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DETERMINATION OF COUMERMYCIN A_1 IN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

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SUMMARY

Coumermycin A_1 is an antibiotic isolated from *Streptomyces hazeliensis* var. *hazeliensis* nov. sp. as a sodium salt which exhibits antistaphylococcal activity. A sensitive and selective high-performance liquid chromatographic method was developed for the determination of the compound and three known homologues which are extracted from plasma buffered to pH 6.5 into methyl-*tert.*-butyl ether—2-propanol (97.5:2.5), the residue of which is dissolved in the mobile phase and analyzed by automated reversed-phase high-performance liquid chromatography using UV detection at 330 nm for quantitation. Novobiocin is used as the internal standard. The method was used to determine the plasma concentration—time profile of coumermycin A_1 in the dog following a single intravenous administration of a 12 mg/kg dose of a solubilized dosage form of the bulk drug substance.

INTRODUCTION

Coumermycin A_1 (I, Fig. 1) is an antibiotic isolated from *Streptomyces hazeliensis* var. *hazeliensis* nov. sp. as a sodium salt which exhibits antistaphylococcal activity in vivo [1—6]. Coumermycin A_1 is a bishydroxy coumarin with two weakly acidic groups which are widely separated spatially in the molecule and therefore ionize simultaneously with an approximate pK_a of 6 [2]. The three pyrrole groups are weakly acidic, pK_a > 11, and may decrease the solubility of the compound due to intramolecular hydrogen bonding. The compound is only very slightly soluble in water at 25°C.

The pharmacokinetic profile of coumermycin A_1 was determined in four

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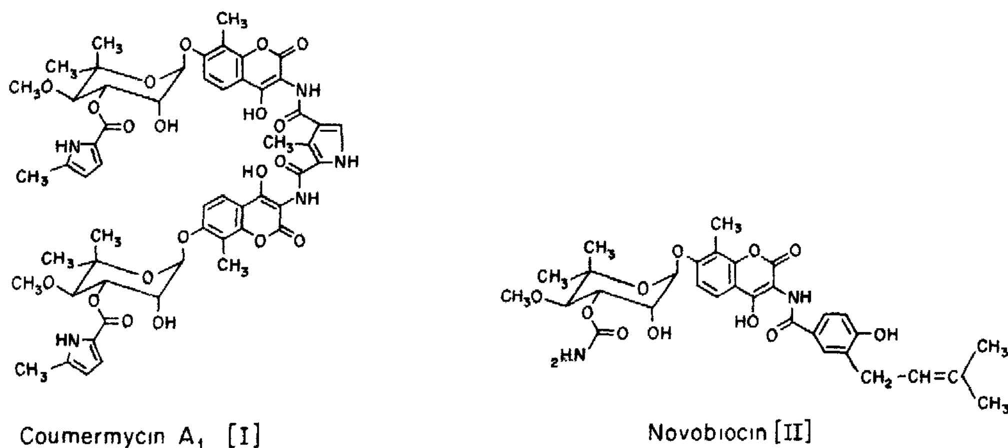

Fig. 1. Chemical structures coumermycin A_1 (I) and novobiocin (II) (internal standard) referred to in the text.

human subjects [7] using a microbiological cup plate assay employing *Staphylococcus aureus* HLR No. 82, to determine serum concentrations. The sensitivity of the method is 80 ng/ml of biological fluid [8].

The presence of biologically active homologues of I in the administered pharmaceutical product, and the possibility of biotransformation of I to several potential metabolites, prompted the development of a sensitive and selective high-performance liquid chromatographic (HPLC) assay capable of resolving compound I from known homologues (Fig. 2). Compound I and its homologues are extracted from plasma buffered to pH 6.5 into methyl-*tert.*-butyl ether—2-propanol (97.5:2.5), the residue of which is dissolved in mobile phase and analyzed by automated reversed-phase HPLC using UV detection at 330 nm for quantitation. Novobiocin (II) is used as the internal standard.

The method was used to determine the plasma concentration—time profile of I in the dog following single intravenous administration of a 12 mg/kg dose of a solubilized dosage form of coumermycin from the bulk drug substance.

EXPERIMENTAL

Reagents

All inorganic reagents were of analytical-reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.). The inorganic reagents include: 1.0 *M* phosphoric acid and the phosphate extraction buffer (400 ml 1 *M* potassium dihydrogen phosphate + 400 ml dipotassium hydrogen phosphate + 250 g potassium chloride, to form a saturated solution, adjusted to pH 6.5).

