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PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE STABILITY OF NOVOBIOCIN IN MASTITIS PRODUCTS STERILIZED BY ^{60}Co IRRADIATION

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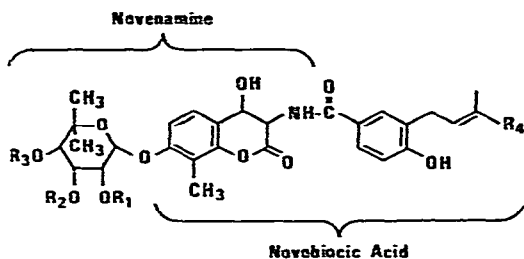(Received September 23rd, 1981)

SUMMARY

An isolation procedure and a high-performance liquid chromatographic technique for the quantitative analysis of novobiocin in mastitis products has been developed. The recovery of novobiocin from peanut oil suspensions was 101.2 % with a relative standard deviation of 1 %. The stability of novobiocin to the ^{60}Co irradiation sterilization process was examined. At irradiation dose levels of 1.5 to 4.9 Mrad, the degree of novobiocin degradation was only 3–4.5 %. An increase in the concentrations of two minor indigenous components was observed. However, no new compounds were generated as a result of the sterilization process.

INTRODUCTION

Many drugs, drug preparations and medical devices must be sterilized to ensure a minimum risk of transfer infection. A method of growing acceptance for the sterilization of bovine mastitis products, such as those containing novobiocin [*e.g.*, Albadry® and Albacillin® (Upjohn)], is the post-preparative irradiation of the packaged drug with cobalt-60, an intense source of γ-rays. The question of major concern is the effect this radiation has on the stability of the primary active constituent, novobiocin. Therefore a method of analysis, with a sensitivity of ± 1 %, was required to monitor the effects of this irradiation process on the novobiocin component primarily, and its related minor constituents secondarily.

There are several analytical techniques published in the literature for the detection of novobiocin, either in drug preparations or as residues in biological media. These techniques include microbiology[1–3], paper chromatography[4], thin-layer chromatography[5], gas chromatography[6] and, most recently, high-performance liquid chromatography (HPLC)[7]. The microbiological methods have traditionally been used to assess the activity or potency of drug preparations; these methods are, however, not sufficiently preciese for measuring small differences in drug content, nor are they of any use for measurement of the minor "inactive" components (see Fig. 1). Of the other methods, HPLC is the most appealing in terms of sensitivity, reproducibility, speed and resolving power to separate and measure all of the drug components.

Fig. 1. Structure of novobiocin and analogues.

Compound	R_1	R_2	R_3	R_4
Novobiocin	H	$\overset{O}{\overset{\|}{C}}NH_2$	CH_3	CH_3
Isonovobiocin	$\overset{O}{\overset{\|}{C}}NH_2$	H	CH_3	CH_3
Hydroxynovobiocin	H	$\overset{O}{\overset{\|}{C}}NH_2$	CH_3	CH_2OH
Descarbamylnovobiocin	H	H	CH_3	CH_3
Desmethyldescarbamylnovobiocin	H	H	H	CH_3

Dihydronovobiocin = Novobiocin + 2 H (reduced isoprene unit)

The initial efforts to develop an HPLC procedure for novobiocin were based on the procedures of Tsjui and Robertson[7], who used a reverse-phase mode of chromatography, with Zipax HCP as the hydrophobic stationary phase and an aqueous methanol–phosphate buffer as the mobile phase. They reported a relative standard deviation of less than 1 % for the analysis of novobiocin drug; in addition, six minor components were readily separated. However, despite the moderate resolving ability of this method, the novobiocin peak was broad, and the overall elution time of 32 min for all components was considered unacceptably long. Therefore, improvements in the techniques were necessary.

In the last few years, ion-pair chromatography has been one of the more popular topics in HPLC. In particular, reverse-phase ion-pair partition chromatography, referred to as paired-ion chromatography (PIC) by Waters Assoc.[8] or "soap chromatography" by Knox and his co-workers[9,10], is finding application in a variety of areas. This technique, in simplified terms, is based on the chromatographic separation of compounds as ionic species paired with counter-ions, or ionic species of opposite electronic charge, contained in the mobile phase. The separation column is usually a C_{18} or ODS reversed-phase material, but other lipophilic packings have been used. The mobile phase is usually an aqueous methanol or aqueous acetonitrile solution of either 1-heptanesulfonate, which provides the counter-ion species for cationic substrates, or a quaternary amine salt for anionic substrates. The ion-pairs that are in equilibrium with the unpaired ions in the mobile phase are more lipophilic and partition into the organic-like stationary phase. Chromatographic separation is achieved

because each ion-pair has a unique partition coefficient. Kissinger[11] suggests that more subtle processes occur in PIC, but a more detailed discussion is beyond the scope of this paper.

