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# Comparative determination of salinomycin by high-performance liquid chromatography, microbiological and colorimetric methods in testing production processes and animal feed preparations*

MILENKO SOKOLIĆ† and MIROSLAV POKORNY

*Department for Development of Fermentative Production, Krka Pharmaceutical, Cesta herojev 45, 68000 Novo mesto, Yugoslavia*

**Abstract**: Salinomycin and other polyether antibiotics usually do not have a strong chromophore, nor electrochemical or fluorescence activity. Consequently, common liquid chromatography detectors cannot be used for the direct determination of these substances and LC methods that utilize a specific post-column reaction with vanillin have been developed to overcome this limitation. In this report, some modifications of perviously published LC methods were made to optimize the analysis of salinomycin. Comparison with standard microbiological and colorimetric methods has been carried out. The LC method was found to provide the best way of precise, and accurate determination in low levels of salinomycin down to the concentration of 1 mg $kg^{-1}$.

**Keywords**: *Salinomycin; LC post-column derivatization; microbiological method; colorimetric method.*

## Introduction

Salinomycin belongs to the polyether antibiotics, a group of naturally appearing ionophore substances that are produced by certain strains of *Streptomyces* species [1, 2]. Salinomycin exhibits a wide range of biological activity and acts antibiotically on the gram-positive bacteria and some *Microbacterium* species. The main therapeutic application is in veterinary medicine for the treatment of coccidiosis, an infective poultry disease, caused by *Eimeria protozoa*. Salinomycin also showed very good results in the intensive animal feeding. When it is added to animal feed in the concentration of 5–100 mg $kg^{-1}$ respectively, it significantly increases resorption of food and body weight of cattle, pigs and chickens. Considerable amounts of salinomycin are used for this purpose worldwide.

Like other polyether antibiotics, salinomycin has very low solubility in water, but it is very soluble in less polar organic solvents. This characteristics can be explained by its cyclic structure and hydrophobic functions being located on the exterior surface. The ultraviolet absorption spectrum of salinomycin showed maxima at 285 nm but of very low intensity [3]: which is why salinomycin cannot be determined by direct spectrophotometric methods. To overcome this limitation, an indirect colorimetric method has been developed involving the Komarowsky reaction [4, 5]. This reaction was first developed as a spot detection test in the thin layer chromatography. Later it was modified for the different quantitative colorimetric determinations [6]. By this analogy, some polyether antibiotics (salinomycin, monensin, narasin) and vanillin, catalysed by anhydrous acidic conditions form a pink coloured product that strongly absorbs in the visible region with maximum absorbance at 515–525 nm.

From its initial introduction salinomycin was also determined by microbiological methods using either an agar diffusion assay with *Bacillus subtilis* [7] or a turbidimetric assay with *Streptococcus faecalis* [8]. Neither microbiological method is sufficiently sensitive or specific to be generally useful.

Increasing use and introduction of different polyether antibiotics in veterinary medicine has established the requirements for more sensitive and specific methods of analysis. For

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† Author to whom correspondence should be addressed.

this purpose different chromatographic methods have been developed, such as thin-layer and thin-layer bioautographic assay [9, 10]. Those methods are more specific but they are time-consuming and the results are only semi-quantitative. Liquid chromatographic methods, known to be specific, sensitive, precise, accurate and easily automated, are candidates for the determination of polyether antibiotics, but direct detection is not possible by UV, EC or fluorescence. In some cases refractive index detection is possible for the analysis of feed premixes samples containing greater than 1 g $kg^{-1}$ salinomycin [11, 12]. For lower concentrations of polyether antibiotics, for example in samples of different animal feeds, these substances have to be derivatized either pre-column or post-column [13, 14].

HPLC methods for determination of polyether antibiotics that utilize post-column reaction based on the previously described vanillin reaction have been developed. After thoroughly studying the kinetics of this colour reaction and the different influencing factors, LC methods have been developed for the determination of salinomycin at concentrations as low as 1 mg $kg^{-1}$ [15, 16].

