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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF NOVOBIOCIN

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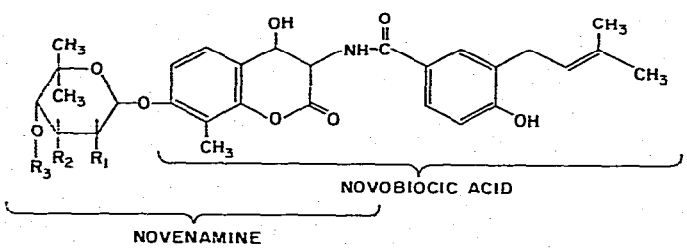
SUMMARY

A high-pressure liquid chromatographic method for the analysis of novobiocin is described. The method uses a 1-m-long Zipax HCP column with a mobile phase of 15% methanol in 0.02 *M* phosphate buffer, pH 7.0, at a flow-rate of 0.85 ml/min (68 atm). Novobiocin, isonovobiocin, dihydronovobiocin, descarbamylnovobiocin, desmethyldescarbamylnovobiocin, novobiocic acid, and novenamine are separated in approximately 30 min. The relative standard deviation for the analysis of novobiocin is less than 1%.

INTRODUCTION

Novobiocin (Fig. 1), an antibiotic produced by *Streptomyces niveus* or related microorganisms, contains isomers and degradation products. The officially accepted assay method, by the Food and Drug Administration, for the potency determination



	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

Fig. 1. Structure of novobiocin.

of novobiocin is the microbiological cylinder cup agar diffusion assay using *Staphylococcus epidermidis* ATCC 12228 as the test microorganism[1]. The microbiological assay method, however, is not a precise method of quantitation and is influenced by various factors[2]. The method is also incapable of detecting the presence of minor degradation compounds and impurities.

Several chemical assay methods are available for the determination of novobiocin[3-11], of which those described in refs. 7, 8, and 11 are capable of differentiating and quantitating novobiocin in the presence of isomers and degradation products. A gas chromatographic method, based on the acetylation of novobiocin and chromatography using an OV-17 column, is also available[12]. Although the precision of the gas–liquid chromatographic method is excellent, relative standard deviation of 0.34%, the method cleaves either a glycosidic or an amide bond, depending upon the derivatization methods used, and is incapable of differentiating isonovobiocin from novobiocin.

High-pressure liquid chromatography (HPLC), one of the latest entries in analytical methodology, has successfully been used for the determination of a few antibiotics[13-16]. This paper reports the separation and quantitation of novobiocin and its isomers and degradation products in 30 min using HPLC.

EXPERIMENTAL

Apparatus

A Laboratory Data Control (LDC, Riviera Beach, Fla., U.S.A.) modular liquid chromatograph equipped with a 254-nm UV monitor (Model 1285), a Milton Roy Minipump (LDC), and a pulse dampener (LDC, Model 709) were used.

An empty DuPont (DuPont, Wilmington, Del., U.S.A.) stainless-steel column, 2.1 × 1000 mm, was first rinsed with tetrahydrofuran (THF) followed by vigorous cleaning of the inside of the tubing with a cotton string pre-soaked with THF to remove loose metal particles. Chloroform was then drawn through the column and the column was dried under a stream of dry nitrogen.

A 10-μm pore size stainless-steel frit was fitted into the inlet end of the column and a hex nut (DuPont, No. 820349) with stainless-steel front and back lock ferrules and a cap (DuPont, No. 201724) were attached to the column. Zipax hydrocarbon polymer (HCP, DuPont) was dry packed into the open end of the column by adding a small amount of Zipax HCP at a time and lightly tapping on the floor. After the column was tightly packed, a 2-μm pore size stainless-steel frit was inserted into the outlet end of the column.

The column thus packed was attached to a DuPont injector port and to the 254-nm UV monitor. The theoretical plates of the column thus prepared were approximately 429 per meter for the novobiocin peak.

Reagents

Mobile phase. 15% methanol in 0.02 *M* monobasic and dibasic sodium phosphate buffer at pH 7.0 was used. Depending upon the performance of the column, the percentage of methanol in the mobile phase may have to be slightly modified to adjust the elution time of the novobiocins.

Column rinse solution. (A) 40% methanol in 0.02 *M* monobasic and dibasic sodium phosphate buffer solution at pH 7.0 and (B) 15% methanol in 0.02 *M* dibasic

sodium phosphate adjusted to pH 2.5 by the addition of phosphoric acid were used.

