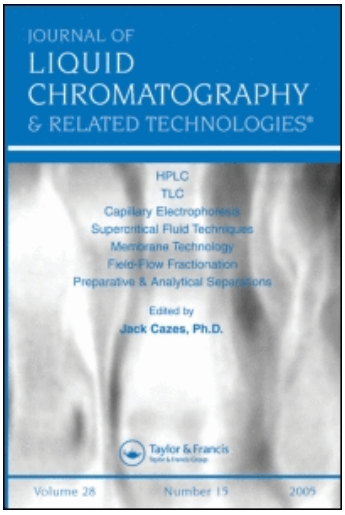

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Microbore hplc determination of polyether antibiotics using postcolumn derivatization with Benzaldehyde reagents

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To cite this Article Fejglová, Z. , Doležal, J. , Hrdlička, A. and Frgalová, K.(1994) 'Microbore hplc determination of polyether antibiotics using postcolumn derivatization with Benzaldehyde reagents', Journal of Liquid Chromatography & Related Technologies, 17: 2, 359 — 372
To link to this Article: DOI: 10.1080/10826079408013357
URL: http://dx.doi.org/10.1080/10826079408013357

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(2), 359-372 (1994)

MICROBORE HPLC DETERMINATION OF POLYETHER ANTIBIOTICS USING POSTCOLUMN DERIVATIZATION WITH BENZALDEHYDE REAGENTS

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ABSTRACT

Monensin, narasin, and salinomycin have been separated by HPLC on microbore column packed with octadecyl-bonded silica gel and detected via postcolumn color reaction with derivative of benzaldehyde in packed-bed reactor. Various benzaldehyde reagents have been tested in batch and 4-dimethylaminobenzaldehyde (DMABA) and vanillin selected as most promising ones. Requirements and characteristics of postcolumn reaction with the two reagents have been investigated and optimized and DMABA has been shown as superior over currently used vanillin reagent. The applicability of postcolumn derivatization with DMABA has been demonstrated by monensin assay in premixes and feeds.

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INTRODUCTION

Monensin, narasin, and salinomycin are fermentation-derived polyether monocarboxylic antibiotics used mainly as feed additives in the prevention of coccidiosis in poultry and as feed efficiency enhancers in cattle. The antibiotic monensin is a mixture consisting of two or more components, i.e. monensin Factor A as the main derivative and monensin Factors B and, if any, C and D.

Typical matrices for the analytical determination of the three antibiotics are fermentation broths, premixes, feeds, and environmental samples. The antibiotics are routinely analyzed by bioassay methods which lack speed and specificity.

Golab et al. (1) introduced a photometric method based upon the reaction of monensin with vanillin in sulfuric acid solution. The method uses Komarowski reaction (2) which is known as a color reaction of higher alcohols with aromatic aldehydes. The color produced is due to aldol condensation products. The reaction of a secondary alcohol (or a similar compound) with benzaldehyde which proceeds in strong acid media of sulfuric acid can be schematically described by the two consecutive steps (3)

$$C_6H_5CHO + R_2CHOH \rightleftarrows C_6H_5CH_2OH + R_2C=O \qquad (A)$$

$$R_2C=O + C_6H_5CHO \rightleftarrows \text{condensation products} \qquad (B)$$

Later on, the reaction with vanillin has been applied to postcolumn derivatization of salinomycin after normal phase HPLC separation (4) and to that of the three antibiotics separated by reversed phase HPLC method (5). Reaction detection with vanillin has become the most used detection means in HPLC of monensin,

narasin, and salinomycin. Recently, high speed liquid chromatographic determination of these antibiotics in feeds using postcolumn reaction with vanillin (6) has been described and the correlation between HPLC determination of monensin and its microbiological assay has been investigated (7).

In this work monensin, narasin, and salinomycin have been separated on a microbore column and derivatized in packed-bed reactor instead in generally used (5-8), up to 9 m long, reaction capillary. Applicability of various benzaldehyde reagents to derivatization reaction has been evaluated and parameters of postcolumn derivatization have been studied.

EXPERIMENTAL

Reagents and Solutions

Stock solutions (0.1 mg/ml) of monensin (960 μg/mg purity), narasin (934 μg/mg; both from Lilly Research Center, USA), and salinomycin (960 μg/mg; Höchst, Germany) were prepared in methanol from their sodium salts and stored under refrigeration.

