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LC determination of salinomycin in fermentation broths and premixes

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Abstract

A simple and rapid high performance liquid chromatography method for the determination of salinomycin in fermentation media of *Streptomyces albus* strains and in premixes has been developed. This method involves reverse-phase separation of the component analysed with UV detection at 210 nm using methanol and 0.2 M acetate buffer pH 5.8 (100:10, v/v) as the mobile phase. The reliability of the method was confirmed by validation. A linear relationship was obtained within range 0.2–2.0 mg ml^{-1} ($r = 0.9999$). The relative standard deviation of methods within-laboratory reproducibility was 1.6%. The estimated quantitation limit of assay was about 32.5 μg ml^{-1}. The method has been successfully used in the determination of salinomycin content in testing production processes and premixes of different commercial brands.

Keywords: Salinomycin; Reverse-phase liquid chromatography; Fermentation broth; Premix; UV detection

1. Introduction

Salinomycin is a carboxylic polyether antibiotic demonstrating ionophoric properties. It has five cyclic ether rings including one dihydropyran ring and two spiroketal groups in a tricyclic unsaturated system (Fig. 1).

The main therapeutic application of salinomycin in veterinary medicine is for the prevention and treatment of coccidiosis in poultry [1]. Salinomycin is produced by a fermentation process using *Streptomyces albus* strain [2].

Several methods for the determination of salinomycin in premixes [3–10], fermentation broths [3–5,8] and animal tissues [11–13] are reported. Microbiological assays using *Bacillus subtilis* in a diffusion method [3] and *Streptococcus faecalis* in a turbidimetric method [4] are time consuming and have lack of specificity and sensitivity. Nonspecific spectrophotometric determination is based upon reaction with vanillin in an acidic medium [5]. Screening methods include thin-layer chromatography–bioautography [6] and enzyme-linked immunosorbent assays [11]. Recently, high perfor-

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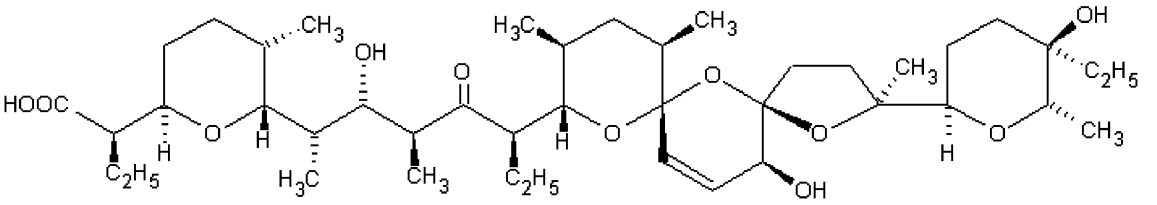

Fig. 1. The chemical structure of salinomycin.

mance liquid chromatography (HPLC) methods became widely used for accurate, precise, sensitive, and selective quantification of salinomycin in such samples. Since salinomycin does not possess any significant UV absorbance, nor electrochemical or fluorescence activity, precolumn or postcolumn derivatization is generally used in order to improve detection. The primary used postcolumn reagent is vanillin [7,8], but *p*-dimethylaminobenzaldehyde is used as a sensitization reagent [9]. The requirement of extra components such a pump, reactor, and heating coil is a disadvantage when compared to conventional HPLC systems. Analysis of UV active derivatives of salinomycin using precolumn derivatization with pyridinium dichromate in poultry skin and fat [12] and 2,4-dinitrophenylhydrazine in premixes [10] were also reported. Very specific and selective determination of salinomycin in poultry tissues and eggs was achieved using mass spectrometry detector coupled with liquid chromatography [13].

This paper describes the development and validation of an HPLC method suitable for fast determination of salinomycin in production fermentation broths and premixes without application of precolumn or postcolumn derivatization. The UV absorption of salinomycin in methanol is poor at 285 nm (ε 108) (corresponding to a carbonyl group) and is unsuitable for the direct detection. The response of salinomycin at low UV wavelengths is about one order higher and therefore detection at 210 nm was proposed. Since at this region many UV-absorbing components presented in analyzed samples could interfere, the selectivity of separation had to be optimized. It is shown that the pH of the mobile phase plays a significant role in this selectivity.

