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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NOVOBIOCIN

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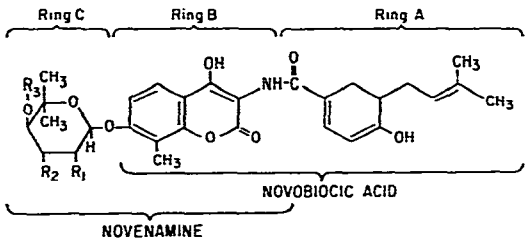
SUMMARY

A stability-indicating normal-phase high-performance liquid chromatographic (HPLC) method for the assay of novobiocin has been developed. The method uses a silica column with a mobile phase composed of butyl chloride–tetrahydrofuran–methanol–acetic acid (88:5:4:3). The amount of acetic acid in the mobile phase significantly influences the resolution of novobiocin and desmethyldescarbamylnovobiocin peaks. Isonovobiocin and degradation compounds of novobiocin have been separated. The standard curve for the quantification of novobiocin was linear in the range of 0.2–0.6 mg novobiocin per ml and the relative standard deviation of the assay was less than 1%. Excellent correlation was obtained between the HPLC and microbiological assay methods. The method was used to assay novobiocin in mastitis products.

INTRODUCTION

Novobiocin (Fig. 1) is an aminocyclitol antibiotic produced by *Streptomyces niveus* or related microorganisms. The official assay method for the potency determi-



	R_1	R_2	R_3
NOVOBIOCIN	OH	$OCONH_2$	CH_3
ISONOVOBIOCIN	$OCONH_2$	OH	CH_3
DESCARBAMYLNOVOBIOCIN	OH	OH	CH_3
DESMETHYLDESCARBAMYLNOVOBIOCIN	OH	OH	H

DIHYDRONOVOBIOCIN (REDUCTION OF ISOPENT-2-ENYL SIDE CHAIN TO ISOPENTYL)

Fig. 1. Structure of novobiocin.

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nation of novobiocin by the Food and Drug Administration is a cylinder cup agar diffusion assay using *Staphylococcus epidermidis* ATCC 12228 as the test microorganism[1]. Since isomers and degradation products of novobiocin are reported as being microbiologically inactive[2–4], the microbiological assay method is a stability-indicating assay method. The microbiological assay method, however, is not a precise method for quantification and is influenced by varieties of factors[5]. The method is also incapable of detecting and quantifying degradation compounds and process impurities.

Several chemical assay methods are available for the determination of novobiocin[6–13]; those listed in refs. 8 and 9 are stability-indicating assay methods. A gas–liquid chromatographic (GLC) method, based on the acetylation of novobiocin and chromatography using an OV-17 column, is also available[14]. Although the precision of the GLC method is excellent (relative standard deviation, R.S.D. = 0.3%), the method cleaves either the glycosidic or the amide bond, depending upon the derivatization method used. The method, moreover, is incapable of differentiating isonovobiocin from novobiocin.

A high-performance liquid chromatographic (HPLC) method for the determination of novobiocin has been reported[4]. Excellent separation of isomers and degradation compounds was obtained. However, the HCP column used was not stable and the column performance deteriorated rapidly upon use. This paper reports the development of a normal-phase HPLC method for separation and quantification of novobiocin, its isomers and degradation compounds.

EXPERIMENTAL

Instruments

An LDC M19-60066-022 high pressure mini-pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to pump the mobile phase at a flow-rate of about 1 ml/min. Analysis was performed using a silica column (Cat. No. SI-5A, Brownlee LiChrosorb SI-100, 5 μm, 250 × 4.6 mm I.D.; Rheodyne, Berkeley, CA, U.S.A.) at room temperature. A 20-μl sample containing about 0.1 mg/ml of novobiocin acid was injected quantitatively onto the column using a Rheodyne M70-10, 20-μl loop injector or Waters intelligent Sample Processor (WISP, Model 710B; Waters Assoc., Milford, MA, U.S.A.). The column effluent was monitored either at 340 nm using an LDC Spectromonitor I variable-wavelength detector or at 254 nm using an LDC UV III monitor fixed-wavelength detector with an attenuation setting of 0.032 a.u.f.s.