In addition, the following organic solvents were used: ethanol (200 proof, Pharmco, Publicker Industries, Philadelphia, PA, U.S.A.), a mixture of methyl-*tert.*-butyl ether—2-propanol (97.5:2.5), methanol and 2-methoxyethanol (from Burdick & Jackson Labs., Muskegon, MI, U.S.A.) Sodium lauryl sulfate

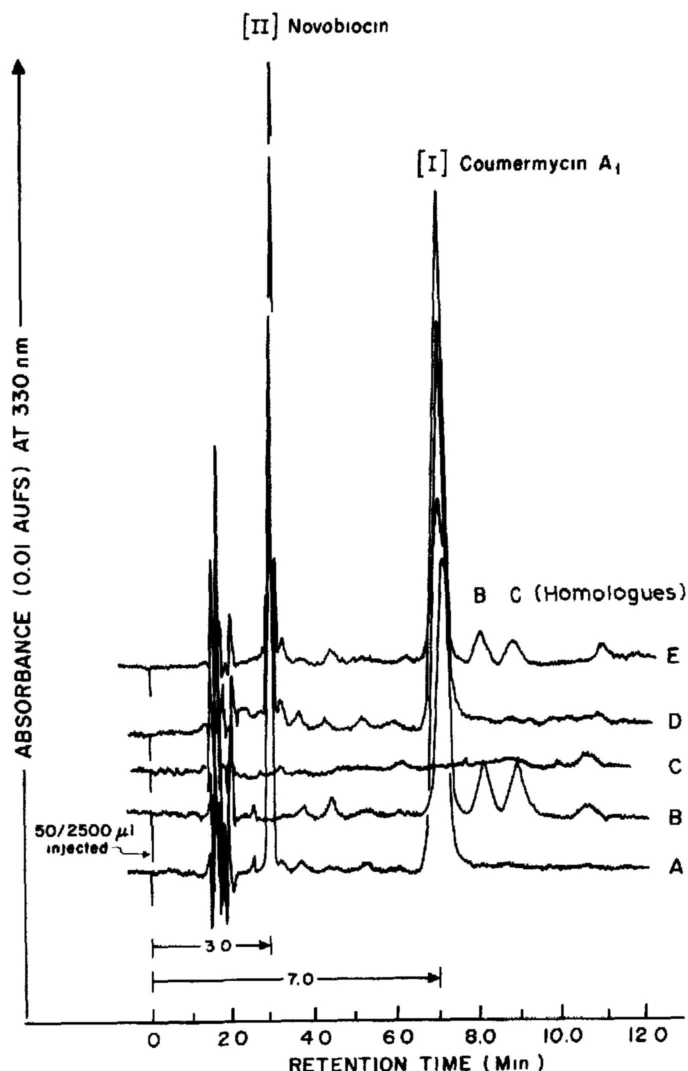

Fig. 2. Reversed-phase chromatograms of (A) authentic standard mixture containing 200 ng I and 50 ng II injected on-column; (B) authentic standards equivalent to 200 ng of I in dosing solution injected showing the presence of homologues (B and C) extracts of 0.1-ml aliquots of (C) control dog plasma and (D) control dog plasma fortified with 10 μg of I and 2.5 μg of II (internal standard) per ml of plasma; (E) dog plasma taken 1.5 h post-intravenous administration of 12 mg/kg of the dosing solution.

(Sigma, St. Louis, MO, U.S.A.) was used in the HPLC mobile phase to improve chromatographic peak shape and resolution.

Analytical standards

Compound I, coumermycin A_1, 5-methyl-1H-pyrrole-2-carboxylic acid diester with N,N'-bis{7-[(6-deoxy-5-C-methyl-4-O-methyl-α-L-lyxo-hexopyranosyl)oxy]-4-hydroxy-8-methyl-2-oxo-2H-1-benzopyran-3-yl}-3-methyl-1H-pyrrole-2,4-dicarboxamide, $C_{55}H_{59}N_5O_{20}$, molecular weight, MW = 1110.1 (anhydrous) was obtained from Sigma. Compound II, novobiocin, N-[7-{[3-O-

(aminocarbonyl)-5,5-di-C-methyl-4-O-methyl-α-L-lyxopyranosyl]oxy}-4-hydroxy-8-methyl-2-oxo-2H-1-benzopyran-3-yl]-4-hydroxy-3-(3-methyl-2-butenyl)benzamide, $C_{31}H_{36}N_2O_{11}$, MW = 612.65 (Merck Index No. 6530) was obtained from Sigma as the sodium salt, MW = 634.6 and was used as the internal standard. These two compounds were used as the analytical standards.

