}) + P_D{}^*(\text{TD purity})$$

P or biopotency conversion factor is the microbiological potency of each component that was obtained by three bioassay methods, individually. Tylosin (T) purity is the content of each component obtained from HPLC analysis. Then, equivalency of theoretical and microbiological potencies for each sample was evaluated using statistical analysis.

Data analysis and potency calculation

The results of agar diffusion and turbidimetric assays according to the BP were statistically analyzed through linear parallel model for potency calculation [16] by the aid of bioassay assist version 3.0.25749 statistical software. The potency of USP turbidimetric assay was calculated by interpolation from a standard curve based on the log concentration-response linear model according to the general chapter of "antibiotics-microbial assays" in USP 37 [14] and by means of Microsoft Excel 2016. Differences were considered statistically significant for $P < 0.05$ by Sigma Plot 12.0 software. The comparison of microbiological and theoretical potencies for equivalency was performed by using the two one-sided test (TOST) (maximum allowed percentage difference, K of 5%) with XLSTAT trial version statistical software.

Table 2 Chromatographic characteristics of system suitability tests for tylosin injection analytical method

Parameter	Value			
	T[a]A	TB	TC	TD
Peak area %RSD[b]	1.28	1.03	1.7	1.8
Retention time %RSD	0.9	0.5	0.8	0.89
Tailing factor	1.3	1	0.7	1.2
Resolution between TA-TD, TC-TB	3.03		2.79	

[a] T, tylosin

[b] RSD, relative standard deviation

Table 4 Content of tylosin components (% *w*/w) in the tylosin base samples determined by HPLC

Sample number	T[a]A	TB	TC	TD
1	98.96	0.34	0.01	0.14
2	96.81	0.08	0.07	2.25
3	96.67	1.27	0.06	1.35
4	97.18	0.48	0.05	1.92
5	80.41	15.02	1.72	1.45
6	97.81	0.89	0.02	0.73
7	94	0.58	0.01	4.65
8	97.2	2	0.01	0.26
9	96.58	0.77	0.03	2.3
10	90.13	0.23	0.06	9.5
11	81.06	0.15	0.03	18.73
12	92	1	0.03	6.5
13	98.41	0.18	0.03	0.91
14	98.21	0.37	0.08	0.7
15	90.97	5.26	1.02	2.27
16	91.42	0.29	5.6	0.84
17	96.81	0.08	0.07	2.25
18	87.5	5.67	0.03	5.35
19	97.18	0.48	0.05	1.92
RS[b]	99.4	0.04	0.04	0.49

[a] T, tylosin

[b] RS, reference standard; Three replicates per sample

Results

Purity analysis of main matrix components of commercial samples and standard tylosin

The results of system suitability tests (SST) of the applied HPLC method were reported in Tables 1, 2, and 3. The results were complying with the mentioned specifications in the monograph of tylosin samples [4].

Composition of different tylosin products and standard was analyzed by HPLC based on the area normalization method. The results are reported in Tables 4, 5 and 6. Tylosin A was the major constituent in all of the samples. Most of the samples contained different amounts of other tylosin factors. The tylosin B content in tylosin phosphate and tartrate samples (Table 6) was higher than that of tylosin base and its preparations (injection). Most of the samples had a low tylosin C content of between 0 and 6.04%. Some of our tested samples (e.g. tylosin base No. 11 and 18) showed significant differences in content of tylosin components relative to those in tylosin reference standard ($P < 0.05$).

Table 5 Content of tylosin components (% w/w) in the tylosin injection samples determined by HPLC

Sample number	T[a]A	TB	TC	TD
1	92.91	1.03	0	5.67
2	97.97	0.96	0.01	0.67
3	83.6	8.56	0.02	7.5
4	90.44	7	1.01	1.1
5	88.81	0.53	0	8.99
6	95.65	2.35	1.24	0.5
7	97.88	1.07	0.02	0.65
8	98.05	0.85	0	0.65
9	98.27	0.19	0.03	0.7
10	96.75	0.69	0.06	2.49
11	84.78	4.86	0.7	9.55
12	97.66	0.59	0	1.52
13	90.43	0.83	6.04	2.61
14	96.44	0.87	0.09	2.51
15	81.25	9.27	0.06	8.35
16	82.62	0.29	0.9	6.84
17	98.15	0.32	0.6	0.93
18	87.91	6.03	2.8	0.67
19	97.97	0.96	0.01	0.67
RS[b]	99.4	0.04	0.04	0.49