Vanillin, (4-hydroxy-3-methoxybenzaldehyde, anal. grade, Loba Chemie, Germany), salicylaldehyde (2-hydroxybenzaldehyde, pure, Reachim, USSR), 4-nitrobenzaldehyde, 4-dimethylamino-benzaldehyde (both pure), 3,5-dibrom-4-hydroxybenzaldehyde, and 3,4-dimethoxybenzaldehyde (both recrystallized, all from Lachema, Czechoslovakia) were employed as derivatization agents.

Distilled methanol and water redistilled in a quartz still were used. Other reagents were of analytical grade purity (Lachema, Czechoslovakia).

Mobile phase was prepared as described previously (7, 8) and consisted of methanol, water and glacial acetic acid at a ratio of 940:59:1.

To prepare a derivatization reagent solution, concentrated sulfuric acid was slowly mixed with methanol under stirring and, after cooling to room temperature, weighted amount of benzaldehyde reagent was added. The solution was freshly prepared daily.

In batch experiments, methanolic solutions contained 5 μg/ml (ca. 7 μmol/l) monensin, benzaldehyde reagent at concentrations from 15 to 20 mmol/l, and 20 mmol/l sulfuric acid. These solutions were heated for one minute in a water bath at 60 °C, cooled to room temperature and their absorbance was measured at the wavelengths of absorption maxima.

Solid samples of premixes (DBBR2; 0.5 g) and medicated feeds (KSBR2; 5 g; all from Institute for State Control of Veterinary Biopreparates and Medicaments, Brno, Czech Republic) were extracted with 15 ml of methanol by mixing 2 h on a shaker and filtered. Extracts of feed additives were transferred into 50-ml volume flasks and diluted with methanol. Feed extracts were evaporated to the volume of approx. 3 ml and diluted in a volume flask with methanol to 10 ml. Volumes of 3 μl of final solutions were injected onto chromatographic column and assayed for monensin using DMABA as derivatization reagent.

Apparatus

Two syringe pumps, model MHPP 20, were used to deliver the mobile phase and reagent solution. Samples were injected using a model LCI 30, six-port injection valve with 3-μl sample loop (all Laboratory Instruments Prague, Czechoslovakia), onto a CGC glass column 150x1 mm i.d. packed with Separon SGX C18 (5 μm) silica gel with octadecyl groups (Tessek Prague, Czechoslovakia). Separations were performed at an ambient temperature.

Effluent and reagent flow were joined in a low dead volume T-piece (Swagelok, USA) and mixed in a home-made, glass, 150x1 mm i.d., packed-bed reactor filled with acid-washed glass beads, 40-70 μm in diameter. The reactor was placed in a thermostated water-bath jacket. A model HP 1050 multiple wavelength detector, equipped with a 1-μl cell, 5 mm in path length (Hewlett-Packard, Japan), was used for detection and data was evaluated by using a model CI 100 integrator and a TZ 4620 recorder (Laboratory Instruments Prague, Czechoslovakia).

Spectrophotometric investigations were done on a model Specord M 40 spectrophotometer (Carl Zeiss Jena, Germany), however, absorption curves of derivatization products were measured on a chromatographic detector introduced above.

Acidity was measured with a model OP-208 pH-meter equipped with an OP-0808-P glass-Ag/AgCl combined electrode using phthalate and phosphate standard buffers (all Radelkis, Hungary).

RESULTS AND DISCUSSION

Batch Experiments

In preliminary experiments, absorption curves of products of derivatization reactions with vanillin, 4-dimethylaminobenzaldehyde (DMABA), 3,4-dimethoxybenzaldehyde (DMOBA), and salicylaldehyde (SA) were measured to determine their absorption maxima listed in Table 1. Reagents with electron-accepting substituents (i.e. 4-nitrobenzaldehyde and 3,5-dibrom-4-hydroxybenzaldehyde) did not react with the antibiotics.