Preparation of standard solutions

Standard solutions were prepared in amberized glassware as follows:

Standard solution A-1. Dissolve 10.0 mg of I in 2 ml of 2-methoxyethanol plus 5 ml of ethanol (coumermycin from other sources may require 1—2 ml of tetrahydrofuran for dissolution) in a 10-ml volumetric flask. Dilute to 10.0 ml with ethanol to yield a solution containing 1.0 mg of I per ml.

Standard solution B-1. Dissolve 10.36 mg of II · Na in 2 ml of 2-methoxyethanol plus 5 ml of ethanol in a 10-ml volumetric flask. Dilute to 10.0 ml with ethanol to yield a solution equivalent to 1.0 mg of II per ml.

Preparation of working standard solutions

Aliquots of solutions A-1 and B-1 are mixed and diluted to 10.0 ml in ethanol as given in Table I.

TABLE I

PREPARATION OF WORKING STANDARD SOLUTIONS

Solution No.	Aliquots of standard (μl)		Final concentration of solution (μg per 100 μl)	
	A-1	B-1	I	II
1	1500	250	15.0	2.5
2	1000	250	10.0	2.5
3	500	250	5.0	2.5
4	250	250	2.5	2.5
5	100	250	1.0	2.5
6	50	250	0.5	2.5
7	25	250	0.25	2.5
8	—	250	—	2.5

Recovered standard curve

Aliquots of 100 μl of solution 1, 2, 3, 4, 5, 6, or 7 are evaporated in separate culture tubes and 0.1-ml specimens of control plasma are added. These calibration samples (plus a control blank without II, the internal standard) are processed along with the unknowns to establish a processed (recovered) standard calibration curve for the direct quantitation of unknowns.

External calibration curve

Aliquots of 100 μl of the above solutions (1—8) are evaporated and re-dissolved in 2.5 ml of mobile phase and then used to establish an external standard calibration curve to determine the linearity and performance of the HPLC system, and the percentage recovery of the recovered standards.

Column

The column used for reversed-phase HPLC was a pre-packed, 10 cm × 8 mm,

Radial-Pak cartridge* containing Nova-Pak® C_{18} (containing a C_{18} phase bonded to 5-μm spherical silica particles) (Waters Assoc., Milford, MA, U.S.A.).

Instrument

The HPLC system consisted of a Model 6000A reciprocating piston pump (Waters Assoc.), a Waters Intelligent Sample Processor (WISP™) Model 710B, a Waters Z-module™ radial compression system (to compress and hold the Radial-Pak cartridge), and an LDC SpectroMonitor III, UV-tunable absorbance detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The isocratic mobile phase consisted of 4.33 g of sodium lauryl sulfate (0.015 mol) dissolved in 100 ml of water and 2.0 ml of 1 *M* orthophosphoric acid (final pH = 2.8), then diluted with an additional 50 ml of water, and mixed with 50 ml of 2-methoxyethanol and 800 ml of methanol. The mobile phase was pumped at a constant flow-rate of 1.8 ml/min, resulting in a pressure of approximately 4 MPa (550 p.s.i.).

The eluent was monitored at 330 nm and the absorbance detector range was 0.010 a.u.f.s. The chart speed on the 10-mV recorder (Model 7132-A, Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP autoinjector was programmed for a 14-min run time per sample using methanol as the rinse solvent. Under these conditions 200 ng of I injected gave ≥ 100% of full scale pen response. The retention times (t_R) of I and II were 7.0 and 3.0 min, respectively, with corresponding capacity factors (k') of 4.0 and 1.1, respectively (Figs. 2 and 3). These compounds are completely resolved from three homologues of I present in the bulk drug, which elute with retention times of 6.0, 8.0 and 9.0 min (k' = 3.4, 4.8, 5.4), respectively (Fig. 4). The minimum detectable amount of I in a plasma extract, at 0.01 a.u.f.s., was 10 ng injected, equivalent to 2.5 μg/ml of plasma, using a 0.1-ml plasma sample and injecting 50/2500 μl for HPLC analysis.

Analytical procedure

Into separate 100 × 13 mm disposable borosilicate culture tubes (Cat. No. 14-962-10C, Fisher Scientific, Pittsburgh, PA, U.S.A.) was added a 100-μl aliquot of standard solution 8 (equivalent to 2.5 μg of II, as the internal standard). The solvent was evaporated at 20—25°C in an N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean dry nitrogen, a 0.1-ml aliquot of unknown plasma sample was added and the contents were mixed for a few seconds with a vortex mixer.