Reagents

Butyl chloride, tetrahydrofuran, and methanol were all UV grade, distilled in glass, obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Acetic acid was AR grade obtained from Mallinckrodt (Paris, KY, U.S.A.).

Mobile phase

The mobile phase used was butyl chloride (50% water saturated)–tetrahydrofuran–methanol–acetic acid (88:5:4:3). Fifty percent water-saturated butyl chloride was prepared by mixing one volume of water-saturated butyl chloride with an equal volume of butyl chloride.

Novobiocin reference standard solution

USP novobiocin acid reference standard was dried for 4 h at 100°C under vacuum (< 5 mmHg). Approximately 10 mg of the dried and cooled reference standard were accurately weighed into a 100-ml volumetric flask. The novobiocin acid was dissolved by adding 10 ml tetrahydrofuran, and the solution was diluted with the mobile phase and sonicated to aid dissolution. The tetrahydrofuran used contained 0.3 mg prednisone per ml, an internal standard suitable for monitoring at 254 mm.

Sample preparation

Novobiocin powder. Approximately 12–14 mg of sodium or calcium novobiocin were accurately weighed "as is" into a 100-ml volumetric flask. A 10-ml volume of tetrahydrofuran was added and the solution diluted to volume with the mobile phase.

Peanut oil based veterinary mastitis products. Each plastet (a unique syringe and needle assembly made entirely of polyethylene) was vigorously shaken with two glass beads for 30 min on a platform reciprocating shaker to obtain a uniform suspension. The contents of each plastet were expelled into a 100-ml volumetric flask. The syringe was weighed before and after to obtain sample weight. A 10-ml volume of tetrahydrofuran was added to the flask and the solution was diluted to volume with mobile phase. This solution was sonicated and shaken on a platform reciprocating shaker at high speed for 5 min. An aliquot was centrifuged in a disposable vial for 5 min at 2000 *g*. A portion of the clear supernatant was then used for analysis. Novobiocin samples thus prepared are stable up to 1 week at room temperature.

Calculations

The amount of novobiocin in the mastitis product can be calculated by

$$\text{Novobiocin (mg per 10 ml)} = \frac{A_{smp}}{A_{std}} \times \frac{C_{std}}{W_{smp}} \times F \times V \times S \times 10$$

where A_{smp} and A_{std} are the peak areas of novobiocin in the sample and the reference standard, C_{std} is the concentration (mg/ml) of the novobiocin reference standard solution, W_{smp} is the weight (mg) of sample, F is the assigned purity of the reference standard, V is the volume of the sample solution (100 ml), S is the specific gravity of the sample, and 10 is a factor to express the result on a 10-ml basis.

RESULTS AND DISCUSSION

Chromatographic separation of impurities and degradation compounds

Commercially available novobiocin contains, in addition to novobiocin, small amounts of isonovobiocin, descarbamylnovobiocin, desmethyldescarbamylnovobiocin, novenamine, novobiocic acid, and rings A, B, and C (Fig. 1). Since efforts to develop an HPLC method using a reversed-phase column to separate isonovobiocin from novobiocin failed, a normal-phase HPLC method for the assay of novobiocin was developed to separate the isomers. Isonovobiocin was reported to have no antimicrobial activity[3]. The HPLC method described in this report is capable of differentiating and quantifying novobiocin and its isomers and degradation products. Authentic samples of these isomer and degradation compounds, available at

