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Short Communication

Determination of salinomycin by high-performance liquid chromatography using a precolumn derivatization technique

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Abstract

An improved method for the determination of salinomycin by high-performance liquid chromatography using precolumn derivatization is described. Salinomycin was derivatized with 2,4-dinitrophenylhydrazine in acidic medium and separated on an ODS column using methanol–1.5% aqueous acetic acid (94:6, v/v) as a mobile phase.

1. Introduction

Salinomycin, a polyether antibiotic, is obtained from a stain of *Streptomyces albus* (ATCC 21838). It has a unique tricyclic spiroketal ring system and a unsaturated six-membered ring in the molecule (Fig. 1).

The UV absorption spectrum of salinomycin in methanol shows very poor absorption at 285 nm (ε 108) and in methanolic sodium hydroxide at 285 nm (ε 218), corresponding to a carbonyl group, making it difficult to determine it by a direct spectrophotometric method. However, a non-specific colour development method based on the reaction of salinomycin with vanillin in presence of sulphuric acid to give a pink colour has been reported [1].

Salinomycin has also been determined by microbiological methods using *Bacillus subtilis* in a diffusion method [2] and *Streptococcus faecalis* in a turbidimetric method [3], but these methods are non-specific, less sensitive and time consuming.

As the use of salinomycin is constantly increasing in veterinary formulations, a suitable and conventional chromatographic method is re-

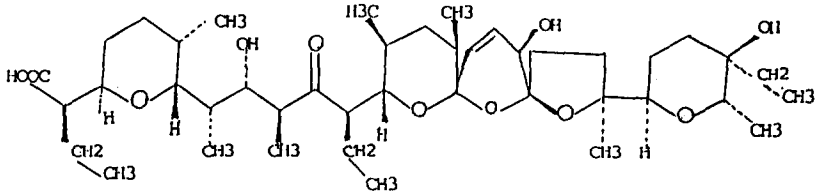

Fig. 1. Structure of salinomycin.

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quired for its precise determination in feed samples. A method based on postcolumn derivatization has been reported [4] but requires extra components such as a pump, postcolumn reactor and heating coil, which is not practicable in conventional chromatography.

The proposed method is based on the precolumn derivatization of salinomycin with 2,4-dinitrophenylhydrazine (DNP) to form a highly UV-active hydrazone derivative. Derivatization was achieved by the method proposed by Siggia [5] and the product was chromatographed directly without work-up.

It is also known that some carbonyl-containing drugs give isomeric 2,4-dinitrophenylhydrazones which are separable by TLC [6] and can also be separated on HPLC using high-resolution columns. The present HPLC method was developed after thoroughly studying the reaction kinetics and other factors affecting the stability of the coloured complex.

2. Experimental

2.1. Chemicals and solvents

Most reagents were of analytical-reagent grade form Merck. Methanol was of HPLC grade form BDH. HPLC-grade water was obtained from an Elgastat UHQ system. Salinomycin sodium reference standard (98%) was obtained from Hoechst. Samples of salinomycin were feed-grade commercial samples containing 6% and 12% of salinomycin.

2.2. Instrumentation

The HPLC system consisted of Gilson Model 302 and 305 pumps, a Gilson Model 116 UV detector operated at 380 nm, a dynamic mixer and a manometric module, all controlled by computer using Gilson 712 software. Samples were injected through a Rheodyne Model 7161 injector fitted with a 20-μl fixed loop. The column used for the separation was Inertsil ODS-2 (150 × 4.6 mm I.D.; 5 μm) from G.L. Sciences. The eluent was methanol–1.5% aqueous acetic acid (94:6, v/v) at a flow-rate of 1.0 ml/min.

2.3. Standard preparation

A solution of about 300 μg/ml of salinomycin was prepared by dissolving about 31 mg of salinomycin sodium reference standard in 100 ml of absolute ethanol. This solution was stable when refrigerated at 4°C and could be used for 2 months.

2.4. Sample preparation

All feed samples were pulverized in a grinder to obtain a homogeneous powder and a suitable amount was mixed with ethanol ultrasonically for 5 min. The solution and filtered through a 0.45-μm filter and derivatized.