Internal standard solution. Approximately 150 ml of anhydrous methanol were added to a 250-ml stoppered graduated cylinder containing 87.5 mg prednisolone. After dissolving, 25 ml of 0.2 *M* sodium phosphate buffer solution at pH 7.0 and sufficient water to bring to volume were added.

Procedures

Preparation of novobiocin standard. Novobiocin acid USP reference standard was used "as is" at 970 μg/mg. Approximately 10 mg of the reference standard was accurately weighed using a Cahn Electrobalance Model G (Cahn Instrument Corp., Paramount, Calif., U.S.A.) and placed in a 10-ml volumetric flask.

Preparation of sample. Approximately 10 mg of novobiocin sample was accurately weighed into a 10-ml volumetric flask.

Prior to the analysis, each standard and sample was dissolved and diluted to volume with the internal standard solution. An ultrasonicator (Ultrasonics, Plainview, L.I., N.Y.) was used to quickly dissolve standard and samples.

Novobiocin chromatographic conditions. Flow-rate of the mobile phase was 0.85 ml/min with a column pressure of approximately 68 atm (1000 p.s.i.). The column temperature was ambient and the chart speed was 0.25 in./min. A 1.0-μl quantity of sample was injected into the column at the electrometer range setting of 0.04 full scale.

Column rinse. After the end of a day's analysis, the column was rinsed with the rinse solution A for at least 1 h followed by the solution B. This procedure was necessary in order to prolong the column life.

RESULTS AND DISCUSSION

Separation of novobiocins

The actual chromatogram of a calcium novobiocin bulk powder sample is shown in Fig. 2. The separation, or resolution, for two adjacent peaks is calculated in terms of the resolution function (*Rs*) using the following formula:

$$Rs = 2(t_2 - t_1)/(W_1 + W_2)$$

Rs is equal to the difference in retention time ($t_2 - t_1$) for the two adjacent peaks divided by the average baseline peak width in time units [1/2 ($W_1 + W_2$)] (ref. 17). As may be seen in Table I, the resolution functions between the majority of the peaks are above 1.2 at a peak area ratio (A_1/A_2) of 1/64 or above; therefore, the peak separations are quite satisfactory.

No detectable amount of degradation of novobiocin occurred in the internal standard solution in 8 h when stored at room temperature since no increase in the percentage of degradation compound was seen by the HPLC. Novobiocin is reported to be relatively stable in an aqueous solution of pH 7.0 when stored at room temperature[18,19].

In order to examine the detection and quantitation of isonovobiocin from novobiocin by the HPLC, novobiocin acid USP reference standard containing 0, 5, 10, 25, and 40% of isonovobiocin (3736-HH-19.3) were prepared and analyzed. Al-

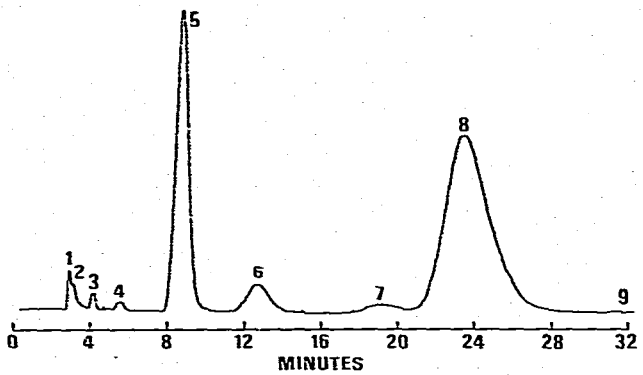


Fig. 2. High-pressure liquid chromatography of calcium novobiocins using a 1-m Zipax HCP column at 0.85 ml/min (1050 p.s.i.) at room temperature. Mobile phase: 15% methanol in 0.01 *M* pH 7.0 phosphate buffer. 1 = Injection peak; 2 = novobiocic acid; 3 = novenamine; 4 = desmethyldescarbamylnovobiocin; 5 = internal standard; 6 = descarbamylnovobiocin; 7 = isonovobiocin; 8 = novobiocin; 9 = dihydronovobiocin.

