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ION-PAIR REVERSED-PHASE CHROMATOGRAPHY OF 7-*R*-O-METHYLNOGAROL AND RELATED COMPOUNDS

QUANTIFICATION OF BULK DRUG AND STERILE POWDER PHARMACEUTICAL FORMULATION

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SUMMARY

7-*R*-O-Methylnogarol is an anthracycline antibiotic showing good antitumor activity and is currently undergoing clinical trials. Development of an ion-pair reversed-phase high-performance liquid chromatography system capable of separating menogarol from its precursors and several related compounds is described in this report. The effects of pairing ion size, pairing ion concentration, and water–acetonitrile ratio, on the chromatographic behavior of these compounds are discussed. This stability-indicating system is used to quantitatively determine the purity of bulk drug and the amount of drug in a freeze-dried sterile powder formulation.

INTRODUCTION

7-*R*-O-Methylnogarol (7-OMEN, menogarol, U-52,047, NSC 269148) (Fig. 1, IR) is an anthracycline antiobiotic which has been shown to be highly active as an antitumor agent[1–4]. It is an analogue of and is produced from the fermentation derived antitumor agent, nogalamycin (Fig. 1, II)[3–6]. It is the most active analogue of nogalamycin of a variety of analogues that were evaluated.

The use of anthracycline antitumor agents is generally limited by two major toxicities: acute myelosuppression and total accumulated dose dependent cardiomyopathy. 7-OMEN was shown to be one fifteenth as potent as doxorubicin in inducing cardiotoxicity in the rabbit[7]. This fact and its good chemotherapeutic activity have encouraged further investigations into its development as an antitumor agent.

This report describes an ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method for quantitatively determining the purity of bulk drug 7-OMEN and the amount of drug in a sterile powder pharmaceutical formulation. The method is stability indicating; capable of separating 7-OMEN from a variety of related compounds. Spectrophotometric detection at 254 nm is employed.

Normal-phase[8–10], reversed-phase[11–18] and ion-pair reversed-phase[19–22]

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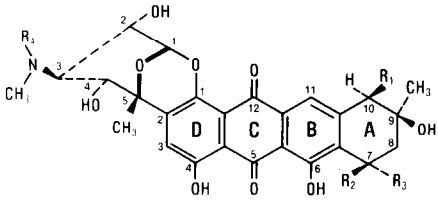
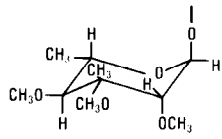
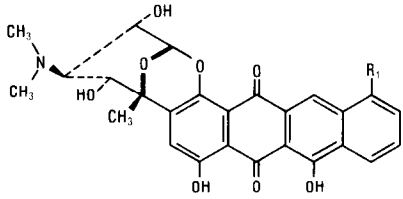




nogalose

		R_1	R_2	R_3	R_4
IR	7—R—O—methylnogarol	H	OCH_3	H	CH_3
IS	7—S—O—methylnogarol	H	H	OCH_3	CH_3
II	nogalamycin	$COOCH_3$	H	nogalose	CH_3
III	nogalamycinic acid	COOH	H	nogalose	CH_3
IV	nogamycin	H	H	nogalose	CH_3
VR	7—R—O—methylnogalarol	$COOCH_3$	OCH_3	H	CH_3
VS	7—S—O—methylnogalarol	$COOCH_3$	H	OCH_3	CH_3
VIR	7—R—nogarol	H	OH	H	CH_3
VIS	7—S—nogarol	H	H	OH	CH_3
VII	7—R—O—methyl—N—demethylnogarol	H	OCH_3	H	H
VIII	7—deoxynogarol	H	H	H	CH_3
IX	7—R—O—ethylnogarol	H	OC_2H_5	H	CH_3



		R_1
X	nogalarene	$COOCH_3$
XI	nogarene	H

Fig. 1. Structures of 7-OMEN and related compounds.

methods have been reported for various anthracyclines. Reversed-phase[15] and ion-pair[21] methods have been reported for nogalamycin. Methods for determining 7-OMEN in plasma employing reversed-phase[16] and ion-pair[22] methods have recently been reported. The method reported here provides more complete resolution of 7-OMEN from its precursors (II, III and IV) and a variety of related compounds.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade. Acetonitrile and tetrahydrofuran were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Ion-pair reagents were from Eastman-Kodak (Rochester, NY, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.). Samples of compounds I–XI were prepared by published methods[4] and characterized

by Dr. P. F. Wiley, The Upjohn Company. Doxorubicin was obtained from the Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.).