2. Experimental

2.1. Chemicals

HPLC grade methanol and ethanol were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Analytical grade acetic acid and sodium acetate trihydrate were obtained from Lachema a.s. (Neratovice, Czech Republic). Water was deionized and distilled. Salinomycin sodium reference standard was prepared in R&D department of Biotika a.s. The identity and purity was employed by comparison with salinomycin purchased from Sigma (Deisenhofen, Germany).

2.2. Standard preparation

The standard solution was prepared by dissolving salinomycin sodium in ethanol to give a concentration of about 1 mg ml^{-1}. This solution was stable for at least 1 month when stored at 4 °C.

2.3. Sample preparation

2.3.1. Fermentation broth

A suitable amount ($\sim$5 g) of fermentation broth was weighed in to a 50 ml volumetric flask and diluted to volume with ethanol. The solution was sonicated for 10 min in an ultrasonic bath. The solution was filtered through a 0.45 μm membrane filter and analyzed. The final concentration of salinomycin was in the range of 0.5–1.5 mg ml^{-1}.

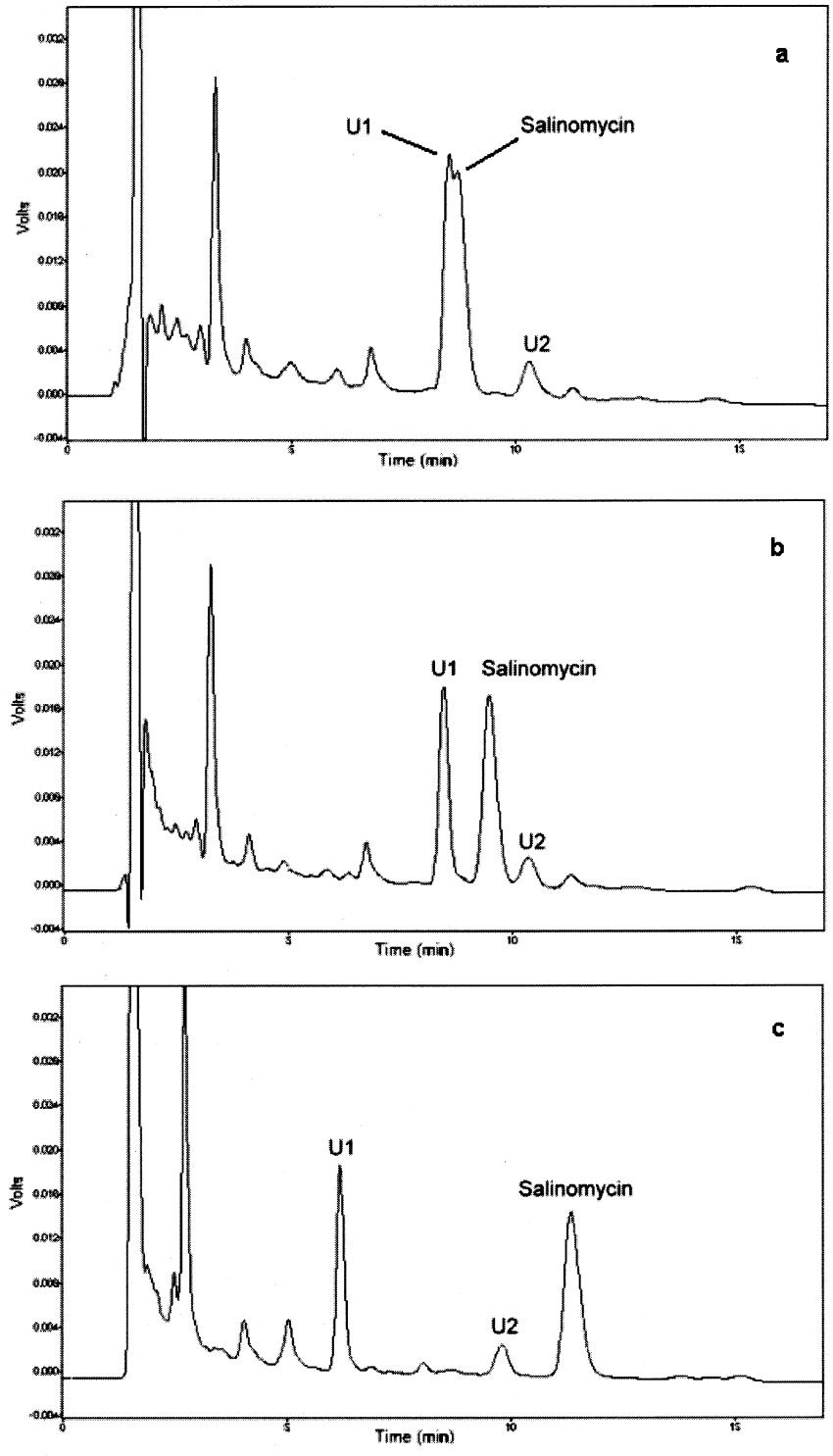
a
U1
Salinomycin
U2
Volts
Time (min)
b
U1
Salinomycin
U2
Volts
Time (min)
c
U1
Salinomycin
U2
Volts
Time (min)