[a] T, tylosin

[b] RS, reference standard; Three replicates per sample

Table 6 Content of tylosin components (% w/w) in the tylosin phosphate and tartrate samples determined by HPLC

Sample number	T[a]A	TB	TC	TD
1	84.9	14.68	0.06	0.3
2	90.7	0.12	0.04	9.14
3	80	19	0.15	0.03
4	86.53	13.26	0.04	0.15
5	86.7	13.14	0	0.15
6	93.95	0.67	0.03	5.35
7	85.23	14.55	0.06	0.15
8	96.14	0.4	0.05	3.36
9	85.44	13.86	0.01	0.61
10	96.53	0.41	0.04	3.02
11	82.71	16.98	0.04	0.26
12	96.7	0.32	0.02	2.76
13	85.93	13.18	0.01	0.8
14	93.35	0.36	0.01	6.27
15	96.58	0.12	0.26	2.95
16	91.6	0.72	0.04	7.64
17	85.64	13.68	0	0.68
18	90.7	0.12	0.04	9.14
19	81.06	18.73	0.03	0.15
RS[b]	99.4	0.04	0.04	0.49

[a] T, tylosin

[b] RS, reference standard; Three replicates per sample

MIC values of tylosin components

As shown in Table 7, similar MIC values were obtained for tylosin components against *K. rhizophila* (0.1 μg/ml) except for tylosin D (1.56 μg/ml) which was the less microbiological active component. The MICs of tylosin components against *S. aureus* showed significant increase in comparison with the MICs obtained against *K. rhizophila* ($P < 0.05$). Tylosin A and C with the MIC value of 0.39 μg/ml showed similar sensitivity to *S. aureus*. The MIC of tylosin B was 0.78 μg/ml and tylosin D with the MIC of 12.5 μg/ml displayed a weak activity against test bacteria. Consequently, *K. rhizophila* and *S. aureus* responded differently to

Table 7 MICs of tylosin components against test organisms

Bacterial strain	MIC of tylosin components (μg/ml)			
	A	B	C	D
S. aureus	0.39	0.78	0.39	12.5
K. rhizophila	0.1	0.1	0.1	1.56

MIC, minimum inhibitory concentration

tylosin components. No growth inhibition was observed in control wells containing 2.5% methanol in phosphate buffer.

In this study, the test bacteria (*K. rhizophila* and *S. aureus*) showed different sensitivity to four tylosin components. Thus, the MICs of A and D components against a series of Gram-positive bacteria were determined to find a test organism with similar sensitivity to tylosin components. As depicted in Table 8, the ratio of MIC of D to A component against these strains were not fewer than that of *K. rhizophila*.

Potency determination of main matrix components

The potencies of tylosin components (A, B, C, and D) were determined using three microbiological assay methods described in the USP and the BP. The ratio of responses (relative potency) of tylosins B, C, and D to that of tylosin A by the three methods as percentage of tylosin A potency were calculated (Table 9). In agar-diffusion method by using *K. rhizophila,* the relative potency of B, C, and D components were estimated 110, 101, and 39%, respectively. The results showed that the relative potencies obtained for B and D components were lower in both turbidimetric methods in comparison with the diffusion assay (79.3 and 22.5% by the USP turbidimetric method, 77.3 and 22.8% by the BP turbidimetric method for B and D components, respectively). The potencies of tylosin C and A were almost equal in all three methods.

Quantitative relationship between content by HPLC and potency of tylosin A, B, C, and D

The microbiological potency of selected samples of tylosin base and tylosin phosphate which contained different ratios of matrix components, was determined by three bioassay methods and and their theoretical potencies were calculated as described above. Equivalency of the results of theoretical and microbiological potencies for each sample was evaluated by TOST and are summarized in Table 10. The theoretical and microbiological potencies were equivalent in all three methods for the samples No. 1 (containing 98.6% tylosin A) and No. 3 (containing tylosin A 91.42%, and tylosin C 5.6%). For the samples No. 2 (containing tylosin D 5.35%, and tylosin B 5.67%) and No. 4 (containing tylosin D 0.03%, and tylosin B 19%) microbiological potencies that were determined by both turbidimetric methods were not equivalent with the theoretical potencies, but they were equivalent in agar-diffusion method.