Apparatus

The chromatographic system consisted of an Altex 110 pump or a Perkin-Elmer Series 4 pump, an LDC Model 1203 UV Monitor III, and a Waters WISP autosampler. DuPont Zorbax® C_8 columns (25 cm × 4.6 mm I.D.) were employed. Detection was performed at 254 nm (λ_{max} = 259 nm, ε = 23,500 l mol^{-1} cm^{-1} for 7-OMEN). The flow-rate was normally set at 2.0 ml/min.

Spiking study

Spiked samples for the sterile powder formulation were produced by placing appropriate amounts of drug in a solution containing the correct proportions of the excipients. These solutions were frozen and lyophilized. These samples were one-tenth the size of the actual formulation, but drug to excipient ratios were identical. Sampling volumes were adjusted accordingly.

Mobile phase

Prepare a mobile phase consisting of 600 ml water, 400 ml acetonitrile, 20 ml tetrahydrofuran, 5 ml glacial acetic acid and 1.44 g sodium dodecyl sulfate. Filter.

7-R-O-methylnogarol

Internal standard solution. Prepare a 0.2-mg/ml solution of melengestrol acetate in mobile phase.

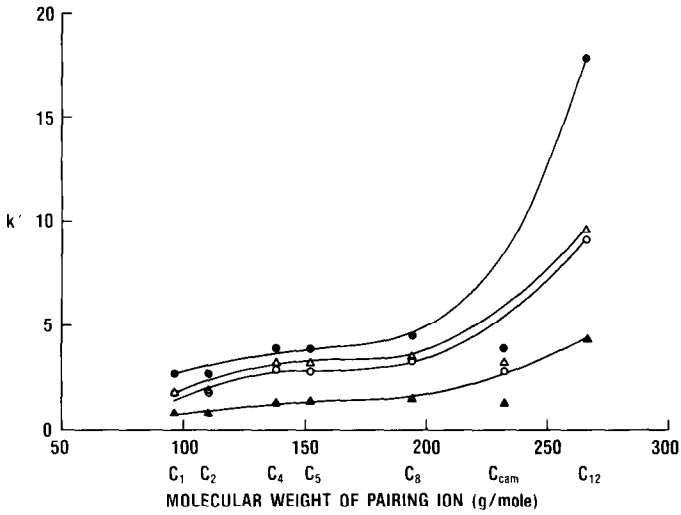


Fig. 2. Effect of pairing ion on retention of 7-OMEN and related compounds. The mobile phase consisted of 600 ml water, 400 ml acetonitrile, 20 ml tetrahydrofuran, and 5 ml glacial acetic acid. The pairing ion concentration was 5 m*M*. Flow-rate = 2.0 ml/min. ●, 7-OMEN (IR); △, nogalamycin (II): ○, nogamycin (IV); ▲, nogalamycinic acid (III). C_{cam} = camphorsulfonate, C_1 = methanesulfonic acid, C_2 = ethanesulfonic acid, etc.

Standard preparation. Accurately prepare a solution of 7-OMEN reference standard of approximately 0.1 mg/ml in internal standard solution. Protect from light.

Sample preparation. Accurately prepare a solution of 7-OMEN sample of approximately 0.1 mg/ml in internal standard solution. Protect from light.

Procedure. Inject 20 μl of each standard and sample preparation.

Sterile powder formulation (50 mg/vial)

Internal standard solution. Prepare a 0.4-mg/ml solution of melegestrol acetate in the following mixture: 80 ml acetonitrile, 20 ml water, 4 ml tetrahydrofuran, 1 ml glacial acetic acid and 0.288 g sodium dodecyl sulfate.