Fig. 2

Table 1
Effect of acetate buffer pH on chromatographic parameters calculated for salinomycin peak

pH of acetate buffer x	k'	Width	Height	Symmetry	Theoretical plates per m
3.10	4.82	–	19 999	–	–
4.00	5.33	0.58	17 273	1.27	28 762
4.51	5.79	0.61	15 550	–	29 714
4.74	6.29	0.64	14 497	1.24	31 594
5.07	6.58	0.66	14 863	1.29	31 370
5.52	6.82	0.68	13 736	1.29	31 568
5.92	6.88	0.68	13 584	1.26	31 746
6.86	6.96	0.69	13 516	1.27	31 692

LC conditions: column, Inertsil ODS-3 (150 × 4.6 mm, 5 μm); guard column, Lichrosorb RP 18 (14 × 4.0 mm, 5 μm); mobile phase, methanol–acetate buffer (0.2 M) pH x (100:10, v/v) at a flow rate of 1.0 ml min^{-1}; detection, UV at 210 nm.

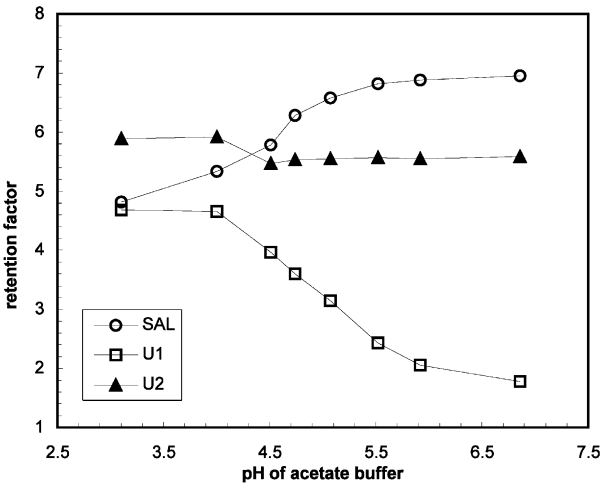

Fig. 3. Effect of pH of acetate buffer on retention factor of salinomycin (SAL) and unknown peaks (U1, U2). LC conditions are the same as in Fig. 2.

2.3.2. Premix samples

Premix samples were pulverised in a grinder to obtain a homogeneous powder. About 1 g was weighed in to a 50 ml volumetric flask and diluted to volume with ethanol. The solution was sonicated for 10 min in an ultrasonic bath. The solution was then filtered through a 0.45 μm membrane filter and diluted with ethanol to obtain a final concentration of salinomycin about 1 mg ml^{-1}.

2.4. Instrumentation

The HPLC system consisted of a Shimadzu (Kyoto, Japan) model LC-10AS single piston pump, a model SIL-10AXL autoinjector, a model CTO-10AC column oven and a model SPD-10AV UV–VIS detector operated at 210 nm all controlled by a computer using Class-VP 4.0 software. Separation was carried on a Inertsil ODS-3 (150 × 4.6 mm, 5 μm) column (GL Science, Tokyo, Japan) coupled with a Lichrosorb RP 18 (14 × 4.0 mm, 5 μm) guard column (Merck, Darmstadt, Germany). The column temperature was maintained at 35 °C. The flow of mobile phase was 1 ml min^{-1} and injection volume was 20 μl.