To validate the results of theoretical potency, five mixed samples from four tylosin components were prepared in the laboratory and their theoretical and microbiological potencies were determined (Table 11). In both turbidimetric assays, the theoretical potency was equivalent with the microbiological potency for the mixed sample M3. In diffusion method, the results of mixed samples M1, M2, and M3 were equivalent. The results of turbidimetric and diffusion-methods for the M4, and M5 were not equivalent.

Discussion

In this study, we evaluated the component ratio of different tylosin samples along with the individual potency of tylosin major components by using three different microbiological assays including agar diffusion, and two turbidimetric methods. The purity analysis showed significant differences between the composition of reference standard and some of the tylosin raw materials and preparations. Bioassay studies revealed that *K. rhizophila* ATCC 9341, and *S. aureus* ATCC 9144 had different sensitivities to tylosin matrix components.

Table 8 MICs of A and D components of tylosin

No.	Bacterial strain	MIC of tylosin component (μg/ml)	
		A	D
1	*Staphylococcus aureus* ATCC 9144	0.39	12.5
2	*S. aureus* ATCC 29737	0.39	12.5
3	*S. haemolyticus* ATCC29970	0.39	50
4	*Kocuria rhizophila* ATCC 9341	0.1	1.5
6	S. aureus ATCC 1112	0.1	25
7	*S. warneri* ATCC 27836	1.5	50
8	*S. xylosus* ATCC 29971	1.5	50
9	*S. epidermidis* ATCC 12228	0.78	12.5
10	*S. saprophyticus* ATCC 15305	1.5	25
11	*Bacillus subtilis* ATCC 6633	0.39	12.5

MIC, minimum inhibitory concentration

Table 9 The relative potencies of tylosin components by agar-diffusion and turbidimetric methods

Assay method	Tylosin component	Potency[a] (95% confidence interval)	Relative potency[b]
USP-Turbidimetric by using *S. aureus*	A	1025.58 (1018.84–1032.31)	100
	B	813.24 (796.60–829.88)	79.3
	C	1021.45 (1013.51–1029.38)	99.6
	D	231.04 (217.80–244.29)	22.5
BP-Turbidimetric by using *S. aureus*	A	1015.99 (988.16–1043.82)	100
	B	785.09 (707.71–875.80)	77.3
	C	1021.61 (1007.42–1035.80)	100.6
	D	231.62 (208.78–254.46)	22.8
BP-Agar-diffusion by using *K. rhizophila*	A	1012.738 (997.289–1028.186)	100
	B	1118.004 (1031.144–1204.864)	110.4
	C	1031.961(1000.621–1063.301)	101.9
	D	395.276 (376.365–414.187)	39

[a] Unit is IU/mg for BP methods and μg/mg for USP method

[b] Relative potency is percentage of potency of each tylosin component to that of tylosin A

The MIC values of tylosin components against *S. aureus* were consistent with their relative potencies obtained from USP and BP turbidimetric assays by using *S. aureus*. Such relationship was observed for the relative potencies obtained from BP agar-diffusion assay by using *K. rhizophila* and the MICs of tylosin components.

Several investigators have examined the potencies of main matrix components of other multi-components antibiotics such as teicoplanin, and neomycin by different bioassay methods. Chang et al. (2013) estimated the activity of main matrix components of teicoplanin on *B. subtilis* by four microbiological assay methods which were different in pH of culture medium or buffer solution. Results of this study showed that there were significant differences between responses of each matrix component in three methods. They concluded that if there were significant differences between matrix components ratio of the reference standard and test sample, then various potencies may be obtained by using different bioassay methods [9]. In other study, Tsujiet et al. demonstrated that relative responses of neomycin C to neomycin B by agar-diffusion and turbidimetric methods were1:3 and 1:2.5, respectively [17]. Sokolski et al. reported that neomycin components (B and C) did not present equivalent responses towards different bioassay systems and the potency of

Table 10 Equivalency of HPLC assay and microbiological potency determination results for selected tylosin samples