## Experimental

*Chemicals and samples*

Methanol, sulphuric acid and glacial acetic acid were of analytical reagent grade (Kemika, Zagreb, Yugoslavia). Water was deionized and distilled. Vanillin (NF food grade) was used (Merck, Darmstadt, Germany). Salinomycin–sodium reference standard 970 μg $mg^{-1}$ was purchased from Hoechst (Frankfurt am Main, Germany). Narasin and monensin standard substances were gifts from LUFA (Kiel, Germany). Different process samples of salinomycin (fermentation broths, feed grade preparations) and salinomycin-spiked animal feed, were supplied by Krka Pharmaceuticals (Novo mesto, Yugoslavia). Some commercial samples of 6 and 12% (w/w) salinomycin feed grade under the trade name Sacox, were obtained from Hoechst, and salinomycin feed grade 10% (w/w) from Kaken Chemical Co. Ltd (Tokyo, Japan).

*LC apparatus, operating conditions and methods*

The LC instrumentation for the post-column derivatization procedure was arranged as indicated and consisted of an LKB pump 2150, an LKB LC Controller 2152, an LKB variable wavelength monitor 2151 (LKB, Bromma, Sweden). The post-column reactor consisted of a Veb MLW U8 temperature-controlled water bath (Freital, Germany), maintained at 80°C and a reactor coil of stainless steel tubing (0.7 mm i.d., 4250 mm length).

Separations were made on a Chromspher C18 column (5 μm, 150 × 4.6 mm i.d.) coupled with reverse-phase pelicular pre-column (75 × 2.1 mm i.d.) (Chrompack, Middelburg, Netherlands). The temperature of the separation was ambient and the eluted components were detected at 520 nm after post-column reaction.

Samples were injected through a Rheodyne Model 7125 injector fitted with 20 μl fixed loop (Rheodyne, Inc., Cotati, CA, USA). Integration was based on peak area measurement on an LKB 2220 recording integrator. An external standard procedure was used for quantitative determinations of salinomycin.

For the comparison of post-column derivatization with other detection possibilities, an LKB 2140 rapid spectral detector and a refractive index detector (Knauer differential refractometer type 198, Berlin, Germany) were used.

Colorimetric determination of salinomycin was tested according to the method mentioned in ref. 6. Microbiological determination was carried out according to ref. 7, after some modification. Instead of *Bacillus subtilis* (ATCC 6633) a strain of *Bacillus cereus* that was found to be more sensitive to salinomycin was used.

*Mobile phases and reagents*

Mobile phase was delivered at 0.6 ml $min^{-1}$ flow rate and consisted of: methanol–water (94:6, v/v). The water contained 1.5% (v/v) glacial acetic acid. Vanillin 4% (w/v) reagent was prepared as follows. Concentrated sulphuric acid (10 ml) was added to 500 ml of methanol while gently stirring and cooling. Vanillin (20 g) was then dissolved.

The delivery system for the vanillin reagent consisted of a vanillin bottle immersed into an ice bath in a Dewar can. In this way the reagent remained stable when protected from light for 12 h. The reagent was pumped by an LKB 2150 HPLC pump at 0.4 ml $min^{-1}$ flow rate. After mixing, the total flow of the reagent stream and column eluent was 1 ml $min^{-1}$.

Vanillin reagent and mobile phase were prepared fresh daily and filtered through 5 μm membrane filter.

*Sample preparation*

The stock solution of salinomycin standard was prepared by dissolving and diluting salinomycin in methanol to 600 μg $ml^{-1}$. This solution was very stable when refrigerated at 4°C and could be used for 60 days.

Salinomycin was extracted from each sample with methanol on the rotary shaker for 30 min. The supernatant liquid was filtered through 0.45 μm membrane filter and further diluted to the appropriate concentration with methanol.

## Results and Discussion

*HPLC method*

The mean advantages of LC with post-column derivatization for the analysis of salinomycin lay in the specific vanillin reaction and the selective separation by the C18 column. In this report the HPLC method for salinomycin determinations was compared with the agar diffusion microbiological and colorimetric methods. Some minor modifications of the previous LC methods [13, 14] were necessary to optimize the separation of salinomycin in different samples.

A vanillin concentration of 4% (w/v) and 80°C temperature in the reaction coil was sufficient to produce satisfactory colour reaction. Total flow rate of 1 ml $min^{-1}$ (0.4 ml $min^{-1}$ of vanillin reagent and 0.6 ml $min^{-1}$ for mobile phase) was selected because it gave an adequate 1.6 min reaction time in the heated coil [16]. Further increase of reaction time required longer reaction tubing and produced broadening of the peaks and deterioration of the separation.