3. Results and discussion

3.1. Development of the method

Several mobile phases used for RP–HPLC analysis of polyether antibiotics have been described. Most of them consisted of methanol diluted with acidified water (e.g. acetic acid) to a

Fig. 2. Effect of mobile phase pH on separation selectivity. *LC conditions:* column, Inertsil ODS-3 (150 × 4.6 mm, 5 μm); guard column, Lichrosorb RP 18 (14 × 4.0 mm, 5 μm); mobile phase, methanol—0.2 M acetate buffer (100:10, v/v), flow rate 1.0 ml min^{-1}, UV detection at 210 nm; analysed sample, ethanol extract of 12% salinomycin premix (Biotika). U1 and U2 unknown peaks. Acetate buffer pH (a) 3.1, (b) 4.0, (c) 5.1.

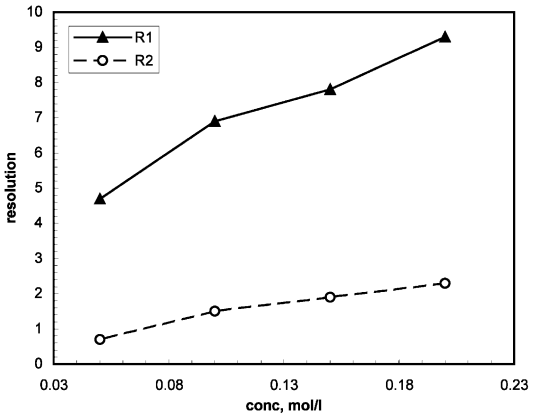

Fig. 4. Effect of acetate buffer concentration on resolution between salinomycin and unknown peak U1 (R1) and between salinomycin and unknown peak U2 (R2). Acetate buffer pH 4.7, other conditions are the same as in Fig. 2.

final methanol concentration 90–95% (vol.) [7,8]. In our experience, this type of eluent was unsuitable for the direct UV detection at 210 nm due to poor resolution of the main peak (salinomycin) from other components present in tested salinomycin premixes. In order to improve the resolution, the selectivity of separation conditions should be improved. At first, the effect of varying the pH of the mobile phase was investigated. The acidified water was replaced by acetate buffer consisted of 0.2 M sodium acetate and 0.2 M acetic acid. The required pH of acetate buffer was obtained by mixing these two solutions in various ratios. The following pH values for the buffer were tried: 3.1, 4.0, 4.5, 4.7, 5.1, 5.5, 5.9 and 6.9. Mobile phase then consisted of 10 volumes of buffer and 100 volumes of methanol. It was found that pH has a