Along with the samples, eight 0.1-ml specimens of control plasma are processed, one to be used as a control blank and seven to be used for the preparation of the recovered standard curve (100 μl of solution 1, 2, 3, 4, 5, 6 or 7 equivalent to 15, 10, 5, 2.5, 1.0, 0.5 or 0.25 μg of I and 2.5 μg of II, respectively, were evaporated in each tube before adding 0.1 ml of control

*A conventional (15 cm × 4.6 mm I.D.) column containing Supelcosil LC-18 (5 μm) (Supelco, Bellefonte, PA, U.S.A.) can also be used at a flow-rate of 1.5 ml/min and a head pressure of 10—12 MPa (1500—1800 p.s.i.). The retention times of the respective compounds are I, 5.4; II, 2.0; and homologue peaks B, 6.2; C, 7.0; and D, 4.6 min. (Courtesy Mr. Keith Ward, Roche-Analytics®, Richmond, VA, U.S.A.).

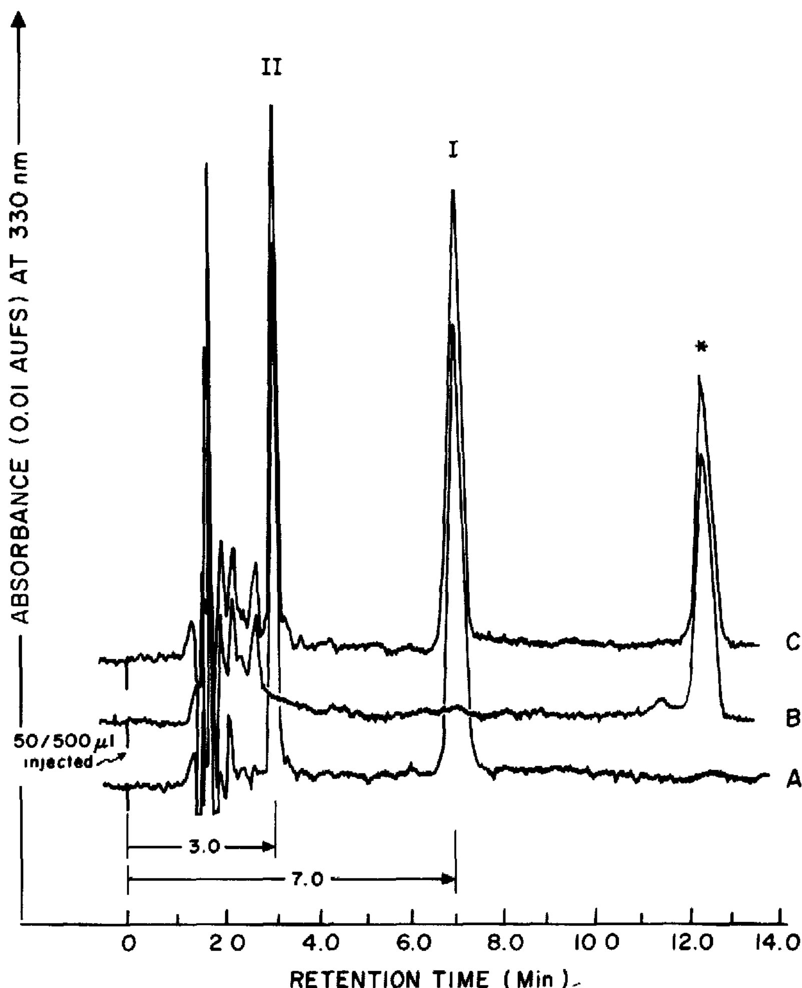

Fig. 3. Reversed-phase chromatograms of (A) authentic standard mixture containing 100 ng I and 50 ng II injected on-column; and extracts of 1.0-ml aliquots of (B) control human plasma and (C) control human plasma fortified with 1.0 µg I and 0.5 µg II (internal standard) per ml of human plasma (* = endogenous impurity).

plasma). These standards are used to establish the processed (recovered) standard curve for the direct quantitation of the unknowns and for the determination of percentage recovery by comparison with the external standard calibration curve.