Standard preparation. Accurately prepare a 0.2-mg/ml solution of 7-OMEN reference standard in internal standard solution. Mix 1:1 with water.

Sample preparation. Dissolve the contents of a vial of sterile powder Menogarol in distilled water. Sonicate and shake as needed to insure complete dissolution. Quantitatively transfer this solution to a 250-ml volumetric flask. Rinse the vial and funnel several times with distilled water and dilute to volume with distilled water. Mix 10 ml of the 7-OMEN solution with 10 ml of internal standard solution.

Procedure. Chromatograph 20 μl of each standard and sample preparation.

RESULTS AND DISCUSSION

The majority of HPLC methods for anthracyclines employ reversed-phase separations. Attempts to chromatograph 7-OMEN and the related compounds shown in Fig. 1 on a straight reversed-phase system gave highly unsatisfactory results. Peaks were broad and tailed badly. Efficiency was very low with only 200–1000 theoretical plates being achieved. This type of behavior was observed on TMS, C_8 and C_{18} columns with the longer alkyl chains giving broader peaks with more tailing. Addition of a low concentration of an ion-pairing reagent such as an alkyl sulfate or sulfonate sharpened the peaks and decreased their capacity factors. As the pairing ion concentration was increased, retention times increased in a manner typical of ion-pair chromatography.

Effect of pairing ion size

The effect of the size of the pairing ion on the retention of several of these compounds was determined. Results are shown in Fig. 2. As the length of the alkyl chain was increased the capacity factors increased. The capacity factors increased gradually in going from methane to pentane sulfonate and then began to show more dramatic increases in going to octane and dodecyl sulfonate. Camphorsulfonate gave capacity factors intermediate to those provided by pentane- and octanesulfonate.

Plotted in Fig. 3 are the height equivalent to a theoretical plate (HETP) values achieved for 7-OMEN with each pairing ion. In this case the efficiency of the system increased (decreasing HETP values) in a continuous manner with the molecular weight of the alkyl sulfonates. This decrease in the HETP values indicates that the overall kinetics of interaction of the solutes with the pairing ions became more rapid as the size of the pairing ion increased. Camphorsulfonate does not fit this relationship on the basis of molecular weight. However, being a bicyclic molecule its molec-

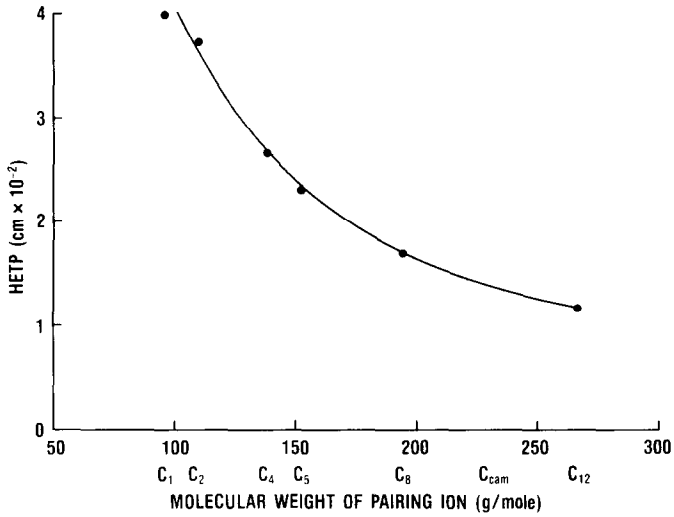


Fig. 3. Effect of pairing ion on HETP for 7-OMEN. Conditions same as for Fig. 1.

ular size is intermedite to that of pentane and octane sulfonate and the capacity factors and efficiency resultant from its use are also observed to be intermediate to those obtained from use of pentane and octane sulfonate. Hence, it would appear that the size of the pairing ion is the critical factor in determining the degree of retention.