EXPERIMENTAL

Apparatus

Pump. A Varian Model 4200 (Varian, Palo Alto, CA, U.S.A.) was set to deliver mobile phase at 1.0 ml/min.

Column. A Microparticulate Zorbax ODS (DuPont, Wilmington, DE, U.S.A.) column (250 × 4.6 mm) was used, preceded by a Whatman Co:Pell ODS pre-column (50 × 2 mm) (Whatman, Clifton, NJ, U.S.A.).

Detector. The detector used was an Altex Model 150 UV Monitor (Altex, Berkeley, CA, U.S.A.) fitted with a 20-µl analytical flow-cell and a 254-nm wavelength kit operated at 0.2 a.u.f.s.

Injector. The Rheodyne Model 701 sample-loop injector used (Rheodyne, Berkeley, CA, U.S.A.) was fitted with a 10-µl loop.

Recorders. A Varian Model A-25 set at 50 mV and 0.1 in./min was used to monitor the novobiocin and internal standard peaks, and an LIC Model 155 mm (Linear Instrument C., Irvine, CA, U.S.A.) set at 1 mV and 20 cm/h was used to monitor the minor components.

Chemicals and reagents

1-Heptanesulfonic acid sodium salt, was obtained from Eastman Organic Chemicals (Eastman Kodak, Rochester, NY, U.S.A.). Methanol and acetonitrile were "distilled in glass" grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

Reference standard solution preparation

Novobiocin and the various reference compounds were Upjohn Co. reference standards. The novobiocin bulk drug was obtained as the sodium salt from three production lots (693FD, 388FG and 392FG). A series of novobiocin standards for HPLC was prepared from a stock solution of *ca.* 140 mg of sodium novobiocin (120 mg "active" novobiocin) in 20.0 ml of CH_3CN-H_2O (3:1) by dilution to concentrations of 5.0, 4.0, 3.0 and 2.0 mg/ml. These solutions were then diluted 1:1 with a solution (1 mg/ml) of 2,6-dichloro-4-nitroaniline [DCNA (Upjohn)] to yield a series of solutions with novobiocin concentrations of 3.0, 2.5, 2.0, 1.5 and 1.0 mg/ml. The 1.5 and 1.0 mg/ml solutions were diluted 1:1 with CH_3CN-H_2O (3:1) to give 0.75 and 0.50 mg/ml solutions. These solutions served to generate the linear regression curve.

For the internal standard solution, approximately 50 mg of DCNA were weighed into a 50-ml volumetric flask and dissolved in CH_3CN-H_2O (75:25); this gave a DCNA concentration of 1 µg/µl.

HPLC mobile phase

Solutions of 1-heptanesulfonic acid sodium salt, 0.005 *M*, in methanol and in water were prepared separately, degassed under vacuum, then magnetically mixed at a ratio of 80:20 (methanol solution to water solution). The mixture was vacuum-filtered through a 4-µm glass-sinter funnel before transfer to the HPLC pump reservoir.

Recovery study

Into each of three 1000-ml separatory funnels was placed 30–32 g of peanut oil, 1500–1700 mg of micronized sodium novobiocin (lot 392FG) —Formulation 1 (to simulate Albadry®)— or 650–750 mg —Formulation 2 (to simulate Albacillin®)— 400 ml of isooctane (distilled in glass) (Burdick and Jackson Labs.) and 400 ml of 75% acetonitrile (non-spectro-grade distilled in glass) (Burdick and Jackson Labs.) in deionized water. The mixtures were vigorously shaken twice for 20–30 sec at 15–20-min intervals. The lower layer was sampled in triplicate by diluting 5.0 ml with 5.0 ml of CH_3CN-H_2O (75:25), then 10 μl of each sample was injected into the HPLC column. The data are presented in Table I.

