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HPLC analysis of salinomycin in human plasma using pre-column oxidation and automated heart cut column switching*

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Abstract: Salinomycin is a polyether antibiotic used to promote growth in cattle and poultry. Workers may be exposed to salinomycin through handling of animal feeds that contain the drug and it is necessary to monitor plasma samples from these workers for salinomycin to ensure safety. A method for analysis of salinomycin in plasma samples was therefore developed. Salinomycin and the internal standard narasin are extracted into iso-octane then subjected to silica gel solid-phase extraction in which the sample is washed with methylene chloride–methanol (98.5:15) then eluted with a 90:10 proportion of the same mixture. Both salinomycin and narasin are oxidized with pyridinium dichromate to form a chromophore absorbing at 225 nm. The concentrated product was injected onto a C_{18} pre-column and heart cut from 1.85 to 3.65 min onto a C_{18} analytical column. The method was shown to be selective for salinomycin and narasin in six blank plasma samples. The method was linear over a range of 15–300 ng ml^{-1} with a detection limit of approximately 5 ng ml^{-1}. The mean absolute recovery was found to be 93.4 and 97.9% for salinomycin and narasin, respectively. The method was accurate to within 5% at all concentrations studied. Within-run and between-run precision were both less than 8% RSD at all concentrations studied and the method was suitable for the purpose of monitoring plasma from exposed agricultural workers.

Keywords: *Salinomycin; column switching; HPLC; narasin.*

Introduction

Salinomycin is a polyether antibiotic used as a grain animal feed additive for its anticocidial activity and its potential to improve growth in cattle and poultry [1]. The chemical structures of salinomycin and the internal standard and their conversion to the chromophore are shown in Fig. 1. Because of the presence of the drug in animal feed (40–60 g ton^{-1}) agricultural workers who handle the feed are subject to exposure and could potentially attain systemic levels of the drug that are measurable in plasma. The present method was developed in order to monitor potential concentrations of salinomycin in the plasma of these workers.

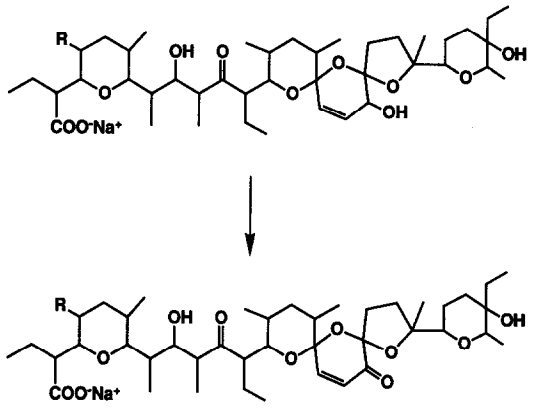

Figure 1
Structure and chemical conversion of the allylic hydroxyl group on sodium salinomycin (R = H) and sodium narasin (R = CH_3) to the ketone chromophore.

Salinomycin has been quantitated in animal tissues by bioautographic thin-layer chromatography [2, 3]; in animal feeds by HPLC with post-column derivatization [4]; and in chicken fat [5] by HPLC utilizing column switching. Column switching HPLC and thin-layer chromatography have been compared for analysis of salinomycin in chicken fat and liver and has been shown to yield similar results [6].

Salinomycin does not possess a useful chromophore or luminophore and is not

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electroactive enough for low level analysis by HPLC. In addition, interferences from the plasma matrix were expected in the short wavelength region of the spectrum which was the region of interest planned for the method. The method of Dimenna *et al.*, was used as a model for development of the plasma assay because it combined the detectability of a strong UV-absorbing ketone oxidation product of salinomycin with the selectivity attainable by heart cut column switching.