Dodecylsulfonate gave the smallest HETP value, appropriate capacity factors and the best overall separation of these compounds; therefore, it was selected for use as a pairing ion for development of an assay for 7-OMEN. At this time sodium dodecyl sulfate (SDS) was evaluated and was found to give equivalent results. Since SDS is more readily available and is less expensive, it was used for all further work.

Effect of pairing ion concentration

The effect of the pairing ion concentration on the capacity factors of 7-OMEN and several related compounds is illustrated in Fig. 4. The behavior of these compounds was typical of all the compounds shown in Fig. 1. 7-OMEN and its related compounds were very strongly retained and tailed badly at very low ($\leqslant$2.5 mM) or zero pairing ion concentrations. In the author's opinion, this is due to the interaction of the positively charged tertiary amine group with unreacted silanols on the surface of the packing material. As pairing ion is added it blocks the surface silanols and/or ties up the protonated amine groups, eliminating any effect this interaction has on the retention of these compounds.

As the pairing ion concentration was increased from 10 mM to 30 mM, the k' values generally increased, as expected for ion-pair chromatography. Over this range peaks were symmetrical with very slight tailing. Above 30 mM k' values began to decrease. Several reports of such behavior have been published in the literature[21,23,24] and the theoretical basis for this behavior has been discussed by Knox and Hartwick[24] and Stranahan and Deming[25]. This phenomenon is thought to be due to a decrease in the surface tension of the mobile phase as the pairing ion con-

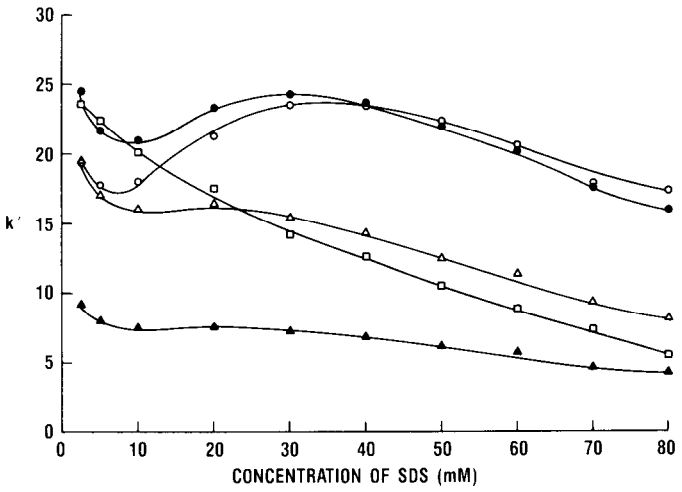


Fig. 4. Sodium dodecyl sulfate (SDS) concentration *versus* capacity factor, k'. Conditions the same as for Fig. 1. ●, 7-OMEN (IR); ○, 7-*R*-O-Methyl-N-demethylnogarol (VII); ▲, nogalamycinic acid (III); △, nogalamycin (II); □, melengestrol acetate.

centration is increased[25]. As the pairing ion concentration increases there is a steady decline in the surface tension. Eventually the reduction in surface tension exerts a great enough influence to predominate over the ionic interaction and retention times begin to decrease. The decrease in surface tension also explains why neutral species show continually decreasing k' values as pairing ion concentration is increased. Melengestrol acetate, a neutral species, exhibits this type of behavior as can be seen in Fig. 4. The behavior of these compounds appears to be well explained by this reasoning.

In the region where k' values were decreasing with increasing pairing ion concentration, the anthracycline peaks gradually broaden, indicating a loss in efficiency. The number of theoretical plates declined by 30–40% as the pairing ion concentration was increased to 80 m*M*.

The elution order of the anthracycline peaks does not change as the pairing ion concentration is increased, except for 7-*R*-O-methyl-N-demethylnogarol (VII). At low pairing ion concentration its capacity factor was less than that of 7-OMEN. At 40 m*M* their k' values were the same and at higher concentrations, VII was more strongly retained than 7-OMEN. This reversal of elution order was not surprising as a secondary amine would be expected to interact somewhat differently than a tertiary amine with the pairing ion.