No.	Method	Content by HPLC assay (% T[a] components)				Total percent	Potency[b] (95% confidence interval)		TOST result (Equivalency test)[c]
		TA	TB	TC	TD		Microbiological	Theoretical	
1	USP-Turbidimetric	98.96	0.34	0.01	0.14	99.45	1025 (1020–1031)	1018 (1017–1019)	E
2		87.5	5.67	0.03	5.35	98.55	806 (787–825)	956 (954–957)	NE
3		91.42	0.29	5.6	0.84	98.15	1015 (1003–1027)	999 (987–1011)	E
4		80	19	0.15	0.03	99.18	903 (894–912)	977 (976–978)	NE
1	BP-Turbidimetric	98.96	0.34	0.01	0.14	99.45	1009 (1000–1018)	1009 (1008–1011)	E
2		87.5	5.67	0.03	5.35	98.55	799 (787–812)	946 (945–947)	NE
3		91.42	0.29	5.6	0.84	98.15	995 (989–1001)	990 (989–990)	E
4		80	19	0.15	0.03	99.18	900 (892–909)	964 (961–966)	NE
1	BP-Agar diffusion	98.96	0.34	0.01	0.14	99.45	1013 (1006–1020)	1007 (1006–1008)	E
2		87.5	5.67	0.03	5.35	98.55	980 (973–987)	971 (971–972)	E
3		91.42	0.29	5.6	0.84	98.15	1006 (995–1018)	990 (988–992)	E
4		80	19	0.15	0.03	99.18	1015 (1009–1020)	1024 (1022–1026)	E

[a] T, tylosin

[b] Unit is IU/mg for BP methods and μg/mg for USP method

[c] E, equivalent; NE, not equivalent

Table 11 Equivalency of HPLC assay and microbiological potency determination results for mixed samples (M1 to M5) with different proportions

Sample	Method	Mixed samples (% T[a] components)				Total percent	Potency[b] (95% confidence interval)		TOST (Equivalency test)[c]
		TA	TB	TC	TD		Microbiological	Theoretical	
M1	USP-Turbidimetric	80	18	1	1	100	1040 (1027–1054)	980 (978–982)	NE
M2		85	10	0.5	4.5	100	1019 (1010–1027)	969 (968–970)	NE
M3		90	1	5	4	100	1005 (998–1012)	991 (990–993)	E
M4		80	0.5	4.5	15	100	825 (784–866)	905 (903–906)	NE
M5		75	10	5	10	100	842 (831–853)	925 (923–927)	NE
M1	BP-Turbidimetric	80	18	1	1	100	1038 (1024–1052)	968 (967–969)	NE
M2		85	10	0.5	4.5	100	1015 (1009–1022)	958 (957–959)	NE
M3		90	1	5	4	100	980 (967–994)	983 (982–985)	E
M4		80	0.5	4.5	15	100	800 (789–810)	897 (894–899)	NE
M5		75	10	5	10	100	830 (824–836)	915 (914–917)	NE
M1	BP-Agar diffusion	80	18	1	1	100	1035 (1020–1051)	1026 (1024–1027)	E
M2		85	10	0.5	4.5	100	1010 (1002–1017)	996 (995–996)	E
M3		90	1	5	4	100	1000 (994–1007)	990 (988–991)	E
M4		80	0.5	4.5	15	100	990 (986–995)	922 (921–923)	NE
M5		75	10	5	10	100	1015 (1007–1023)	962 (961–963)	NE

[a] T, tylosin

[b] Unit is IU/mg for BP methods and μg/mg for USP method

[c] E, equivalent; NE, not equivalent

neomycin, a multi-component antibiotic, depends on the components ratio (B:C) in the test and standard preparations [8]. Sokolski et al. also indicated that negative bias for neomycin C was seen by the commonly used assay procedures for neomycin assay [18]. Kibwage et al. measured the potencies of erythromycins B, C, and D relative to erythromycin A as a reference compound. They observed that these components have lower potencies than erythromycin A [19]. It was reported that different compositions as well as different antimicrobial activities of the matrix components may affect the accuracy of potency determination by the present bioassay methods. Therefore, the present assay method should give equal responses to all of the components to eliminate composition difference between test sample and reference standard [9].