Materials and Methods

Extraction and pre-column oxidation

Standards and controls were prepared by spiking plasma with salinomycin (Hoechst Aktiengesellschaft, D-6230 Frankfurt, Germany) in methanol (5 μg ml^{-1}) at concentrations of 300, 200, 100, 60, 30, 15 and 0 ng ml^{-1} and 250, 75 and 25 ng ml^{-1} for standards and controls, respectively. One millilitre of spiked plasma was added to 100 μl of a 2.0 μg ml^{-1} narasin solution in methanol (Eli Lilly and Co., Indianapolis, IN, USA) in a 20 × 150 nm screw-capped tube. Twenty millilitres of iso-octane was then added and the tubes were rotated for 15 min at 50 rpm. The iso-octane extracts were transferred to 75 ml reservoirs coupled to 0.5 g Bond-Elut™ Silica extraction columns (Analytchem International, Harbor City, CA, USA). The extracts were drawn through the cartridge by vacuum and the columns were washed with 10 ml of methylene chloride–methanol (98.5:1.5). The analyte and internal standard were then eluted from the columns with 6 ml of methylene chloride–methanol (90:10) into 16 × 100 mm Teflon screw-capped tubes. The eluents were evaporated to dryness under dry nitrogen at 65°C for approximately 8 min. Residues were reconstituted with 2 ml of methylene chloride and 5 mg of pyridinium dichromate synthesized as described previously [7], was added. Tubes were then shaken on an Eberback Shaker for 15 min followed by washing with 2 ml of 5% sodium bicarbonate to remove excess reagent. The tubes were vortexed and centrifuged for 2 min at approximately 3000 rpm. Washing and centrifugation steps were repeated twice with 2 ml of 5% sodium bicarbonate and once with water. The remaining washed methylene chloride layer was evaporated to dryness under nitrogen at 65°C for approximately 12 min. The residues were reconstituted in 120 μl of acetonitrile and 100 μl was injected. Unless otherwise indicated, all chemicals were reagent grade from Aldrich and solvents were HPLC grade from Burdick and Jackson.

HPLC system

The chromatographic system equipped for heart cut column switching, consisted of a Hewlett–Packard Model 1050 Gradient Pump (Hewlett–Packard, Avondale, PA, USA) used for pre-column mobile phases, a Gilson Model 302 Pump (Gilson Electronics, Middleton, WI, USA) for the analytical column mobile phase; a Gilson model 231 autosampler; a model 1050 UV absorbance detector (Hewlett–Packard) set at 225 nm, 0.02 a.u.f.s. and 1 s response time; and an Autochrome model 401 6-port switching valve equipped with a model 201 solenoid interface. Chromatographic data was acquired and analysed using the Maxima 820 Workstation (Waters Division of Millipore Corp., Milford, MA, USA). Valve switching events were controlled by contact closures on the autosampler.

Samples (100 μl) were injected onto the pre-column (Waters Nova-Pak, C_{18}, 7.5 cm × 3.9 mm i.d., 4 μ) with a mobile phase containing 0.1% H_3PO_4 (10%) and acetonitrile (90%) flowing at 2.0 ml min^{-1}. Valve switching times were established at the beginning of each analytical run with diversion of the pre-column eluate onto the analytical column 0.25 min before elution of a pure standard of oxidized salinomycin and switched back to waste 0.25 min after elution of a pure standard of oxidized internal standard. These switching events occurred approximately 1.85 and 3.65 min following injection. Following the valve switching events, the pre-column was purged with a mobile phase of 15% tetrahydrofuran and 85% acetonitrile from 5 to 12 min following injection. The pre-column mobile phase was returned to the original composition from 12.1 to 15 min following injection. The mobile phase for the analytical column (Beckman Ultrasphere ODS C_{18}; 25 cm × 4.6 mm i.d. 5 μ at 2.0 ml min^{-1}), consisted of 4% double distilled water and 96% acetonitrile with 100 μl of concentrated phosphoric acid added per litre of mobile phase. The analytical column was protected by a dry packed guard column (Waters BondaPak 37–50 μ).

Quantitation

The output of the detector was transferred through interface to the chromatography workstation. Digitized peaks were integrated, and a weighted (1/concentration) least squares regression equation was generated by comparing the known salinomycin concentrations of standards with the ratio of salinomycin peak areas