Effect of water: acetonitrile ratio

Fig. 5 illustrates the effect of varying the water–acetonitrile phase ratio on the capacity factors of various compounds. From 80 to 35% acetonitrile (0.2–0.65 water–acetonitrile phase ratio) all compounds tested gave continuously increasing k' values. VanLancker *et al.*[21] reported that the capacity factors of adriamycin, adriamycinol, and nogalamycin passed through a minimum in this range of acetonitrile content in a straight reversed-phase system. In this work where the charged amine

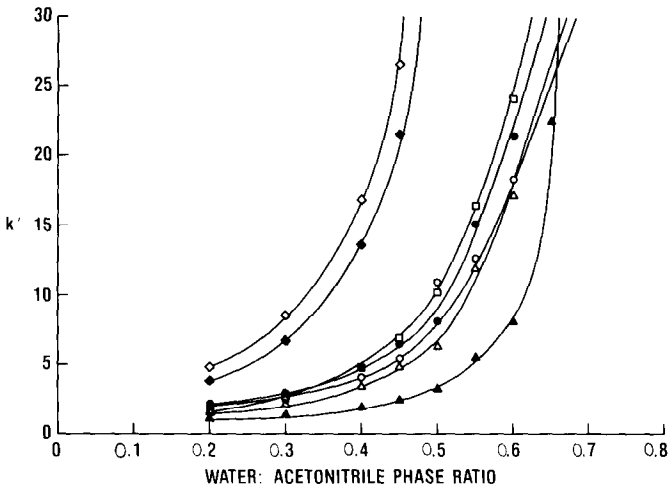


Fig. 5. Effect of variation in the water–acetonitrile phase ratio on the retention of 7-OMEN and related compounds. The mobile phase contained 20 ml tetrahydrofuran and 5 ml glacial acetic acid per l and was 5 m*M* in SDS. Flow-rate = 2.0 ml/min. ●, 7-OMEN (IR); ○, 7-*R*-O-methyl-N-demethylnogarol (VII); △, nogalamycin (II); ▲, nogalamycinic acid (III); ◇, nogalarene (X); ◆, nogarene (XI); □, melengestrol acetate.

groups were blocked by pairing ion from interacting with the surface silanol groups, no increase in retention time was observed at high acetonitrile levels. This supports the contention of VanLancker *et al.*[21] that the interaction of the amine groups with the surface silanols was the cause of the increase in retention time that they observed at high acetonitrile levels.

Effect of organic modifier

The effect of replacing acetonitrile with methanol or tetrahydrofuran was investigated. Both methanol and tetrahydrofuran were observed to give slightly different selectivities. For example, in a methanol–water system nogalamycin was observed to elute after the 7-OMEN peak. With tetrahydrofuran (THF) several compounds coeluted with 7-OMEN. Acetonitrile provided the best overall selectivity for the compounds studied, separating more of them from each other than either methanol or THF. THF was observed to give appreciably narrower peaks, however. Addition of 1–3% THF to the water–acetonitrile mobile phase sharpened the peaks, reducing the HETP by a factor of *ca.* 2. This amount of THF did not effect the elution order of the peaks but does decrease the capacity factors of all compounds by 5–10%.

Varying the amount of glacial acetic acid added to the mobile phase from 2 to 20 ml/l has essentially no effect on the chromatographic behavior of the system. Some acetic acid is necessary to insure that the amine groups are charged and to help solubilize these anthracyclines.

Mobile phase

Based on the preceding investigations a final mobile phase consisting of 600 ml water, 400 ml acetonitrile, 20 ml THF, 5 ml glacial acetic acid and 1.44 g (5 m*M*)