To demonstrate the specificity of the vanillin reaction the post-column reactor was removed and a diode array detector used in the UV range from 200 to 300 nm. As it was outlined in ref. 3, a low absorbance of salinomycin itself was expected at 285 nm. But at this spectral range, no peak for salinomycin was detected. A small peak, barely distinguishable from the baseline noise was diluted at 200–220 nm (Fig. 1), but this absorptivity was insufficient for routine analyses. UV-absorbing compounds in salinomycin feed grade are visible under the same conditions and some partly coelute with salinomycin itself (Fig. 2). In contrast, the UV-absorbing components from the samples did not react with vanillin and did not, therefore interfere with salinomycin determination at 520 nm (Fig. 2b). Sensitivity of the vanillin post-column reaction at 520 nm was much higher than with refractive index detection (Fig. 3).

As the water content in the mobile phase preparation was the main factor that influences

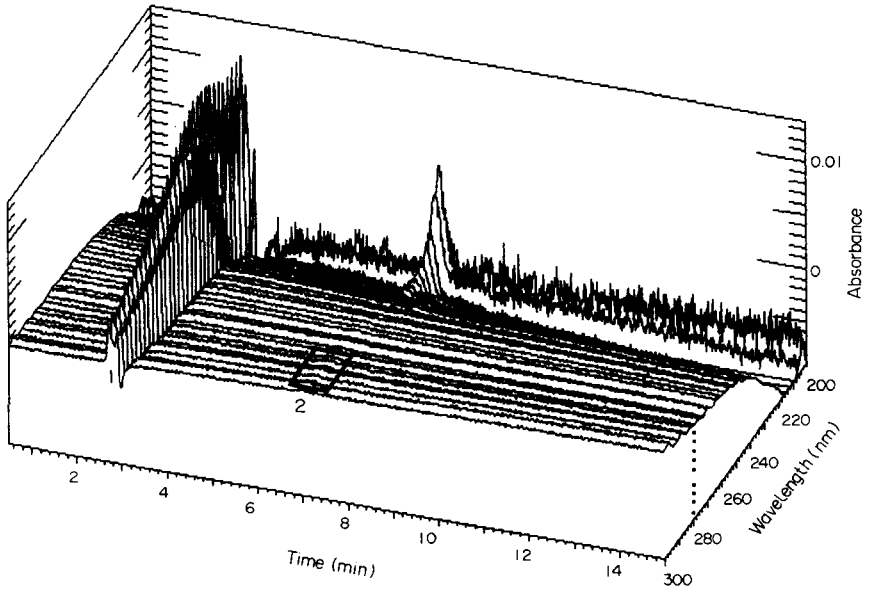


**Figure 1**
Diode array topogram of salinomycin standard solution (60 μg $ml^{-1}$), without post-column reactor. The wavelength range was 200–300 nm. 1, solvent peak; 2, position where salinomycin was expected.