Absorbance of reaction products was rapidly increasing with a reaction time up to ca. 40 s while practically constant absorbance values were observed after 1-min or longer reaction. This was not valid for DMABA, where absorbance values were increasing during a period of 2 min. Absorbance was also increasing with benzaldehyde reagent and sulfuric acid concentrations and becomes independent of them if these were higher than 20 mmol/l. Different behavior was observed by the use of DMABA, where absorbance reached a maximum values at 15 mmol/l DMABA, and then was sharply decreasing up to 40 mmol/l, and at higher DMABA concentrations remained at low levels.

Calibration curves of individual antibiotics measured at conditions used in previous batch experiments were linear in the range from 1 to 15 μg/ml. Values of y-intercept were low, ranging from -34 to 13 mAU. The slope values of calibration curves were decreasing from DMABA, vanillin, and DMOBA to salicylaldehyde and, thus, DMABA was shown as a reagent more sensitive than

TABLE 1

Conditional Molar Absorption Coefficients Determined from Calibration Curves at Wavelengths of Absorption Maxima.

Concentration of antibiotics 1-15 μg/ml, 15 mmol/l vanillin, 17 mmol/l DMABA, 16 mmol/l DMOBA, and 20 mmol/l salicylaldehyde (SA); 20 mmol/l sulfuric acid. Reaction time 1 min at temperature of 60 °C.

	Vanillin	DMABA	DMOBA	SA
Monensin [a]	3.77 (520)	5.39 (585)	3.28 (511)	2.15 (480)
Narasin	3.72 (525)	4.08 (598)	3.31 (512)	1.61 (498)
Salinomycin	3.85 (521)	5.12 (597)	3.35 (513)	1.93 (502)

Values in 10^{-4} l mol^{-1} cm^{-1} (nm). [a] Based on molecular weight of monensin Factor A.

vanillin in spite of proceeding the reaction for only 1 min. The values of conditional molar absorption coefficients determined from the slopes are introduced in Table 1. For postcolumn derivatization, DMABA and vanillin were chosen as the most promising reagents.

Postcolumn Derivatization

The effect of mobile phase and derivatization reagent flow rates and, thus, also the effect of column efficiency and that of reaction time on detector response were studied using a reagent solutions containing 0.34 mol/l DMABA or 0.33 mol/l vanillin and

0.75 mol/l sulfuric acid. The derivatization reaction was performed at 75 °C. Injected samples contained 0.1 mg/ml of each antibiotic. Products of derivatization were monitored at 592 (DMABA) or 520 nm (vanillin). The total flow rate was varied from 15 to 75 μl/min at the ratio of mobile phase/reagent flow rate equal to 2:1. At higher total flow rates, corresponding to a hold-up time between column outlet and detector from 50 to ca. 85 s, peak heights were increasing linearly and a sharper increase was observed for DMABA than for vanillin reagent. Further, the increase of peak heights was lower and, finally, at hold-up time from ca. 2 to 3 min, the peak heights of individual antibiotics practically did not change. As an optimum, a reaction time of 110 s, i.e. a total flow rate of 35 μl/min was chosen. A total dead volume in the T-piece, packed-bed reactor, and connecting tubing was calculated as 64 μl.

At the total flow rate of 35 μl/min the ratio of mobile phase/reagent flow rate was varied from 4:1 to 1:4. The best detector response was found at flow rates of 20 and 15 μl/min of mobile phase and reagent solution, respectively.

The increase of peak heights of individual antibiotics with increasing reaction temperature is demonstrated in Fig. 1. Formation of bubbles in a reactor was observed at temperatures higher than 80 °C. A temperature of 75 °C was chosen as optimum for both derivatization reactions.

The effect of DMABA and vanillin concentrations on the detection of antibiotics is shown in Fig. 2. In the concentration range studied, the peak heights were increasing linearly with vanillin concentration while by DMABA application practically