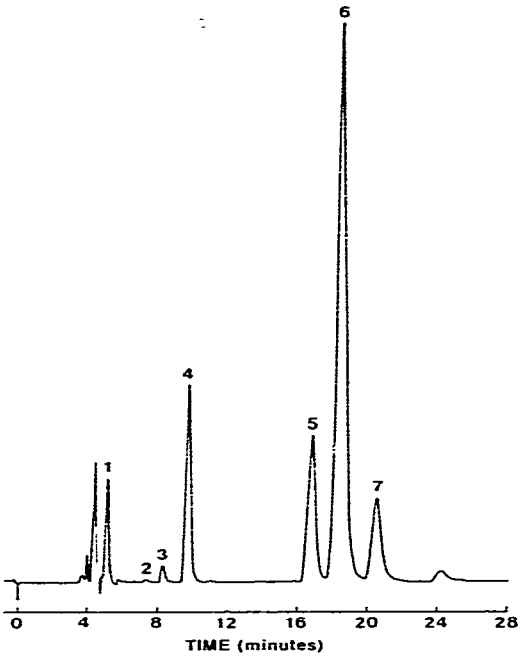


Fig. 2. HPLC Separation of novobiocin from its isomers and degradation compounds. Peaks: 1 = ring B; 2 = novobiocic acid; 3 = ring A amide; 4 = descarbamylnovobiocin; 5 = isonovobiocin; 6 = novobiocin; 7 = desmethyldescarbamylnovobiocin.

The Upjohn Company, were spiked into a solution of sodium novobiocin bulk powder and identified on the chromatogram on the basis of relative retention. A chromatogram indicating the separation of various degradation products can be seen in Fig. 2. The relative retention of these isomer and degradation compounds are presented in their order of elution in Table I. Among the components of the mobile phase, acetic acid significantly influenced the resolution of novobiocin and desmethyldescarbamylnovobiocin peaks. An increase of acetic acid from 1.0% to 3.0% changed the peak resolution from 0.41 to 1.44.

Unless specifically derivatized, the ring C cannot be detected when monitored by an UV spectrophotometer. Detection of the ring A and/or novobiocic acid should be indicative of the ring C formation. Under the chromatographic conditions used,

TABLE I

RELATIVE RETENTION OF NOVOBIOCIN AND IMPURITIES

Compound	*Relative retention*
Ring B	0.20
Ring A acid	0.27
Novobiocic acid	0.44
Ring A amide	0.48
Descarbamylnovobiocin	0.53
Isonovobiocin	0.91
Novobiocin	1.00
Desmethyldescarbamylnovobiocin	1.10

novenamine does not elute from the column. Formation of novenamine should coincide with that of the ring A; the latter can be monitored by HPLC (Table I, Fig. 2). Fig. 3 is a typical chromatogram of a production lot of sodium novobiocin indicating the presence of isomers and impurities.

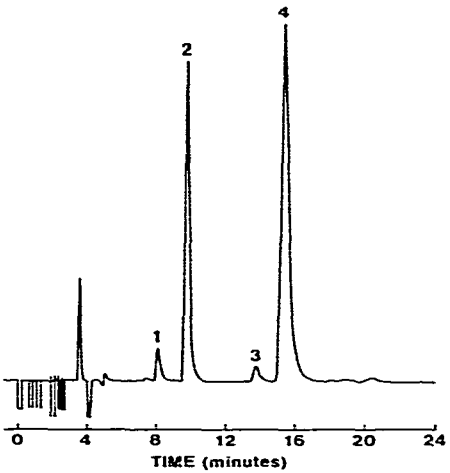


Fig. 3. HPLC chromatogram of sodium novobiocin with prednisone as the internal standard. Peaks: 1 = descarbamylnovobiocin; 2 = internal standard; 3 = isonovobiocin; 4 = novobiocin.

Assay of novobiocin bulk drug

Prednisone was selected as the internal standard suitable for monitoring at 254 nm. As shown in Fig. 3, prednisone elutes after descarbamylnovobiocin but well ahead of isonovobiocin. The impurities and degradation compounds in novobiocin do not interfere with prednisone.