In this study, theoretical and microbiological potencies were equivalent in each method for the samples with the more consistent composition in comparison with our reference standard (sample No. 1) and high tylosin A and C contents (sample No. 3). The tylosin A and C showed similar high antimicrobial activities in three bioassay methods. For the samples with lower content of A component and higher B and D amounts (sample No. 2 and 3), the theoretical and microbiological potencies were not equivalent in turbidimetric methods, but they were equivalent in agar-diffusion assay which may be due to the higher potency of B and D components in agar-diffusion in comparison with turbidimetric assays. These findings were confirmed by our experiments on mixed tylosin samples. In the present study, sample with high amount of tylosin A (M3) were equivalent in both turbidimetric and diffusion assays. Therefore, it seems that the equivalency of theoretical potency and microbiological potency will be obtained for the samples that contained similar total amount of components with high antibacterial activity (tylosin A and C) with the reference standard. In diffusion method, the results of mixed samples M1, M2, and M3 with low amount of tylosin D, more than 80% of tylosin A, and more than 95% of the total A, B, and C components were equivalent. As shown previously, tylosin A, B, and C had similar antibacterial activity against *K. rhizophila* in agar diffusion method. The sample M4 which contained 80% of A component and 85% of the total A, B, and C components were not equivalent due to the presence of high tylosin D content (15%). The theoretical and experimental potencies for the sample M5 with 10% of tylosin D, 75% of tylosin A, and 90% of the total A, B, and C components were not equivalent. In our microbiological potency determination, we used a tylosin reference standard that contained 99.4% of tylosin A, the total of tylosin A, B, and C content of 99.48%, and very low amount of tylosin D (0.04%). As presented above, the equivalency of theoretical potency and microbiological potency in agar-diffusion method could be obtained for the samples that contained similar total amount of tylosin A, B, and C with the reference standard. Our results indicated that due to the different test organism sensitivity of the components and assay conditions, we couldn't find a single factor for converting the content of each

component determined by HPLC to its bioequivalent potency. Moreover, the potency factor calculated for each microbiological assay is applicable while the test conditions remained unchanged within a laboratory. This factor is laboratory-dependent and might not be used in other laboratories unless they validate their microbiological method to deliver reproducible results. Similarly, the effect of test conditions on potency factor determination for erythromycin has been reported by other investigators [19, 20].

Another way that was proposed to address the different sensitivity of each component to test microorganism, is the use of standard with similar composition as that of the sample [21]. It will never be possible to obtain a standard with the same matrix components ratio for all of the samples on the market. This problem could be solved by setting limits for the amount of low potency components in the standard and test samples. By considering individual limits, the reproducibility and accuracy of the microbiological assay could be improved. The USP and the BP does not limit the individual content of tylosin B, C, and D, and samples with different compositions are compared with a reference standard mixture of known composition. In this way, Ph. Eur. limits the content of neomycin C to 3–15% and also, erythromycin B and C to 5%. Furthermore, USP has a limit of about 12% for erythromycin B and 5% for C [19, 22, 23].

Many researches pointed out that microbiological assays might not give more exact results for multicomponent antibiotics than physicochemical methods such as HPLC [20]. Therefore, it seems that replacement of microbiological assays by physicochemical methods without using a conversion factor is more reasonable. This goal could be achieved by defining individual limits for low and high active components in the antibiotic specifications.

Conclusion

In view of these results, we concluded that the potency of tylosin components were affected by the species of the test organism and the conditions of the microbiological assay. Due to the different antibacterial sensitivity of tylosin components, it is not possible to find a single factor for converting the content of each component determined by HPLC to its bioequivalent potency. Since the reference standard and test samples have no consistency in their matrix components ratios, individual limits could be defined for the content of low active components and the total of components with similar high activity. Finally, to define a desired composition for a multicomponent antibiotic, in addition to the bioassay results, the antimicrobial activity against clinical isolates, pharmacokinetics characteristics, and the toxicity of each component should be considered.

Authors' contributions This study was PhD thesis of KhH who did all the practical experiments. KhH contributed in the conception, design of the study, data collation and manuscript preparation. NS contributed in the conception, the design of the study, gave revisions of the manuscript and supervised the study. MA contributed in the design of the study and supervised the study. All authors read and approved the final manuscript.

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Compliance with ethical standards

This study was approved by the Ethics Committee of the Tehran University of Medical Sciences, Tehran, Iran (ethics committee reference number: IR.TUMS.VCR.REC.1395.26).

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interest.

Abbreviations *DMT*, Demecinosyl-tylosin; *OMT*, O-mycaminosyl-tylonolide; *BP*, British pharmacopoeia; *USP*, United states pharmacopeia; *HPLC*, High pressure liquid chromatography; *EP-CRS*, European pharmacopoeia chemical reference substance; *EDQM*, European directorate for quality medicines & healthCare; *MIC*, Minimum inhibitory concentration; *MHB*, Mueller–hinton broth; *TOST*, Two one-sided test

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