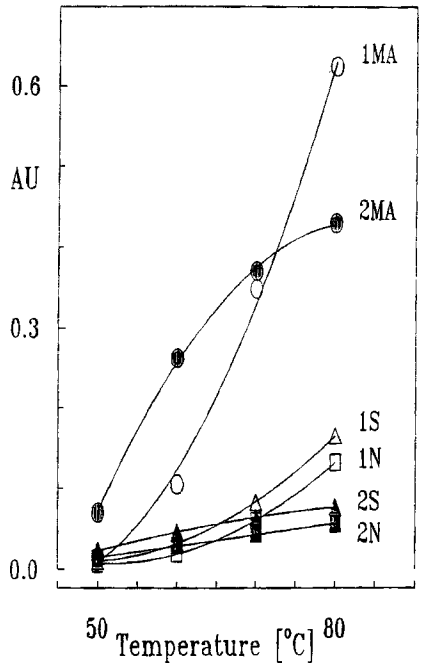

FIGURE 1 Peak heights vs. reactor temperature. Acidity of postcolumn reagent, 0.75 mol/l H_2SO_4; reaction time, 110 s; injected sample, 0.1 mg/ml each antibiotic; detected at 592 or 520 nm after derivatization with DMABA (open symbols) or vanillin (closed symbols). Curves: 1MA and 2MA - monensin Factor A (0.5 mol/l DMABA and 0.66 mol/l vanillin in the reagent solution), 1S and 2S - salinomycin, 1N and 2N - narasin (0.24 mol/l DMABA or vanillin).

constant values were obtained at c(DMABA)>0.6 mol/l. If the concentration of benzaldehyde reagent was higher than 0.7 mol/l, the baseline was noisier and, therefore, concentrations of 0.5 mol/l DMABA and 0.66 mol/l vanillin were chosen as a good compromise between signal and noise level. Concentration of sulfuric acid in reagent solutions was varied from 0.15 to 2.0 mol/l. Peak heights

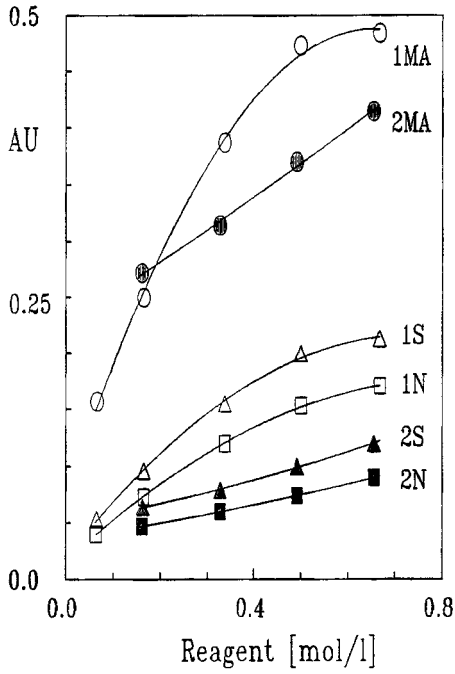

FIGURE 2 Peak heights vs. derivatization reagent concentration. Reactor temperature 75 °C, other conditions given at Fig. 1. Open symbols: derivatization with DMABA - curves 1MA (monensin Factor A), 1N (narasin), and 1S (salinomycin); closed symbols: with vanillin - curves 2MA, 2N, and 2S.

increased with increasing acidity, however, at $c(H_2SO_4) > 1.1$ mol/l the increase was already low and, simultaneously, higher blank absorbance of vanillin reagent was observed. Sulfuric acid concentration of 1.2 mol/l was taken as optimum in both reagent solutions.

Calibration curves, peak height [AU] vs. antibiotic concentration [μg/ml], measured under optimized conditions were linear up to 1 mg/ml. Some parameters of calibration curves are

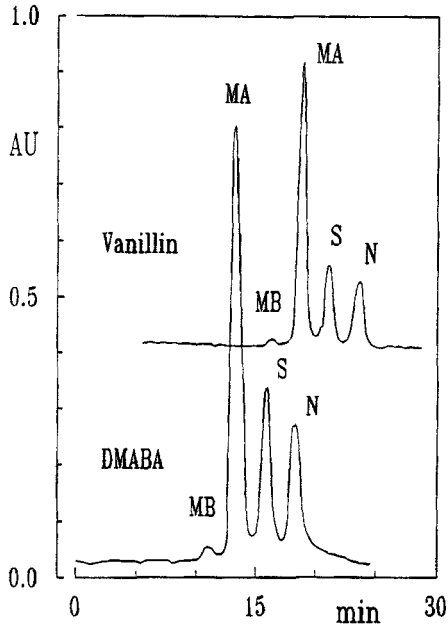

FIGURE 3 Chromatograms of monensin Factor A (MA), monensin Factor B (MB), narasin (N), and salinomycin (S) detected via postcolumn reaction with DMABA or vanillin. Injected sample, 0.1 mg/ml each antibiotic (monensin A and B as total monensin). Other conditions as in Table 2.