A standard curve for the assay of novobiocin was constructed using a Novobiocin Acid Reference Standard between the range of 0.2 to 0.6 mg/ml. The standard curve is linear (correlation coefficient of 0.99998) with a linear regression of $y = 361x + 1.04$. A small bias observed at the y intercept when $x = 0$ can be considered as well within error of the assay.

The effect of the use of the internal standard on the precision of the assay was evaluated from the R.S.D. obtained by analyzing six individually weighed samples of sodium novobiocin powder and diluting in a solution containing the internal standard. As shown in Table II, the R.S.D. of the assay, calculated without the use of the internal standard, was 0.93%. The R.S.D. of 1.62% was obtained when the internal standard calculation format was used. This apparent increase in the R.S.D. of the assay by the internal standard calculation method can be explained by the design of the method, which requires use of the peak area ratio of the two peaks, novobiocin and prednisone, for calculation. Therefore, the assay variance become additive due to the two independent variables. The potency of the novobiocin powder did not differ significantly whether or not the internal standard was used for calculation. The potency was 809 μg/mg and 811 μg/mg with and without the use of the internal standard, respectively (Table II).

The sensitivity of the assay is approximately 40 ng novobiocin per column injected. The novobiocin sample prepared in the mobile phase is stable for approxi-

TABLE II

EFFECT OF THE INTERNAL STANDARD ON PRECISION OF THE HPLC ASSAY FOR NOVOBIOCIN POWDER

Sample no.	*Weight of novobiocin (mg 100 ml)*	*Peak area*		*Ratio*	
		Novobiocin	*Internal standard*	*No internal std. Novo peak area/wt.*	*With internal std. Novo/wt./int. std.*
1	10.03	18,369	9736	1831.4	0.1881
2	10.24	18,419	9876	1798.8	0.1821
3	10.32	18,710	9837	1813.0	0.1843
4	9.98	18,298	10112	1833.5	0.1813
5	10.04	18,141	9865	1806.9	0.1832
6	10.19	18,265	9989	1792.5	0.1794
			R.S.D.	0.93%	1.62%
			Potency	811 μg/mg	809 μg/mg

mately 1 week when tightly capped and stored at room temperature (peak height of novobiocin: initial, 46.6; 1 week later, 46.5). No decrease in the novobiocin peak nor appearance of degradation peaks was noted.

Correlation between HPLC and microbiological assay methods

Novobiocin is normally quantified by the microbiological cylinder cup agar diffusion method using *Staphylococcus epidermidis* ATCC 1228[1]. This method, however, is incapable of detecting and quantifying the presence of minor degradation products and impurities, which are microbiologically inactive. Several lots of commercially available novobiocin powder were assayed using the HPLC method and the results were compared with those of the microbiological assay method (Table III).

TABLE III

COMPARISON OF HPLC AND MICROBIOLOGICAL ASSAY METHODS FOR ANALYSIS OF NOVOBIOCIN

Lot no.	*Potency (μg/mg)*	
	HPLC	*Microbioassay*
1	810	831
2	885	862
3	865	903
4	844	886
5	861	855
6	856	870
7	871	863
8	829	823
9	809	807
10	801	793
11	809	781

Analysis of the data indicated that there is no statistically significant difference between the HPLC and the microbiological assay data.

In order to evaluate further the correlation between the HPLC and the microbiological assay methods, sodium novobiocin was intentionally degraded by acid, base, and ^{60}Co treatments. These degraded samples were analyzed by HPLC and by the microbiological assay methods. HPLC data indicated that acid treatment degraded novobiocin and formed at least five peaks, one of which has a relative retention identical to novobiocin acid. A mild base hydrolysis formed a substantial quantity of descarbamylnovobiocin with a lesser amount of novobiocin acid. Isonovobiocin was relatively stable to the treatment. Stronger base treatment caused complete degradation of the novobiocin peak and degradation of descarbamylnovobiocin with substantial increase of ring A.