TABLE I

RESOLUTION FUNCTION (*Rs*) BETWEEN THE TWO ADJACENT NOVOBIOCIN PEAKS

Peak	*Rs*	A_1/A_2
Solvent peak		
Novobiocic acid	0.6	1/1
Novenamine	2.4	1/1
Desmethyldescarbamylnovobiocin	2.4	1/1
Internal standard	3.2	1/64
Descarbamylnovobiocin	2.5	6/1
Isonovobiocin	2.7	3/1
Novobiocin	1.2	1/40

TABLE II

IMPURITIES IN THE USP NOVOBIOCIN ACID REFERENCE STANDARD AND IN ISONOVOBIOCIN (3736-HH-19.3) AS DETERMINED BY HPLC

	Composition (%)	
	Novobiocin acid	*Isonovobiocin*
Novobiocin	98.1	0
Isonovobiocin	0.2	66.3
Descarbamylnovobiocin	0.3	0.4
Desmethyldescarbamylnovobiocin	0.3	8.0
Novobiocic acid	0.08	17.6
Novenamine	0.08	7.8
Dihydronovobiocin	1.2	0

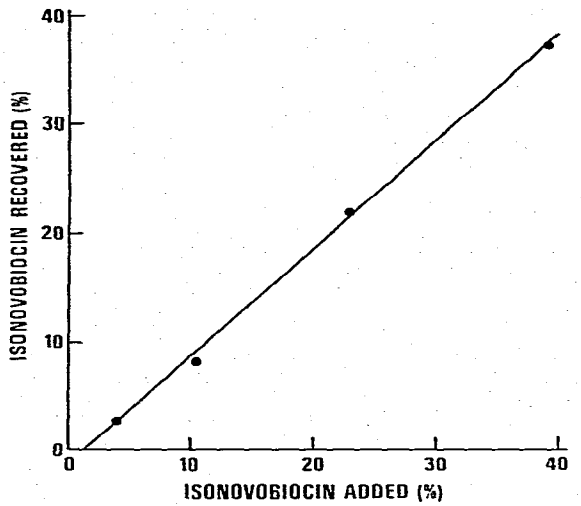


Fig. 3. Correlation between the isonovobiocin added and the isonovobiocin recovered from novobiocin by HPLC.

though the novobiocin acid used was relatively pure, the isonovobiocin contained many impurities (Table II). Therefore, the ratio of novobiocin and isonovobiocin in samples was corrected to compensate for these impurities. The results shown in Fig. 3 indicate that the recovery of isonovobiocin is linear (correlation coefficient, 1.000) with a linear regression of $y = 0.991\ x - 1.231$. The bias in recovery is due to the incomplete separation between isonovobiocin and novobiocin peaks (Table I and Fig. 2).

Quantitation of novobiocin

Various amounts of the USP novobiocin acid reference standard, ranging from 0.194 mg/ml to 2.0 mg/ml, were prepared and analyzed. The standard curve thus prepared was linear (correlation coefficient of 0.9999) with a linear regression of $y = 4.09x - 0.42$. A novobiocin concentration of 1.0 mg/ml was selected for

TABLE III

PRECISION OF THE NOVOBIOCIN, USP NOVOBIOCIN ACID REFERENCE STANDARD, ANALYSIS BY HPLC

Weight of novobiocin (mg/ml)	*Area*		*Area weight ratio*
	Novobiocin	*Internal standard*	
0.9980	913.5	369.7	0.2476
1.0020	997.8	405.0	0.2459
1.0038	945.7	377.9	0.2493
0.9968	970.0	391.2	0.2488
1.0098	977.2	388.2	0.2493
1.0062	1125	450.5	0.2482
1.0060	1073	431.8	0.2470
	Relative standard deviation 0.51%		

TABLE IV

ANALYSIS OF NOVOBIOCIN BULK POWDER

Lot No.	*Potency (μg/mg)*		*Composition (%) by HPLC*						
	Microbioassay	*HPLC*	*Novobiocin*	*Isonovobiocin*	*Desmethyl-descarbamyl-novobiocin*	*Descarbamyl-novobiocin*	*Novobiocic acid*	*Novenamine*	*Dihydronovobiocin*
Na-novobiocin									
1	872	906	92.1	1.5	0.1	3.9	0.1	0.4	2.0
2	876	841	94.6	1.6	0.1	3.1	0.1	0.1	0.6
3	874	823	90.7	2.1	0.1	3.8	0.7	0.4	2.3
4	854	867	92.0	1.9	0.1	3.6	0.1	0.4	1.7
5	865	842	87.9	2.9	0.1	7.4	0.1	0.7	1.0
6	841	884	93.6	1.5	0.1	3.3	0.1	0.3	1.3
7	895	889	91.2	2.5	0.1	3.5	0.3	0.3	2.1
8	912	885	92.7	1.7	0.1	3.9	0.4	0.5	0.7
9	887	898	92.7	1.2	0.2	3.5	0.1	0.5	1.8
10	819	829	88.8	1.9	0.1	6.3	0.1	0.9	1.9
11	830	853	90.5	1.7	0.3	5.6	0.1	0.8	1.2
12	865	826	89.8	1.5	0.1	6.9	0.1	0.5	0.9
		Average	91.4	1.8	0.1	4.6	0.1	0.5	1.5
Ca-novobiocin									
1	927	893	96.9	1.1	0.2	1.3	0.3	0.7	0.1