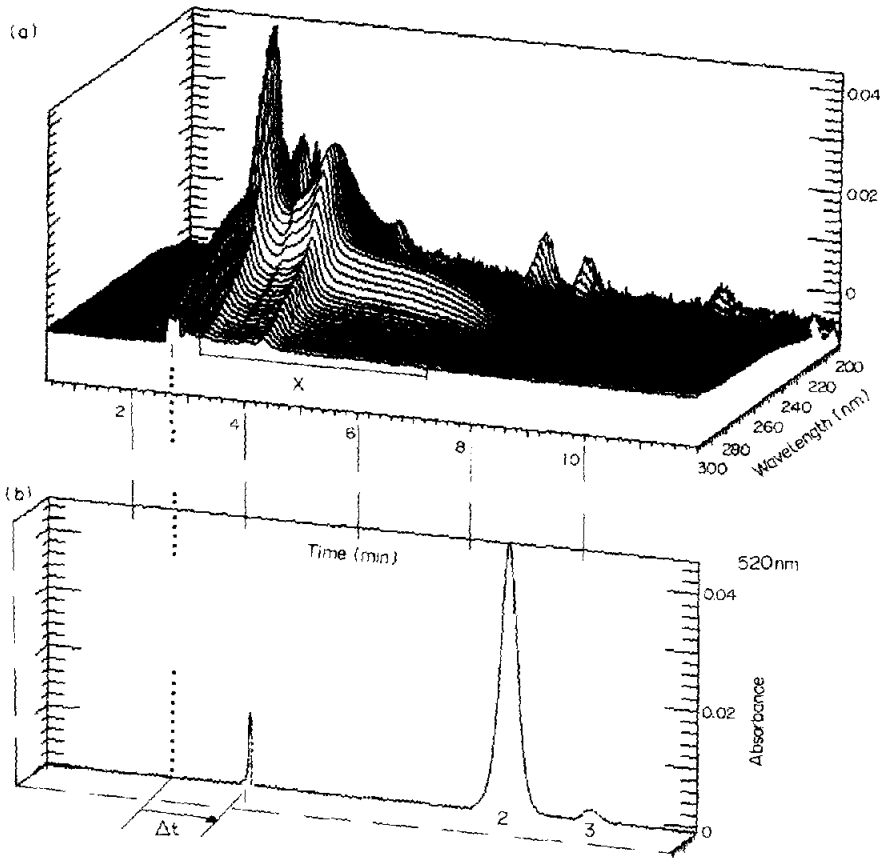


**Figure 2**
Diode array topogram of the salinomycin 6% (w/w) feed grade preparation without post-column reactor. The wavel range was 200–300 nm. (b) Chromatogram of the same sample after connection of the post-column reactor. 1, s peak; X, unidentified UV absorbing components; $\Delta t$, delayed retention equal to the post-column reaction ti salinomycin; 3, unidentified component that reacts with vanillin. Detection: 520 nm.

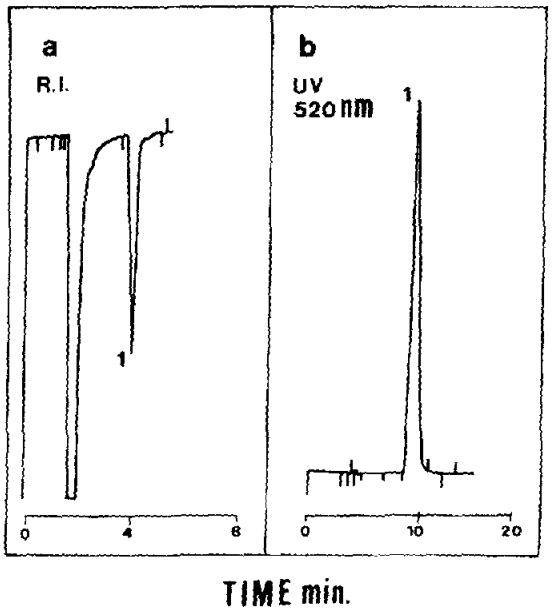


**Figure 3**
Comparison of different detection systems used for salinomycin determination. (a) Direct refractive inde maximal sensitivity range (0.125). Sample of salinomycin standard 600 μg $ml^{-1}$; flow was 2.0 ml $min^{-1}$; ter ambient. (b) Indirect UV detection at 520 nm, after post-column vanillin reaction. Sample of salinomycin st $ml^{-1}$. 1 represents salinomycin.

retention and separation of polyether antibiotics, experiments in which the water content in mobile phase was varied from 6 to 10% (v/v) (Fig. 4). With 10% water in mobile phase, base-line separation of monensin and salinomycin was obtained with some delayed retention. But increase of water content in the mobile phase caused reduced sensitivity (Fig. 4d). For the routine analyses of salinomycin without other polyether antibiotics being present, a mobile phase with 6% (v/v) water was preferred. With these LC conditions the minimum detection quantity (3 × signal-to-noise ratio) of salinomycin is 5–10 ng. The method also expressed satisfactory day-to-day reproducibility. Repeated analysis of the same sample of salinomycin feed grade gave a RSD of 3.49% ($n$ = 6).