Potencies of these degraded samples were calculated from the residual quantities of novobiocin detected by HPLC and the values were compared with those of the microbiological assay method (Table IV). In general, agreement between the two assay methods are good for base degraded samples. The higher HPLC potency obtained on the acid degraded sample may be attributed to incomplete separation of the novobiocin peak from one of the degradation peaks, and to the low quantity of novobiocin, which was outside the linear portion of the standard curve for the microbiological assay method.

TABLE IV

ASSAY OF INTENTIONALLY DEGRADED NOVOBIOCIN SAMPLES

Treatment	*Potency (μg/mg)*	
	HPLC	*Microbiological*
Control	811, 809	879, 816, 758 767, 907, 859 ($\bar{x}$ = 831)
Acid with heat	18	2.5, 1.8
Mild base	14	18, 12
Strong base with heat	0	0.05, 0.04

Novobiocin powder was submitted to Isomedix, Inc. (Morton Grove, IL, U.S.A.) for ^{60}Co-irradiation at various doses up to 5.87 Mrad. These samples were analyzed by the HPLC and the microbiological methods (Table V) and the results indicated excellent correlation between the HPLC and the microbiological assay methods.

The results of these studies confirm the published reports that the isomers and degradation compounds of novobiocin are microbiologically inactive[2–4]. Thus, the HPLC method for the determination of novobiocin provides not only a potency value which correlates well with the microbiological assay method, but also provides opportunity to detect and quantify the degradation compounds and impurities present.

Identification of a degradation compound

Upon examination of chromatograms of irradiated and non-irradiated sodium

TABLE V

ANALYSIS OF ^{60}Co-IRRADIATED SODIUM NOVOBIOCIN POWDER

Irradiation dose (Mrad)	*Potency (μg/mg)*	
	HPLC	*Microbiological*
0	815	816
0.6	784	754
1.76	758	757
2.85	746	697
3.45	736	722
4.78	720	690
5.87	724	728

novobiocin powder, it became evident that one peak (peak No. 2) which was originally present in bulk powder increased upon irradiation (Fig. 4). Dry heat treatment of the bulk powder also formed this peak (Fig. 4). The relative chromatographic retention of this peak (RT, 0.48) is close but not identical to that of novobiocic acid (RT, 0.44) (Table I); moreover, this peak lacks absorptivity at 340 nm. Novobiocic acid absorbs strongly at 340 nm.

In order to identify the peak, sodium novobiocin was refluxed for 30 min in ethyl acetate to enrich the compound. Alternatively, the compound was enriched by two-phase extraction using water and ethyl acetate. The compound is extremely solu-

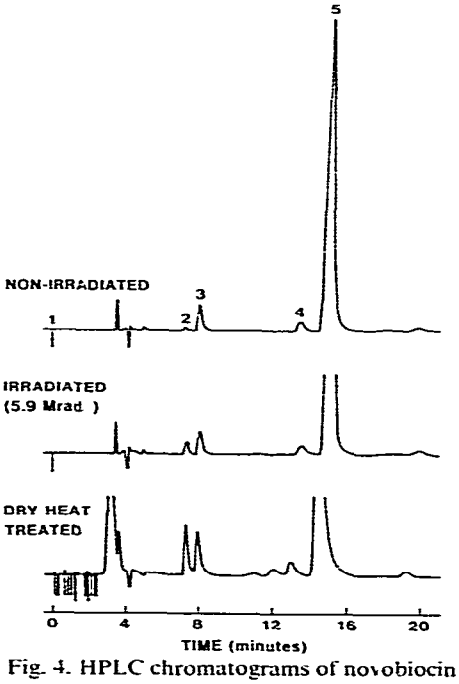
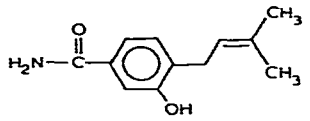


Fig. 4. HPLC chromatograms of novobiocin indicating increase in the Ring A amide by ^{60}Co-irradiation or dry heat treatment. Peaks: 2 = ring A amide; 3 = descarbamylnovobiocin; 4 = isonovobiocin; 5 = novobiocin (1 = injection point).

ble in ethyl acetate while sodium novobiocin is soluble in water. The enriched sample was purified by semi-preparative scale HPLC. The purified sample was freeze-dried and analyzed by high-resolution mass spectrometry. The mass spectrum (Fig. 5) identified the compound as the ring A amide (mol.wt. found, 205.1087; theory for $C_{12}H_{15}N_1O_2$, 205.1103):



Ring A amide

The mass fragmentation pattern also confirmed this identification.