introduced in Table 2. The chromatograms presented in Figure 3 illustrate the separation of monensin, narasin, and salinomycin onto microbore column and differences in their detection via postcolumn reaction with DMABA or with vanillin. The detection method based on the reaction with DMABA was evaluated for the determination of monensin in premixes and medicated feeds. Recoveries which are listed in Table 3 ranged from 88.4% to 102.6% across the different sample types and lower values were found for feed samples.

TABLE 2

Slope Values of Calibration Curves and Sensitivities for Individual Antibiotics.

Eluent, 94% v/v methanol containing 0.1% v/v acetic acid, flow 20 μl/min; postcolumn reagent, methanolic solution of 0.50 mol/l DMABA or 0.66 mol/l vanillin, 0.75 mol/l sulfuric acid, flow 15 μl/min; derivatization, 110 s at 75 °C; sample, 3 μl, 20-100 μg/ml of each antibiotic; detection, 592 (DMABA) or 520 nm (vanillin).

Antibiotic	DMABA		Vanillin	
	ϵ [a]	S [b]	ϵ [a]	S [b]
Monensin A	7 780	1.7	5 190	2.0
Narasin	2 700	2.3	1 320	3.9
Salinomycin	3 610	2.2	1 600	3.3

Values of y-intercepts were between -1.9 and 9.8 mAU (DMABA) or -0.1 and 15.2 mAU (vanillin). [a] Slope values as conditional molar absorption coefficients [l mol^{-1} cm^{-1}]; [b] Sensitivities at a signal-to-noise ratio of 2 [μg/ml].

TABLE 3

Determination of Monensin A by Microbore HPLC Using Derivatization with DMABA.

Sample [a]	Monensin A [μg/g]	Recovery [d] [%]
1 [b]	70.3 ± 4.6	93.7
2 [b]	49.5 ± 3.7	88.4
3 [b]	9 063 ± 181	102.6
4 [c]	10 061 ± 408	100.6

[a] Feeds (1, 4) and premixes (5, 6); [b] 3 weighted amounts, 2 injections; [c] 3 weighted amounts, 5 injections; [d] Based on bioassay results. Other conditions as in Table 2.

Higher concentrations of sulfuric acid and benzaldehyde reagent, longer reaction time and, on the other hand, lower reaction temperature than recommended previously (6-8) have been employed in detection methods described. However, by using the microcolumn HPLC system, reagent consumption and, thus, analysis costs have been substantially lower. Linear working range has been found to be one order of magnitude broader than reported earlier (6). Applying DMABA to derivatization, higher sensitivities, particularly for narasin and salinomycin, than with vanillin, which has been widely used to this purpose, have been attained. Postcolumn reaction with DMABA offers a viable method for efficient and sensitive detection of monensin, narasin, and salinomycin.

REFERENCES

1. T. Golab, S. J. Burton, R. E. Scroggs, J. Assoc. Off. Anal. Chem., 56: 171-173 (1973).

2. A. Komarowski, Chem. Ztg. 27: 807, 1806 (1903).

3. F. R. Duke, Anal. Chem. 19: 661 (1947).

4. J. T. Goras, W. R. LaCourse, J. Assoc. Off. Anal. Chem., 67: 701-706 (1984).

5. W. J. Blanchflower, D. A. Rice, J. T. G. Hamilton, Analyst, 110: 1283-1287 (1985).

6. M. R. LaPointe, H. Cohen, J. Assoc. Off. Anal. Chem., 71: 480-484 (1988).

7. J.M. Rodewald, J. W. Moran, A. L. Donoho, M. R. Coleman, J. Assoc. Off. Anal. Chem., 75: 272-279 (1992).

8. J. M. Rodewald, D. S. Skinner, R. H. Edwards, Determination of Monensin RDD by HPLC Using Post-Column Derivatization, Report No. AM-AA-CA-J199-AA-755, Agricultural Anal. Chem., Lilly Research Laboratories, Greenfield, USA, 1984.

Received: March 1, 1993
Accepted: July 8, 1993