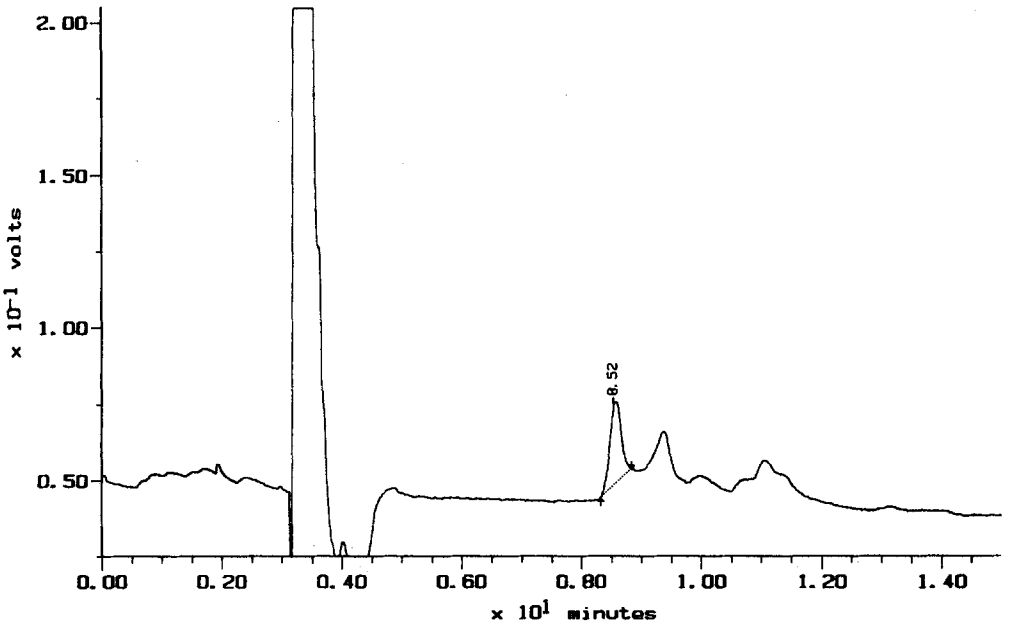

Figure 2
Chromatogram of blank plasma.

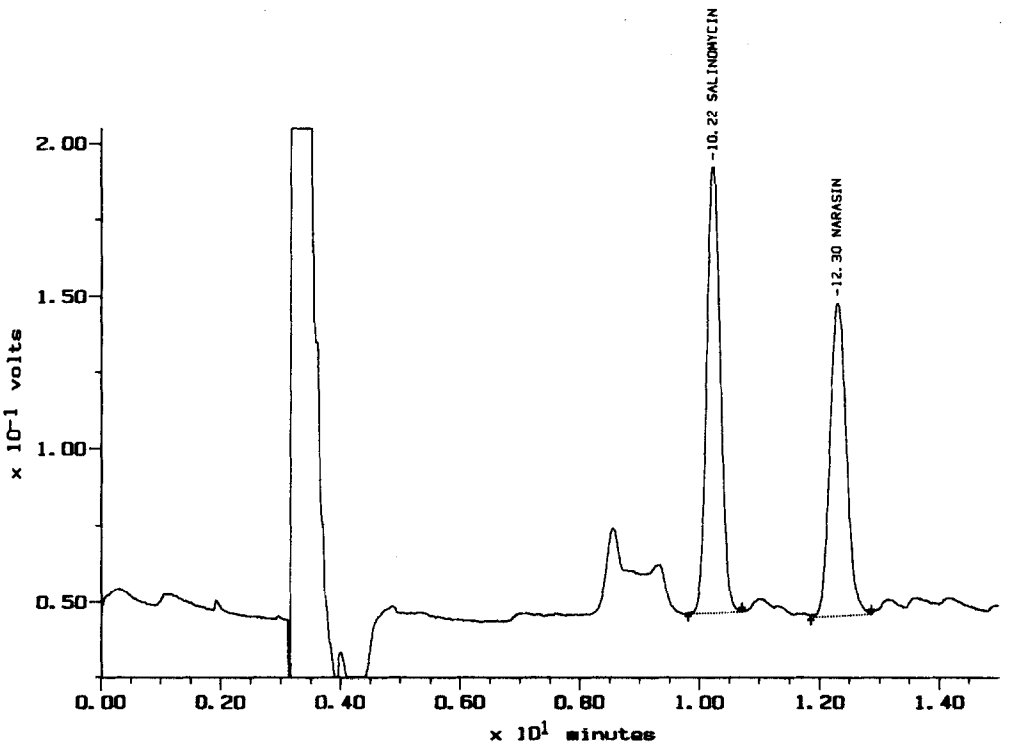

Figure 3
Chromatogram of blank plasma spiked with 300 ng ml^{-1} of salinomycin.

to the internal standard. Unknown and control concentrations were reverse predicted using the regression equation slope and intercept.

Results and Discussion

Selectivity and detectability

The method was evaluated for selectivity by analysis of six human blank plasma samples. None of the six samples yielded peaks that would interfere with quantitation of either oxidized salinomycin or narasin at their respective elution times of 10.2 and 12.3 min. An example chromatogram from an extracted blank sample and a sample spiked with 300 ng ml^{-1} of salinomycin are shown in Figs 2 and 3, respectively. The limit of quantitation was based on a signal to noise ratio of approximately 5 in order to provide precise quantitation. This level was set at 15 ng ml^{-1}, the concentration of the lowest standard. A chromatogram spiked at the limit of quantitation is shown in Fig. 4. The precision of reverse predicted concentrations of the lowest standard was determined to be 4.8% relative standard deviation (n = 12) showing excellent precision at the established quantitation limit.