TABLE I

RECOVERY OF NOVOBIOCIN FROM PEANUT OIL

Sample	Fortification level, mg	Amount recovered*, mg	Recovery, %
A	1358	1373	101.1
B	1459	1484	101.7
C	1255	1251	99.7
D	598	604	100.9
E	557	567	101.8
F	648	662	102.2
		Mean	101.2 ± 0.9

* Mean of three determinations.

Extraction of drug from plastet samples

Four plastets (a plastic syringe-like device for intramammary injection of drug into the udder) were taken at random from each packet of 12 plastets, weighed as one sample to the nearest 0.01 g, then expressed into a 1000-ml separatory funnel. Then 400 ml of isooctane [saturated with CH_3CN-H_2O (75:25)] and 400 ml of CH_3CN-H_2O (75:25) (saturated with isooctane) were added, and the mixture was vigorously shaken twice for 20 sec each time at 15–20-min intervals. A 10–15 ml portion of the lower layer was drained into a 20-ml screw-cap vial, and duplicate HPLC samples were prepared by diluting 3.0 ml with 3.0 ml of the internal standard solution.

Data calculation

A baseline projection was carefully drawn along the bottom of the chromatogram peaks, and the height of both the DCNA and the novobiocin peaks were measured in mm with a plastic ruler and estimated to the nearest 0.1 mm; the ratio of the peak heights (novobiocin to DCNA) was then calculated. The series of standards produced a standard curve whose slope, ordinate intercept, correlation coefficient and various other parameters were calculated by using linear regression analysis. The drug content in each sample, calculated as mg of novobiocin per g of sample, was calculated from the standard curve data.

RESULTS AND DISCUSSION

In our initial attempts to study novobiocin by HPLC, we used the commercially available permanently bonded columns ODS, cyano, C-8 and ETH. In no instance was a proper blend of resolution of the minor components listed in Fig. 1 and acceptable novobiocin peak shape observed with typical reversed-phase solvents. However, when the mobile phase was modified with the ion-pairing agent sodium 1-heptanesulfonate, at a conc. of 0.005 M, peak shape and resolution of the minor components greatly improved. A sample chromatogram is shown in Fig. 2. The minor component isonovobiocin was never observed in any chromatogram containing novobiocin because of the overwhelming amount of the latter and their close chromatographic proximity. As far as is known, this was the only minor component whose fate and quantity remained unsolved by this HPLC method.
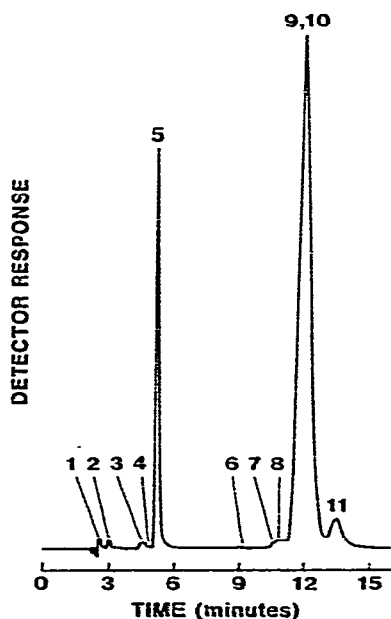

Fig. 2. Chromatogram of novobiocin (lot 388FG); 25 μg on column. HPLC conditions: 0.005 M sodium 1-heptanesulfonate in methanol–water (80:20) at 1 ml/min on a Zipax ODS column (250 × 4.6 mm); recorder A-25 at 50 mV and 0.1 in./min. Peaks: 1, novenamine; 2, Unknown 1: 3, Unknown 2: 4, hydroxynovobiocin; 5, DCNA (internal standard); 6, novobiocic acid; 7, desmethyldescarbamylnovobiocin; 8, dihydronovobiocin; 9, novobiocin; 10, isonovobiocin; 11, descarbamylnovobiocin.

The commercial mastitis products studied in this work were peanut oil suspensions of novobiocin and usually contained a modifier to stabilize this suspension. Therefore, a quantitative extraction of the drug and the drug components from the peanut oil gel suspension was needed. Novobiocin and the various related components are soluble only in highly polar organic solvents, such as acetonitrile. In contrast, the peanut oil is highly lipophilic and will preferentially partition into a non-polar solvent, such as a hydrocarbon. Thus, this isolation could readily be accomplished by a simple partition between CH_3CN–H_2O (75:25) and isooctane.