TABLE I

CAPACITY FACTORS (k') OF MENOGAROL AND RELATED COMPOUNDS

Compound		k'
IR	7-*R*-O-Methylnogarol	21.4
IS	7-*S*-O-Methylnogarol	14.6
II	Nogalamycin	17.2
III	Nogalamycinic acid	8.1
IV	Nogamycin	16.4
VR	7-*R*-O-Methylnogalarol	20.2
VS	7-*S*-O-Methylnogalarol	15.8
VIR	7-*R*-Nogarol	7.9
VIS	7-*S*-Nogarol	5.9
VII	7-*R*-O-Methyl-N-demethylnogarol	18.3
VIII	7-Deoxynogarol	17.3
IX	7-*R*-O-Ethylnogarol	32.1
X	Nogalarene	106.0
XI	Nogarene	130.5
	Doxorubicin	17.6
	Melengestrol acetate	24.1

sodium dodecyl sulfate was selected for use in quantitative determinations of 7-OMEN. The capacity factors of the compounds studied in this system are listed in Table I. The separation provided by this system for 7-OMEN from several of the compounds shown in Fig. 1 is illustrated in Fig. 6.

Nogarene and nogalarene are very strongly retained on this system. In samples of 7-OMEN containing greater than 5% of either of these compounds, these peaks may interfere with succeeding runs. For such samples chromatographic run time must be extended to allow these peaks to elute before another sample is injected. At

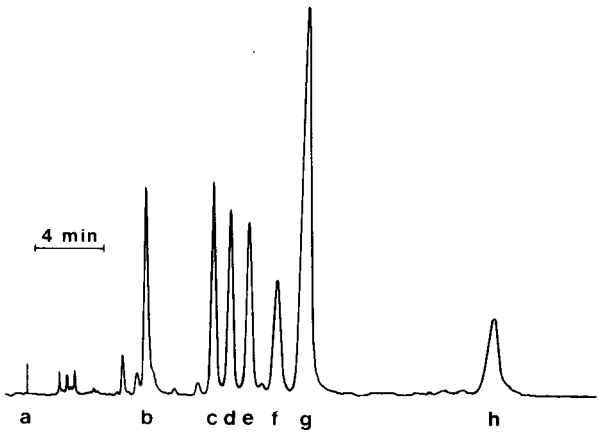


Fig. 6. Chromatogram of 7-OMEN and related compounds. Conditions same as for Fig. 1. Pairing ion was SDS. (a) Injection; (b) 7-*R*-nogarol (VI R); (c) 7-*S*-O-methylnogarol (IS); (d) nogamycin (IV); (e) nogalamycin (II); (f) 7-*R*-O-methyl-N-demethylnogarol (VII); (g) 7-*R*-O-methylnogarol (IR); (h) 7-*R*-O-ethylnogarol (IX).

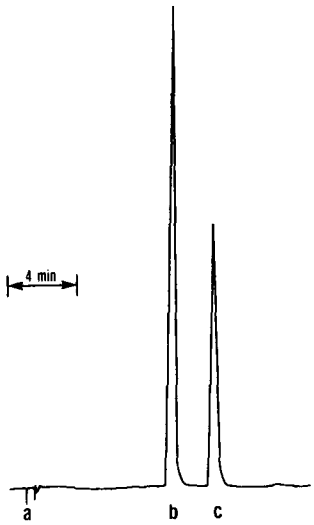


Fig. 7. Sample preparation of sterile powder formulation. Conditions same as for Fig. 6. (a) injection; (b) 7-*R*-O-methylnogarol (IR); (c) internal standard, melengestrol acetate.

levels below 5%, the nogarene and nogalarene peaks appear as little more than slow undulations in the baseline and do not affect quantitation.

This chromatographic system can also be used to quantitate the level of precursors, process impurities, and degradation products in 7-OMEN. However, it is

TABLE II

LINEARITY OF MENOGAROL BULK DRUG ASSAY

Spike (%)	*Amount added (mg/ml)*	*Peak area*		*Peak height*	
		Amount found (mg/20 ml)	*Recovery (%)*	*Amount found (mg/20 ml)*	*Recovery (%)*
50	1.018	1.007	98.9	0.988	97.0
		1.022	100.4	0.984	96.7
75	1.559	1.574	101.0	1.552	99.6
		1.607	103.1	1.549	99.4
100	2.047	2.042	99.8	2.044	99.8
		2.051	100.2	2.046	100.0
125	2.505	2.541	101.4	2.519	100.6
		2.495	99.6	2.519	100.6
150	3.159	3.220	101.9	3.161	100.1
		3.139	99.4	3.134	99.4
Mean (%)			100.6		99.3
R.S.D. (%)			1.3		1.4
Slope		1.006		1.010	
Intercept		$-0.53 \cdot 10^{-4}$		−0.026	
Correlation coefficient		0.9994		0.9998	