Recovery and calibration

Absolute recovery of the extraction procedure was measured by comparing peak heights from plasma spiked with 20, 75 and 250 ng ml^{-1} of salinomycin and 100 μg ml^{-1} of narasin to that of unextracted pure standard injected directly following the oxidation procedure. The mean absolute recoveries measured in this way were 81.7, 105.2, 93.1 and 97.9% for salinomycin spiked at 20, 75, 250 ng ml^{-1} and narasin spiked at 100 ng ml^{-1}, respectively (n = 6). The calibration curve was found to be linear throughout the range of 15–300 ng ml^{-1} which was established as the range of interest. The mean slope, intercept and correlation coefficient of six calibrations was 199.4 ± 5.6; −1.4 ± 0.6 and 0.9993 ± 0.0004, respectively. Mean reverse predicted concentrations from six calibration curves measured at 15, 30, 60, 100, 200 and 300 ng ml^{-1} were 4.7, 2.3, 0.8, 3.2, 0.6 and 1.8% different from their spiked concentrations, again indicating linearity of the calibration curve. A plot of log concentration versus log weighted response for mean calibration standard data (n = 6) demonstrated a slope of 1.01 which again validates the appropriateness of the weighted linear model [8].

Accuracy and precision

Accuracy of the method was evaluated by calculation of the per cent difference of assayed values from spiked control concentrations (%DFA). The controls were evaluated on six different runs and the %DFAs were found to be 4.5, 1.6 and 3.9% for the 20, 75

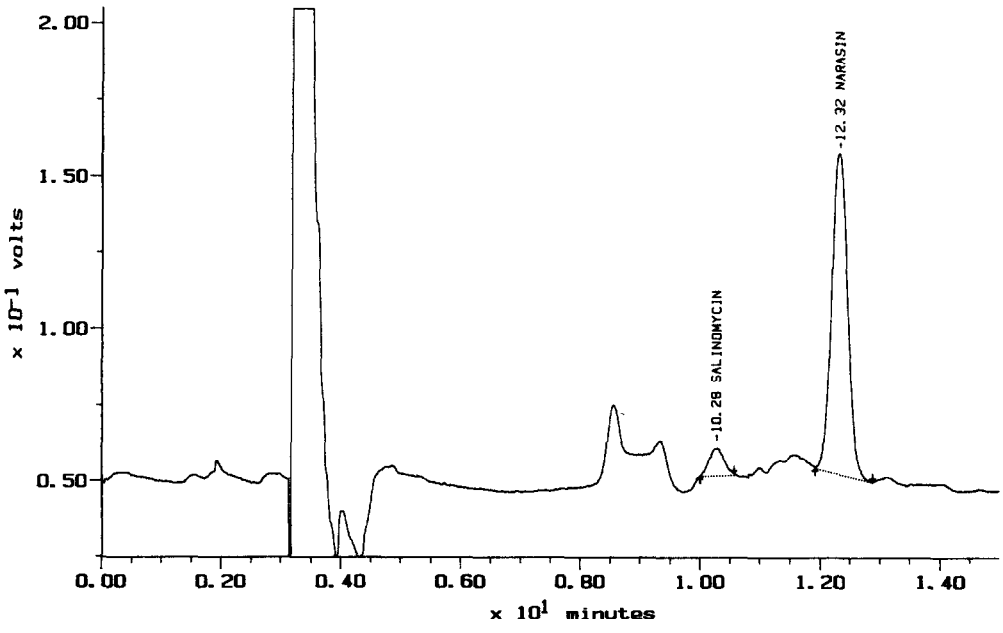

Figure 4
Chromatogram of blank plasma spiked with 15 ng ml^{-1} of salinomycin.

Table 1
Precision of the salinomycin method (n = 6)

	Spiked conc. (ng ml^{-1})	Mean assayed conc. ± SD (ng ml^{-1})	RSD (%)
Within-run	20	17.9 ± 0.4	2.2
	75	75.7 ± 1.1	1.5
	250	247.8 ± 3.3	1.3
Between-run	20	19.1 ± 1.4	7.2
	75	76.2 ± 2.1	2.7
	250	240.3 ± 8.3	3.5

and 250 ng ml^{-1} controls, respectively. Additionally, 10 samples with concentrations that were blinded to the analyst were assayed and found to provide a mean %DFA of 2.5%. The largest single %DFA was 12.3% measured at 25 ng ml^{-1}. Precision of the method was evaluated on both a within-run and between-run basis to evaluate both 'ideal' precision and expected real world precision, respectively [9]. The results of this analysis are shown in Table 1 and demonstrate excellent precision of the method, with all relative standard deviations (%RSD) less than 10%.

Conclusions and sample analysis

The described method has been shown to be sufficiently accurate and precise and conforms to suitability criteria established for the discipline at the Joint Conference on Analytical Methods Validations [10]. The method utilizes an oxidation step to enhance detectability to the low ng ml^{-1} range and column switching to provide the necessary selectivity while maintaining a 13 min chromatographic analysis time. The method was used to evaluate duplicate samples taken from 13 agricultural workers who work with salinomycin doped feed. It was found that none of the 13 workers demonstrated salinomycin levels above the limit of quantitation which was the desired result.

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