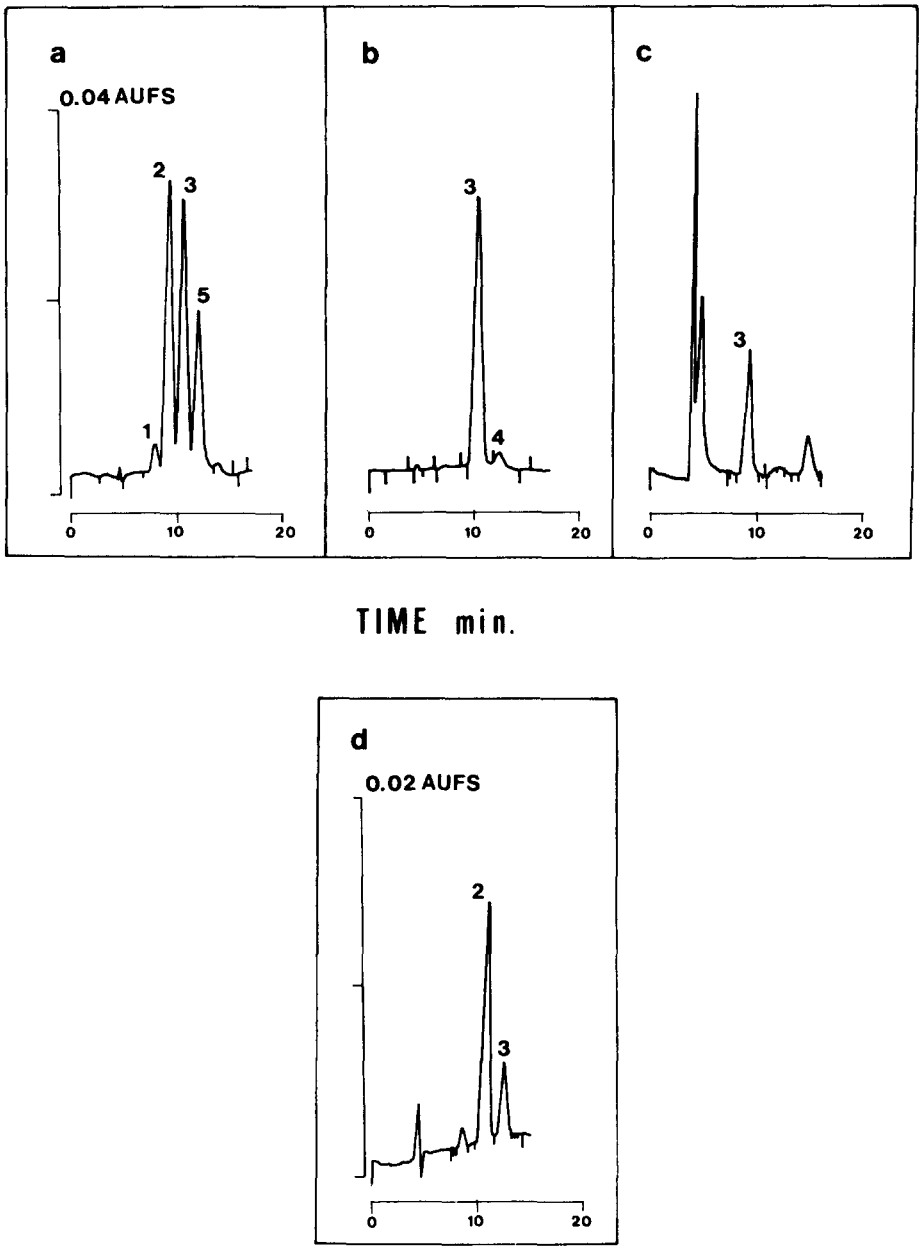


**Figure 4**
Typical chromatograms after vanillin post-column reaction. (a) Standard preparation mixture of monensin, salinomycin and narasin. (b) Salinomycin fermentation broth. (c) Animal feed with declared amount of salinomycin 50 mg $kg^{-1}$. (d) Animal feed with declared amount of monensin and salinomycin 100 and 50 mg $kg^{-1}$, respectively. In (a), (b) and (c) mobile phase contains 6% and in (d) 10% (v/v) of water. 1, Component of monensin; 2, monensin; 3, salinomycin; 4, unidentified component; 5, narasin.