TABLE III

LINEARITY OF THE S. PO. MENOGAROL ASSAY

Spike (%)	*Amount added* (mg)*	*Peak area*		*Peak height*	
		Amount found (mg)	*Recovery (%)*	*Amount found (mg)*	*Recovery (%)*
50	2.389	2.297	96.1	2.312	96.8
				2.332	97.6
100	5.000	5.025	100.8	5.045	100.9
		4.974	99.5	4.965	99.3
150	7.110	6.956	98.0	6.961	97.9
		6.992	98.3	7.030	98.9
Mean (%)			98.5		98.6
R.S.D. (%)			1.8		1.5
Slope		0.986		0.991	
Intercept		0.003		−0.017	
Corr. coeff.		0.9994		0.9996	

* Spiking study performed with samples 1/10 the size of S. Po. formulation. Sample volumes reduced accordingly.

recommended that the acetonitrile content in the mobile phase be increased by 10% for this application. This decreases the retention times of nogarene and nogalarene to reasonable values. Separation of the other compounds is reduced but is adequate for most applications. Peak areas are then used to quantitate these compounds.

Quantitative determination of bulk drug and sterile powder formulation

Samples for the quantitative determinations of 7-OMEN bulk drug and sterile powder formulation were prepared as described in the experimental section. Fig. 7

TABLE IV

REPRODUCIBILITY OF THE MENOGAROL BULK DRUG ASSAY

	Sample ID	*Amount added (mg/20 ml)*	*Peak area*		*Peak height*	
			Amount found (mg/20 ml)	*Recovery (%)*	*Amount found (mg/20 ml)*	*Recovery (%)*
Day 1	1	2.025	2.089	103.2	2.017	99.6
	2	1.942	2.006	103.3	1.948	100.3
	3	2.302	2.266	98.4	2.304	100.1
Day 2	1	1.925	1.934	100.5	1.912	99.3
	2	2.373	2.348	98.9	2.377	100.2
	3	2.258	2.248	99.6	2.267	100.4
Mean (%)				100.6		100.0
R.S.D. (%)				2.1		0.4

TABLE V

REPRODUCIBILITY OF THE S. PO. MENOGAROL ASSAY, LOT A (50.00 mg/VIAL)

		mg Found	
		Peak area	*Peak height*
Day 1		50.7	50.7
		49.5	50.1
		49.5	50.5
		49.1	50.4
		49.9	50.4
		49.0	49.9
Day 2		49.8	50.7
		49.9	50.2
		49.4	50.5
	Mean	49.6	50.4
	R.S.D. (%)	1.02	0.54
	Average recovery (%) (of theory)	99.3	100.7

shows a chromatograph of a sample preparation of sterile powder 7-OMEN. There were no interferences from the excipients. The linearity of this assay was established by preparing and analyzing spiked samples covering the range 50–150% of the specified analytical concentration. For bulk drug this corresponds to solution concentrations of 0.05–0.15 mg/ml and for the sterile powder to 25–75 mg/vial. Results of these spiking studies for bulk drug and sterile powder are listed in Tables II and III, respectively. The slopes, intercepts and correlation coefficients for values obtained by both peak area and peak height are also listed in these tables. Excellent linearity and recovery is observed.

Replicate samples of the bulk drug and sterile powder formulations were analyzed on two days. Results are given in Tables IV and V for the bulk drug and sterile powder, respectively. The relative standard deviation for bulk drug was 2.1% by peak area and 0.4% by peak height. For the sterile powder the R.S.D. was 1.0% by peak area and 0.5% by peak height. Either peak heights or areas can be used for quantitation.

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