To test this procedure for efficiency of novobiocin extraction and for HPLC compatibility, a recovery study was run on suspensions of novobiocin in peanut oil. The results, presented in Table I, clearly show that novobiocin was quantitatively extracted. Two levels of fortification were studied at or near the levels in the commercial products; the overall recovery was $101.2 \pm 1.0\%$.

The simple extraction procedure gave quantitative extraction of all the minor constituents present in the bulk drug. Close inspection of the chromatograms in Fig. 3, derived from bulk drug itself and the same bulk drug suspended in peanut oil, then extracted, showed no observable differences. It was also clear that the peanut oil did not cause any interference in the chromatograms.
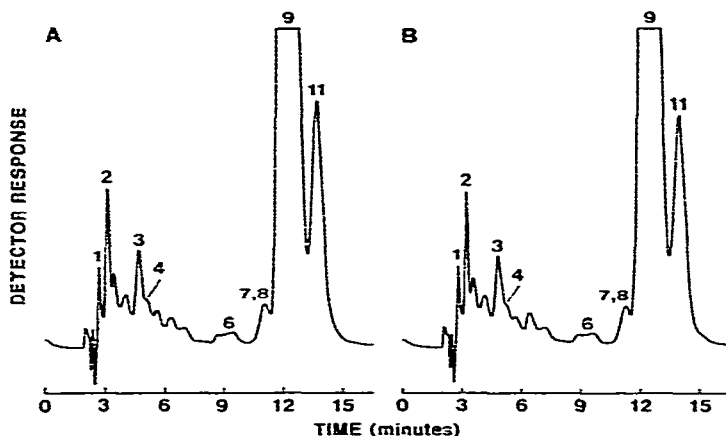

Fig. 3. Chromatograms comparing (A) peanut-oil-suspended novobiocin extracted with CH_3CN-H_2O (75:25) with (B) novobiocin drug standard. Recorder 155 at 1 mV and 40 cm/h. Peaks as in Fig. 2.

^{60}Co Irradiated samples

The mastitis products are marketed in a plastic syringe-like device called a plastet. Each plastet contains *ca.* 9 g of the peanut oil suspension of novobiocin and is individually wrapped in plastic and placed in a thin-walled cardboard box (12 plastets per box). Ten of these boxes are packed into a heavier cardboard box, and this box is irradiated with ^{60}Co. The dose levels depend on the length of exposure and the distance from the source, and are generally expressed as a range, since the amount of irradiation which reaches the center of the box is less than that striking the periphery. Detailed descriptions of the process were given by Pope *et al.*[12].

Four sets of samples were irradiated at five dose ranges, and the relative amounts of novobiocin were measured; the results are summarized in Table II. Irradiation at doses of 0.5 to 1.3 Mrad produced a slight decrease of novobiocin of 1 to 1.5%. A slightly higher dose range (1.55 to 1.77 Mrad), however, produced a 3.2% decrease. A jump to the 2.60–4.88 Mrad range generated only a 4.0 tot 4.4% loss. A plot of the percentage of unchanged novobiocin as a function of mean ^{60}Co dose levels (Fig. 4) clearly shows a non-linear bi-phasic plot. Since dose levels above 5 Mrad were not investigated, we shall not conclude that a terminal plateau was reached.

TABLE II

PERCENTAGE DECREASE OF NOVOBIOCIN IN MASTITIS PRODUCTS AS A FUNCTION OF ^{60}Co IRRADIATION*

Dose range	Form. 1**	Form. 1***	Form. 2**	Form. 2***	Mean
0.0	100.0	100.0	100.0	100.0	100.0
0.50–0.57	99.6	98.4	98.9	99.0	99.0
1.05–1.32	99.0	98.4	98.1	98.3	98.5
1.55–1.77	96.2	97.4	97.0	96.5	96.8
2.60–3.13	94.5	96.0	96.9	96.7	96.0
3.94–4.88	94.9	95.7	95.6	96.0	95.6

    * Relative to the 0.0-Mrad sample in each set. Each value is the mean of six determinations.
    ** Sodium novobiocin lot 693 FD.
    *** Sodium novobiocin lot 388FG.