*Analysis of samples*

For the comparison and evaluation of methods, different samples of salinomycin fermentation broth, feed grade preparation and animal feed with added salinomycin were tested (Tables 1 and 2). In the typical chromatograms of salinomycin fermentation broth and feed grade preparations some other vanillin-reacting components also appear, but in low amounts (Figs 2b and 4b). In the non-chromatographic colorimetric determination of salinomycin, these compounds give an absorbance at 520 nm, which may be a reasonable explanation for the higher results of the colorimetric method (Table 1). Nevertheless, regression analysis data showed a significant correlation ($P < 0.001$) between the values obtained with reference LC method (expressed on the $y$ axis) and the colorimetric method ($x$ axis). The regression line (with 95% confidence limits) was: $y = -1.439 + 0.952x$ and the correlation coefficient ($r$) was: 0.983. It is not significantly different from the ideal regression line ($y = x$).

Results of the time-consuming agar diffusion microbiological method also indicate significant correlation with reference HPLC determinations. Regression analysis parameters of the data from Table 1 for HPLC and microbiological method were: $y = 7.693 + 0.922x$, $r = 0.984$ ($P > 0.001$). However, the low sensitivity of the agar diffusion microbiological determination was the main disadvantage of this method. Consequently, determinations of salinomycin in animal feed samples in the usual range of 30–100 mg $kg^{-1}$, are impossible to analyse this kind of technique. On the other hand, medicated chicken feed samples with added 60 mg $kg^{-1}$ salinomycin (Table 2) can be precisely determined by LC. From these results it is also evident that salinomycin express good stability properties in this kind of preparation. After a period of 120 days at ambient temperature salinomycin contents were not significantly different from the initial analyses. In one sample, the content of salinomycin at the initial level was below the lower assay limit for medicated animal feed (±20% of labeled amount) [17]. As in other samples the content of salinomycin did not change significantly after storage.

**Table 1**
Intra-assay variations for the analytical methods applied to salinomycin feed grade determinations

| Sample* | Salinomycin (g $kg^{-1}$) LC | Microbiological | Colorimetric |
|---|---|---|---|
| 1 | 57.5 | 54.8 | 61.7 |
| 2† | 59.4 | 59.6 | 60.1 |
| 3 | 64.9 | 64.0 | 68.1 |
| 4 | 87.3 | 83.4 | 96.4 |
| 5 | 86.4 | 87.9 | 98.0 |
| 6 | 87.0 | 82.0 | 95.0 |
| 7 | 96.0 | 95.0 | 99.0 |
| 8 | 97.9 | 90.2 | 108.0 |
| 9‡ | 102.0 | 107.8 | 110.0 |
| 10† | 124.3 | 127.7 | 124.3 |

*Each result represents average of two separate determinations of the same batch.
†Sample; Hoechst (under trade name Sacox).
‡Sample; Kaken Chem. Co. All other samples were from; Krka Pharmaceuticals.

**Table 2**
LC analyses of chicken feed samples with 60 mg $kg^{-1}$ salinomycin added

| Sample* | Initial | 30 days | 90 days | 120 days |
|---|---|---|---|---|
| | Salinomycin (mg $kg^{-1}$) (% of labelled amount) | | | |
| 1 | 56 (93) | 54 (90 | 53 (88) | 54 (90) |
| 2 | 53 (88) | 50 (83) | 52 (86) | 52 (86) |
| 3 | 61 (102) | 59 (98) | 55 (92) | 53 (88) |
| 4 | 55 (92) | 53 (88) | 53 (88) | 51 (85) |
| 5 | 45 (75)† | 42 (70)† | 40 (67)† | 41 (68)† |

Analytical requirement for content of salinomycin: 60 mg $kg^{-1}$ with assay limits (±20% of labeled amount).
*Each result represents average of two separate determinations of the same sample.
†Below the lower assay limit.

## Conclusions

It can be concluded from the comparison of the three salinomycin methods that LC is preferred for the analysis of salinomycin samples. The method is especially suitable for testing samples of animal feeds with low concentrations of salinomycin and in combination with other polyether antibiotics. For routine determinations in biotechnological processes of salinomycin production, with adequate concentration of analyte, standard calorimetric and microbiological agar diffusion methods may also be useful.

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