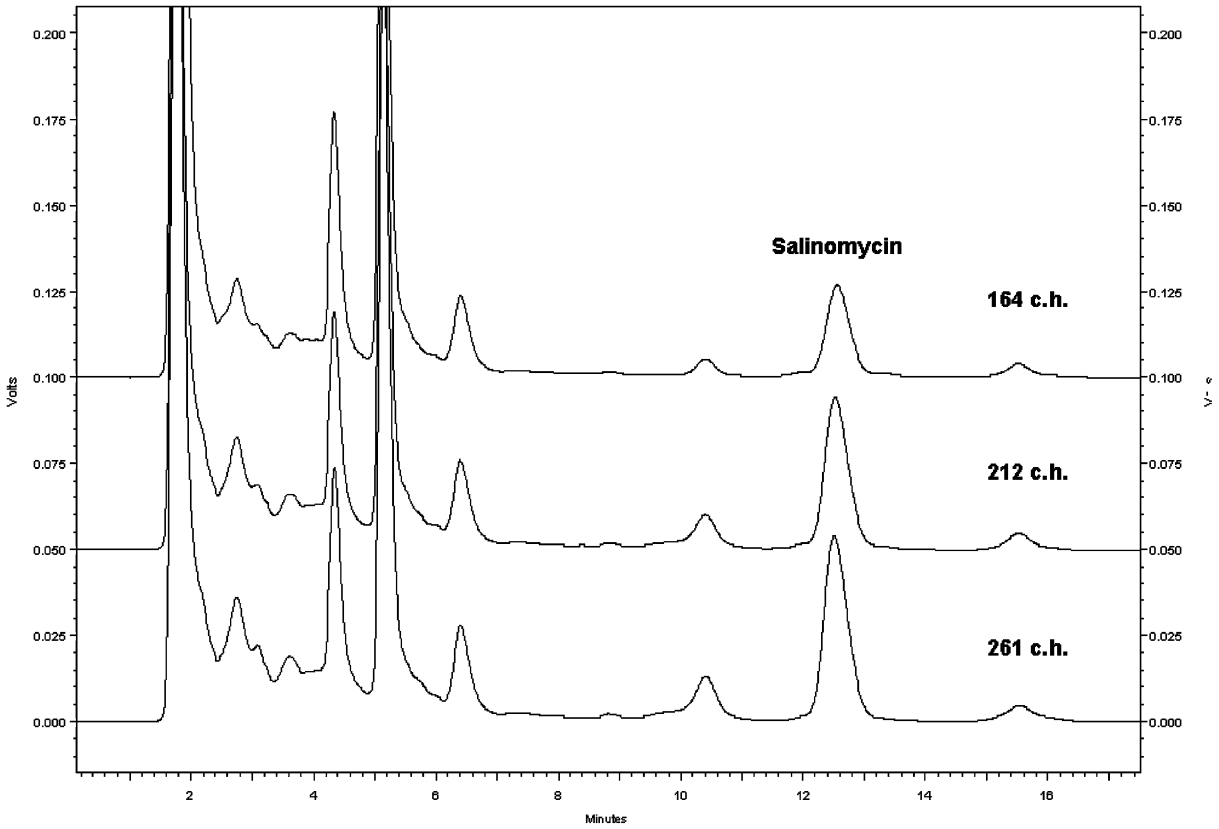

Fig. 5. Representative chromatograms of ethanol extracts of fermentation broth at different cultivation hours ((a) 164, (b) 212 and (c) 261 cultivation hour) at suggested LC conditions. Column, Inertsil ODS-3 (150 × 4.6 mm, 5 μm); guard column, Lichrosorb RP 18 (14 × 4.0 mm, 5 μm); mobile phase, methanol—0.2 M acetate buffer pH 5.8 (100:10, v/v), flow rate 1.0 ml min^{-1}, UV detection at 210 nm.