      Some changes in the minor components were observed, as seen in Fig. 5, which compares non-irradiated sample with 4.0-Mrad-irradiated sample. There was an increase in the components designated Unknown 1 and Unknown 2 (peaks 2 and 3), respectively. Best estimates, based on peak-height measurements, suggested a 1.5 to 2.5-fold increase in their relative concentrations following irradiation. Perhaps more important, no new compounds were generated by the irradiation process which were not previously present as natural by-products of production or natural degradation. All of the production lots analyzed have contained various amounts of these minor components. Thus, all compounds, including Unknowns 1 and 2, must be defined as indigenous components.
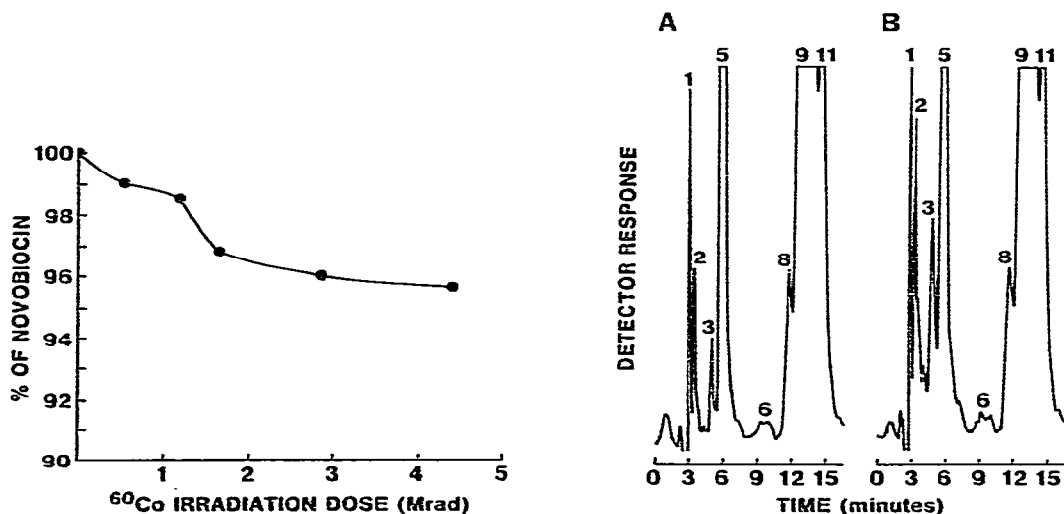

Fig. 4. Dose-response curve of the relative decrease of novobiocin in mastitis products as a function of ^{60}Co-irradiation dose during sterilization.

Fig. 5. Chromatograms comparing the relative intensities of the minor components of novobiocin in mastitis products irradiated with ^{60}Co at an average dose of 0.0 Mrad (A) and 4.0 Mrad (B). Recorder 155 at 1 mV and 20 cm/h. Peaks as in Fig. 2.

One last point should be made. The peanut oil carrier evidently plays only a mediating effect on the irradiation degradation of novobiocin. Irradiation of the raw micronized drug showed an identical degradation pattern and complement of minor components. The decrease in novobiocin was only slightly greater, with a concurrent increase in Unknowns 1 and 2, but again no new components were observed. The results also suggest that the irradiation of the peanut oil does not contribute any observable interference in HPLC.

REFERENCES

1 A. Morris, A. D. Russell and I. L. Thomas. *Experientia*, 23 (1967) 244.
2 Code of Federal Regulations. Title 21. *Food and Drugs*, 1973.
3 L. A. Ouderkirk. *J. Ass. Offic. Anal. Chem.*, 59 (1976) 1122.
4 V. Betina, *J. Chromatogr.*, 15 (1964) 379.
5 R. Bossuyt, R. van Renterghem and G. Waes, *J. Chromatogr.*, 124 (1976) 37.
6 O. Nishijima, O. Matano and T. Kashiwa. *Nogaku Kensasho Hokoku*, 11 (1971) 133; *Chem. Abstr.*, 78 (1973) 47841.
7 K. Tsuji and J. H. Robertson. *J. Chromatogr.*, 94 (1974) 245.
8 "*Paired-Ion Chromatography, an Alternative to Ion Exchange*." Publ. F61, Waters Associates, Milford, MA, 1976.
9 J. H. Knox and G. R. Laird. *J. Chromatogr.*, 122 (1976) 17.
10 J. H. Knox and J. Jurand. *J. Chromatogr.*, 125 (1976) 89.
11 P. T. Kissinger. *Anal. Chem.*, 49 (1977) 883.
12 D. G. Pope, K. Tsuji, J. H. Robertson and M. J. DeGeeter. *Pharm. Technol.*, 2 (1978) 30.