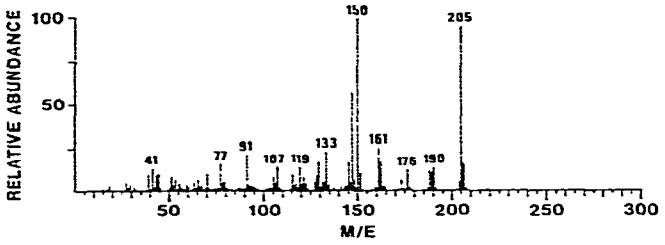


Fig. 5. Mass spectrum of Ring A amide.

Analysis of mastitis product

When the HPLC procedure was applied to analyze novobiocin in a peanut oil based mastitis product containing prednisolone, the steroid was inadequately separated from novobiocin for precise quantification. Efforts to improve separation by the modification of chromatographic conditions were not successful. Use of ion-exchange resins and solvent extraction methods were also examined without success. Interference from prednisolone was eliminated by monitoring column effluent at 340

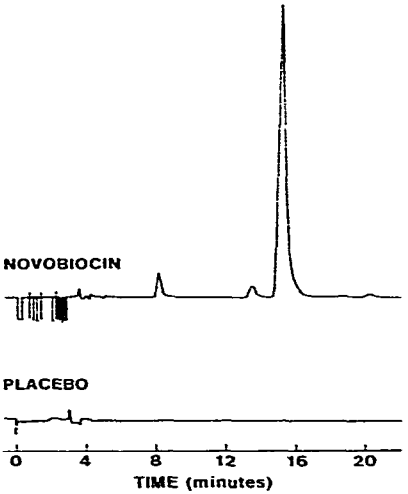


Fig. 6. HPLC chromatograms of novobiocin in peanut oil-based mastitis product and of placebo.

nm. Novobiocin has a maximum absorbance at 340 nm while prednisolone has a maximum at 254 nm and no absorbance at 340 nm.

HPLC chromatograms of novobiocin in mastitis product and of the placebo are shown in Fig. 6. A suitable internal standard to monitor the column effluent at 340 nm is being investigated. Prednisone, however, may satisfactorily be used as the internal standard if a rapid scanning spectromonitor is used to monitor at both 254 nm and 340 nm. The sensitivity of the assay is approximately 10 ng novobiocin per injection when monitored at 340 nm. This is approximately a four-fold increase in sensitivity when compared to monitoring at 254 nm.

The average recovery of novobiocin when spiked at 70–120% of the label was 100.8% with a relative standard deviation of 0.6% (Table VI).

TABLE VI

RECOVERY OF SODIUM NOVOBIOCIN SPIKED IN PLACEBO OF A MASTITIS PRODUCT

Sodium novobiocin (percent label)	*Weight added (mg/10 ml)*	*Weight recovered (mg/10 ml)*	*Recovery (%)*
67.6	80.44	80.72	100.4
68.1	81.04	81.47	100.5
76.7	91.26	91.14	99.9
76.5	91.02	91.88	100.9
84.4	100.52	100.44	99.9
85.2	101.47	102.29	100.8
92.6	110.21	111.60	101.3
92.6	110.24	110.85	100.6
100.8	120.03	121.27	101.0
101.0	120.19	121.27	100.9
120.7	143.7	146.3	101.8
121.0	144.1	146.6	101.7
		Average	100.8%
		R.S.D.	0.60%

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