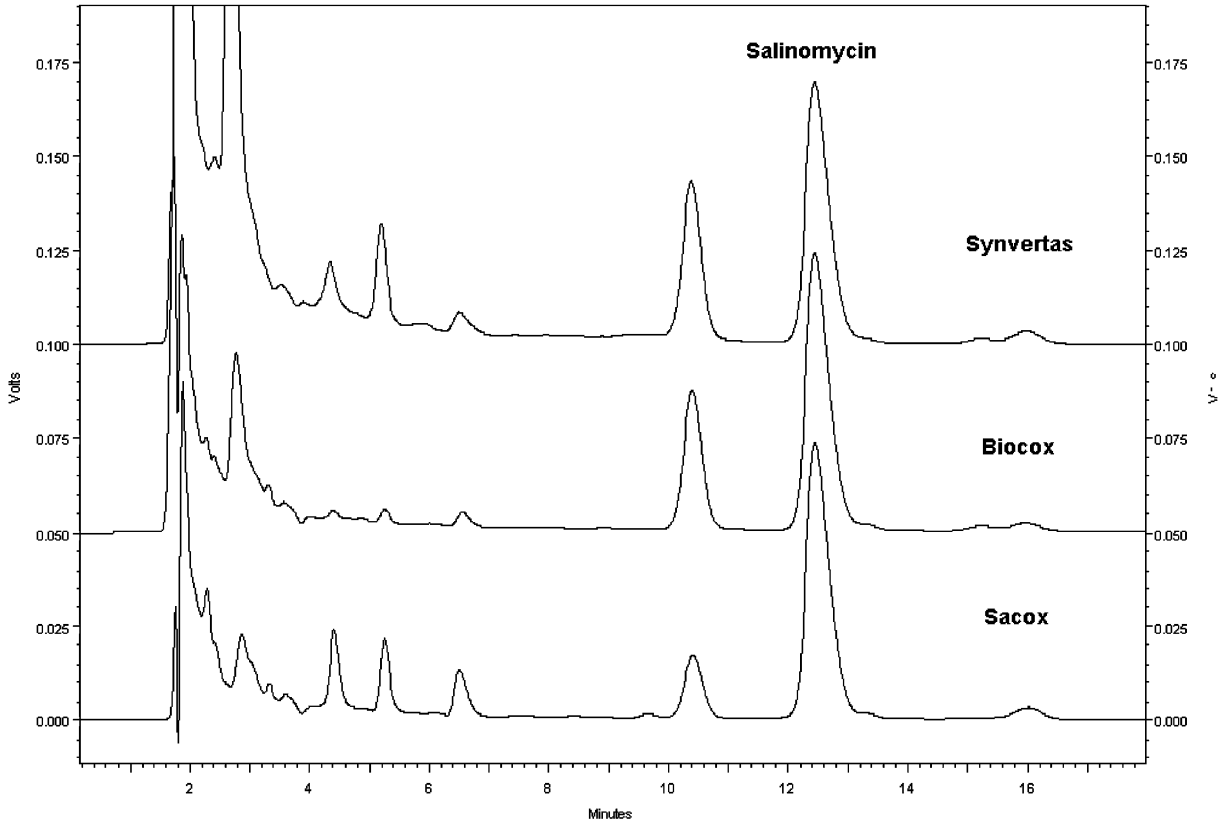

Fig. 6. Representative chromatograms of ethanol extracts of 12% salinomycin sodium premixes obtained from three different producers (Synvertas, Biotika, Slovakia; Biocox, Hoffman-La Roche, USA; Sacox, Hoechst, Germany). LC conditions are the same as in Fig. 5.

Table 2
Results of assay salinomycin sodium in 4 different commercial brands

Premix	Concentration declared % (w/w)	Concentration found % (w/w)
Sacox (Hoechst, Germany)	12.0	12.5
Biocox (Hoffman-La Roche, USA)	12.0	12.5
Synvertas (Biotika, Slovakia)	12.0	12.5
Salinopharm (Pharmachim, Bulgaria)	12.0	11.7

significant influence on the retention of a majority of the eluted peaks, especially on salinomycin and peaks eluting near salinomycin. With increasing pH, the retention factor of salinomycin also increased (Table 1) from $k' = 4.8$ at pH 3.1 to $k' = 6.6$ at pH 5.1, with no further increase at higher pH values.

However, k' of an unknown coeluting peak (U1) was about the same between pH 3 and 4, but at higher pH decreased rapidly. Also $k'$of a retention factor of the second closely eluted peak (U2) decreased slightly, as shown in Fig. 2.

Resolution between salinomycin and two unknown peaks was acceptable at pH 4.7 and higher (Fig. 3).

Next, the effect of different acetate buffer concentrations on the resolution between salinomycin and the unknown peaks (U1 and U2) was investigated. The following concentrations were used: 0.05, 0.1, 0.15 and 0.2 M (pH of buffer was 4.7). Acceptable separation of salinomycin from

Table 3
Intra-assay precision of the method

Sample	Premix 1% ($n = 6$)	Premix 12% ($n = 6$)	Premix 25% ($n = 6$)
Mean (g kg^{-1})[a]	9.7	122.7	258.2
Standard deviation (S.D.) (g kg^{-1})	0.13	0.73	1.32
Relative standard deviation (R.S.D.) (%)	1.35	0.59	0.51
Confidence (at 95% level) (g kg^{-1})	0.14	0.77	1.64

[a] Concentration of salinomycin in premix.

these unknowns was achieved by using higher concentrations of buffer ($\geqslant 0.1$ M) (Fig. 4).

In accordance with these results, the optimal mobile phase contains 100 volumes of methanol and 10 volumes of 0.2 M acetate buffer, pH 5.8. Typical chromatograms of an ethanol extract of the fermentation broth collected at 164, 212 and 261 cultivation hours analysed under proposed chromatographic conditions are shown in Fig. 5.

Fig. 6 shows a comparison of chromatograms of 12% salinomycin premixes obtained from three different producers (Biotika, Hoffman-La Roche, Hoechst), analysed under the proposed chromatographic conditions. The assay values are presented in Table 2.