2.5. Derivatization reagent

A 60-mg amount of 2,4-dinitrophenylhydrazine was dissolved in 100 ml of methanol.

2.6. Linearity

Different aliquots (1, 2, 3, 4 and 5 ml) from the standard solution were taken in 10-ml volumetric flasks and diluted to 5 ml with ethanol. A 1-ml volume of the reagent was added followed by 1 drop of concentrated hydrochloric acid. All the flasks were kept at 50°C for about 3 min, cooled to room temperature, diluted to 10 ml with absolute ethanol and injected in duplicate. All the concentrations were found to be in the linear range with the linear regression line $y = -0.2240 + 0.04909x$ [where y is area counts and x is the concentration (ppm)] and a correlation coefficient (r) of 0.9996.

3. Results and discussion

3.1. Derivatization mechanism and kinetics

The ideal derivative is one that is formed rapidly under mild conditions, preferable at

lower temperatures with good sensitivity and without significant side-product formation.

With carbonyl-containing drugs, most derivatization reactions proceed with the initial attack of a nucleophilic reagent followed by dehydration of the intermediate adduct. In the presence of acid, the dehydration step becomes fast and the nucleophilic attack controls the reaction rate. The carbonyl carbon bears a partial positive charge, and addition of the nucleophile to the carbonyl group resembles protonation of the nucleophile. Therefore, basicity can be a good measure of nucleophilicity. A reagent with a negative charge is invariably a powerful nucleophile and among neutral compounds, the reactivity decreases in the order N > O > S nu-

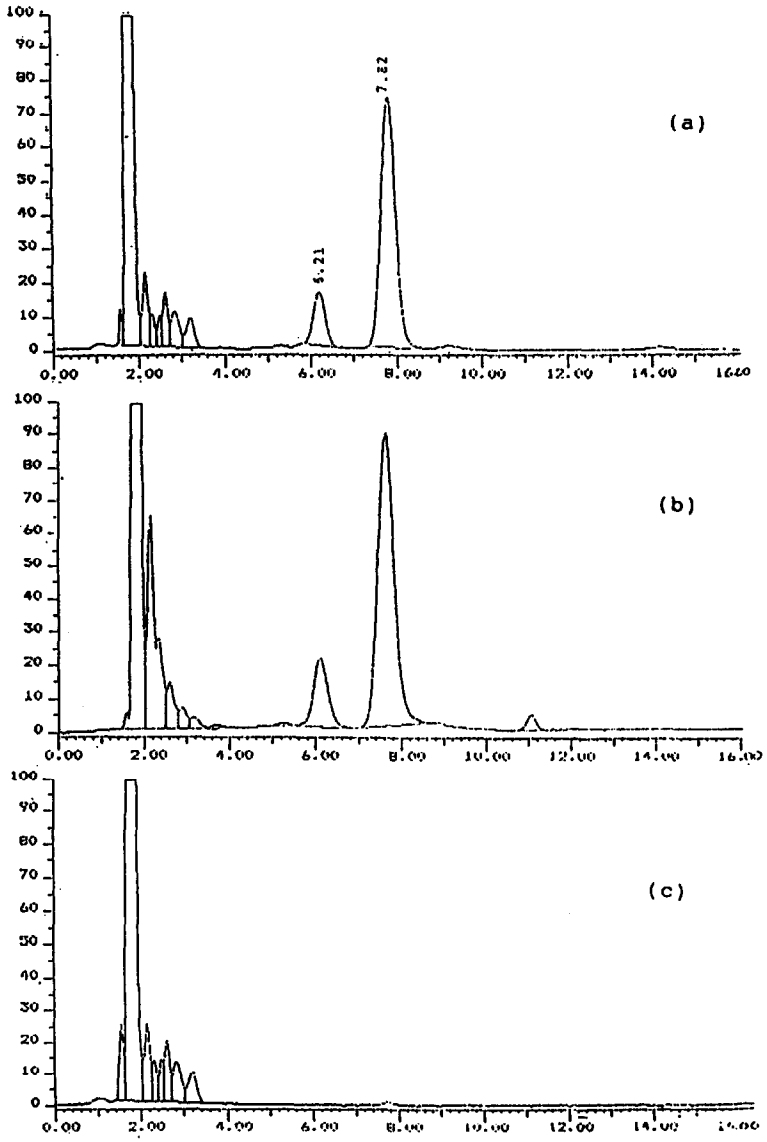

Fig. 2. Typical chromatograms after derivatization. (a) Salinomycin reference standard with unknown peak at 6.2 min and salinomycin at 7.8 min; (b) feed sample containing 12% of salinomycin; (c) blank run with all reagents but without salinomycin. Time scale in min. y-Axes are in $mV \cdot 10^{-1}$.