quantitative analysis. The HPLC method is sensitive to approximately 30 ng novobiocin per sample injected.

The precision of the HPLC method was determined by comparing seven replicate preparations of the USP reference standard. Table III indicates that the relative standard deviation of the novobiocin determination is 0.51%.

Twelve current lots of sodium and one calcium novobiocin sample were analyzed (Table IV). The potency was calculated using the USP novobiocin acid reference standard "as is" at 970 μg/mg. Since isomers and degradation products of novobiocin are reported as being microbiologically inactive[20,21], they were excluded from the potency calculation. The drug content thus calculated was compared to the potency determined by the microbiological agar diffusion assay method[1]. The calculated values showed no statistically significant differences from those of the microbiological assay method.

Determination of impurities and degradation products

The impurities and degradation products of two lots of sodium novobiocin bulk powders, as determined by the HPLC method, were compared with results obtained by paper chromatography[5] (Table V). The values obtained by the two methods agreed very well.

TABLE V

ANALYSIS OF IMPURITIES AND DEGRADATION PRODUCTS IN NOVOBIOCIN POWDERS BY HPLC AND PAPER CHROMATOGRAPHY (PC)

	Lot 1		*Lot 2*	
	HPLC	*PC*	*HPLC*	*PC*
Novobiocin	87.9	87.3	93.6	93.5
Isonovobiocin	2.9	3.6	1.5	2.0
Descarbamylnovobiocin	7.4	7.8	3.3	3.1
Others	1.8	1.3	1.6	1.3

As an average, current lots of sodium novobiocin contain 91.4% novobiocin, 1.8% isonovobiocin, 1.5% dihydronovobiocin, 4.6% descarbamylnovobiocin, 0.1, 0.5, and less than 0.1% novobiocic acid, novenamine, and desmethyldescarbamylnovobiocin, respectively. On the other hand, five year old sodium novobiocin samples contained significantly higher amounts of desmethyldescarbamylnovobiocin and novobiocic acid (Table VI). The amount of isonovobiocin was not significantly different. The similar trend was observed in calcium novobiocin. These data, together with those of the accelerated stability study, indicate that the novobiocin powder in a dry state degrades by cleavage of the ether linkage of the novenamine moiety and that transcarbamylation is not the major degradation route.

TABLE VI

NOVOBIOCIN POWDER STORED AT ROOM TEMPERATURE

Lot No.	*Age (years)*	*Potency (μg/mg)*		*Composition (%) by HPLC*						
		Microbioassay	*HPLC*	*Novobiocin*	*Isonovobiocin*	*Desmethyl-descarbamyl-novobiocin*	*Descarbamyl-novobiocin*	*Novobiocic acid*	*Novenamine*	*Dihydro-novobiocin*
Na-novobiocin										
1	5	870	809	87.3	2.3	1.0	6.6	1.3	1.0	0.4
2	5	818	804	86.3	2.6	0.8	6.6	1.6	1.2	0.6
3	5	846	801	90.9	1.6	0.6	5.2	0.6	0.6	0.2
4	5	867	859	91.7	0.9	0.9	3.9	1.1	1.1	0.4
5	5	854	852	89.8	0.8	0.8	5.6	0.9	1.0	0.1
			Average	89.2	1.6	0.8	5.6	1.1	1.0	0.3
Ca-novobiocin										
1	6	839	832	87.3	4.4	1.1	5.1	0.8	0.9	0.4
2	7	915	834	93.4	1.9	0.6	3.3	0.8	0.2	<0.1
3	7	897	892	93.0	2.3	0.6	2.8	0.8	0.6	<0.1
4	7	855	899	91.4	1.9	0.8	4.1	0.7	0.9	0.2
5	7	896	892	94.1	0.8	0.9	1.1	0.8	0.6	0.7
			Average	91.8	2.3	0.8	3.3	0.8	0.6	0.3

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