3.2. Validation

The proposed chromatographic method was assessed for precision (intra-assay precision and within-laboratory reproducibility), linearity, limit of quantification and accuracy.

3.2.1. Intra-assay precision

The intra-assay precision of the method was studied on the three premix samples with different concentrations of salinomycin (1, 12, 25%, w/w). The approximate salinomycin concentrations in the analyzed solutions were 0.16, 0.96 and 1.50 mg ml^{-1}. The intra-assay precision was then determined by replicate injections of six ethanol extracts of each sample. Analysis of each sample was performed under the same conditions, utilizing the same equipment and chemicals, within a 24-h period. The relative standard deviations ranged from 0.51 to 1.35%. Results are shown in Table 3.

3.2.2. Within-laboratory reproducibility

The within-laboratory reproducibility of the method was assessed during 2 days. On each day the same premix sample (12%, w/w) was six times analysed by different analysts at the same equipment. The approximate salinomycin concentration in the analyzed solutions was about 1.0 mg ml^{-1}. Results are shown in Table 4.

An one-way ANOVA was carried out to determine statistical difference between two sets of data. According to calculated results, the difference between the sets was statistically not significant at 95% confidence level (F_{value} (0.29) $< F_{crit}$ (4.96), P-value (0.6) > 0.05).

3.2.3. Linearity

The linearity of the method was obtained by analysis of a series of salinomycin standard con-

Table 4
Within-laboratory reproducibility of the method

	Analyst 1 ($n = 6$)	Analyst 2 ($n = 6$)	Within-laboratory reproducibility ($n = 12$)
Mean (g kg^{-1})[a]	122.4	121.8	122.1
Standard deviation (S.D.) (g kg^{-1})	0.97	2.76	2.00
Relative standard deviation (R.S.D.) (%)	0.79	2.26	1.64
Confidence (at 95% level) (g kg^{-1})	1.02	2.90	1.27

[a] Concentration of salinomycin in premix.

centrations in methanol: 0.21, 0.52, 1.04, 1.56, and 2.07 mg ml^{-1}. Standard solutions were injected three times for each concentration level.

The obtained calibration curve was subjected to linear regression analysis: $y = 382010x + 1840.6$, where y, mean peak area; x, concentration in mg ml^{-1}; correlation coefficient $r = 0.9999$. The standard error of the slope was 973.5, of the intercept 1240.2, and of estimate $S_{y,x} = 1.0$. The correlation coefficient demonstrated linearity of the method over the examined concentration range.

3.2.4. Limit of quantitation

The limit of quantitation was calculated from calibration curve according equation [14]:

$$LOQ = 10^{*}\sigma(i)/S$$

where $\sigma(i)$ is the standard deviation of the estimation of the intercept and S is the slope of the calibration curve.

The calculated concentration was 32.5 μg ml^{-1}. In order to validate this value, six sample solutions of approximately this concentration were prepared from a fermentation broth extract and analyzed. The mean value was 36.1 μg ml^{-1}, standard deviation 2.10 μg ml^{-1}, relative standard deviation 5.82%, and confidence (at 95% level) 2.61 μg ml^{-1}.

3.2.5. Accuracy

The accuracy of this method was verified by comparison of results with those of a validated HPLC method using postcolumn derivatization (vanillin reagent). Chromatographic conditions of the postcolumn derivatization method were the same as in Ref. [8]. Method was validated in the QC department of Biotika a.s. and is currently used for determination of salinomycin in premix samples. Analyzed samples consisted of four fermentation broths at different cultivation hours and six salinomycin premixes (12–25%, w/w) obtained from different producers. The obtained sets of data were subjected to a paired t-test ($n = 10$). According to the calculated results, the difference between the sets was statistically not significant at 95% confidence level (t_{value} (-0.93) $< t_{crit}$ (2.26), P-value two-tail (0.37) > 0.05). Linear regression analysis of the results obtained by the UV method (y) versus results obtained by the postcolumn derivatization method (x) gave the following equation: $y = 1.0447x - 2.343$, with a correlation coefficient of 0.9975.

4. Conclusion

The developed and validated HPLC method described is simple (no derivatization necessary) and rapid (no need for sample clean up). This method is shown to be precise, linear, and accurate. It is currently use in laboratory scale for monitoring salinomycin productions.

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