cleophiles. For this reason, hydrazines are most suited for the derivatization of carbonyl compounds.

To study the kinetics of the reaction, the reagent was allowed to react with salinomycin at 50°C for different intervals of time varying from 1 to 10 min and chromatographed. The area counts obtained with 1- and 10-min reacted solution were almost the same, indicating a spontaneous rection. However, in absence of hydrochloric acid the reaction proceeds very slowly.

3.2. Chromatography

Using the conditions mentioned under Experimental, the peak of salinomycin was well resolved and eluted at about 7.8 min along with an additional unknown peak at 6.2 min, even with the standard (Fig. 2a). A blank reaction mixture without salinomycin was also injected to make sure that the unknown peak was not from any other source (Fig. 2b).

To check the precision of the chromatographic conditions, the same derivatized solution was injected ten times and the relative standard deviation (R.S.D.) was 0.6%.

3.3. Method validation

To check the stability of the derivatized solution at ambient temperature, the same reaction mixture was chromatographed every 30 min for 4 h, and no degradation was observed. Moreover, a sample injected 24 h after derivatization showed only 1% degradation.

The sensitivity of the method is fairly high. At 380 nm, the limit of quantification was about 25–50 ng of salinomycin. For experimental purposes all the measurements were made at 380 nm as this was the maximum value that could be selected on the instrument used, but the derivatized complex showed maximum absorbance at about 419 nm with an increased sensitivity of about 11-fold (Fig. 3). Therefore, selecting 419 nm as the wavelength, a 2.5–5.0-ng amount of salinomycin can easily be detected under these conditions.

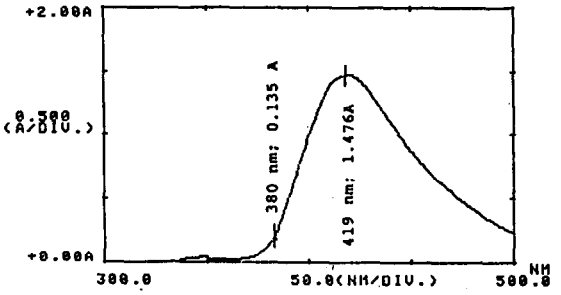

Fig. 3. UV absorption spectra of derivatized salinomycin against blank showing the difference jn, absorbance values at 380 and 419 nm (path length = 1 cm; $c = 90$ μg/ml).

For evaluation of the method, different commercial samples containing 6% and 12% of salinomycin were tested (Table 1). The samples were analysed using the external standard method. The repeatability of these samples was well within limits with R.S.D. = 0.20–2.7%.

4. Conclusions

The simplicity of derivatization and the strong absorption of these derivatives indicate that 2,4-dinitrophenyhydrazine should be broadly applicable as a derivatization agent in the HPLC of carbonyl-containing drugs. The method is suitable for testing salinomycin in all kind of samples even at low concentrations and free from other UV-active interferences.

Table 1
Determination of salinomycin in commercial feed samples

Sample	Salinomycin content (%, w/w)		R.S.D. (%)
	Claimed	Found	
1[a]	6	6.15	0.46 ($n = 2$)
2[a]	12	11.55	2.7 ($n = 3$)
3[a]	12	12.11	1.8 ($n = 3$)
4[a]	12	11.98	0.20 ($n = 3$)
5[b]	6	6.21	1.27 ($n = 4$)
6[b]	6	6.06	2.3 ($n = 3$)
7[b]	6	5.71	2.2 ($n = 3$)

[a] Hoechst.
[b] Kaken Pharmaceutical.

5. Acknowledgements

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6. References

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