To each tube, 1.0 ml of pH 6.5 phosphate extraction buffer was added; followed by the addition of 4.5 ml of the extraction solvent [methyl-*tert*.-butyl ether—2-propanol (97.5:2.5)]. Each tube was stoppered with polyethylene caps (Plugtite Cat. No. 127-0019-100, Elkay Products, Shrewsbury,

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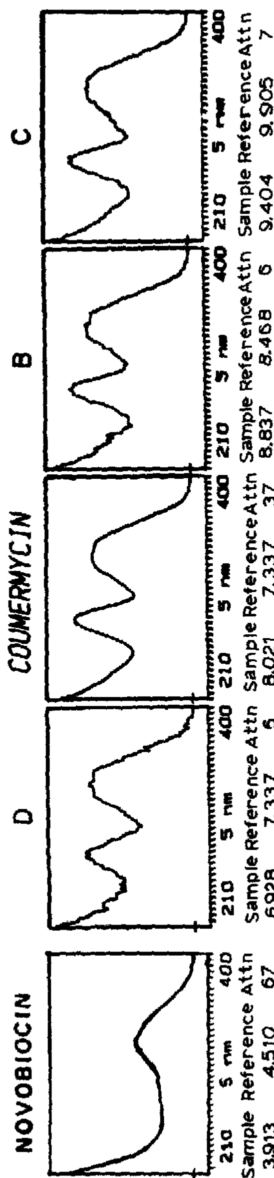
Fig. 4. Computer-generated reversed-phase chromatogram of an injection of an aliquot of a solution of bulk drug equivalent to 1.0 µg I (coumermycin A_1) showing the presence of three known homologues B, C, D with their respective UV spectra (upper panel), taken on the fly using an HP 1040A diode array UV detector.


NOVOBIOCIN
210 5 nm 400
Sample Reference Attn
3.913 4.510 67
D
210 5 nm 400
Sample Reference Attn
6.928 7,337 5
COUMERMYCIN
210 5 nm 400
Sample Reference Attn
8.021 7.337 37
B
210 5 nm 400
Sample Reference Attn
8.837 8.468 6
C
210 5 nm 400
Sample Reference Attn
9.404 9.905 7
hp 1040A
Inj Time 10:18
Attn [mAU]: 31.5 (27.0)
Zero%: 10%
Signal: B: 5,8 Set M
Date: 02/24/1984
Wavelength [nm]
1. 210, 4
2. 230, 4
3. 254, 4
4. 280, 80
5. 280, 4
6. 320, 20
7. 450, 30
8. 550,100
I
B
C
D
NOVOBIOCIN, II
ABSORBANCE
0
5
10

MA, U.S.A.) and the tubes were shaken on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at approximately 70 strokes per min for 5 min. The samples were centrifuged at 2100 rpm (1100 *g*) for 10 min at 10—15°C in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC, Needham, MA, U.S.A.). The upper organic phase was transferred into a clean culture tube, and the lower aqueous mixture extracted again with an additional 4.5 ml of solvent as before. The organic extracts were combined and evaporated at 20—25°C in an N-EVAP evaporator (Organomation Assoc.) under a stream of clean, dry nitrogen. The residue was dissolved in 2.5 ml of mobile phase (using a Vortex Mixer initially and then sonication for 2 min in an ultrasonic cleaner bath to ensure complete dissolution).

The samples were then transferred to standard 4-ml glass vials (Waters part No. 72710). Each vial was sealed with an H-style vial cap (Waters part No. 73010) fitted with a self-sealing septum. The autoinjector (WISP 710B) was programmed to inject a 50-μl aliquot out of a total volume of 2.5 ml for HPLC analysis. Typical chromatograms are shown in Fig. 2.

Calculations

The concentration of I in the unknowns was determined by interpolation from a least-squares regression equation (weighted linear equation: $y = ax + b$) of the calibration data of the recovered standards processed along with the unknowns using peak height ratio of I/II versus concentration of I per ml of plasma.

Modified assay for increased sensitivity

The sensitivity of the assay can be increased to 50 ng/ml (if needed for clinical drug monitoring purposes) by increasing the plasma sample volume extracted from 0.1 to 1.0 ml with the following modifications. Aliquots of 10 μl of each of the working standard solutions 1—6 are added into separate culture tubes. Aliquots of 1.0 ml of control human plasma and 1.0 ml of phosphate buffer, pH 6.5 are added and the samples were extracted twice with 4.5-ml aliquots of the extraction solvent mixture as before. After centrifugation the respective extracts are combined, evaporated to dryness and the residue dissolved in 0.5 ml of mobile phase and solubilized by ultrasonication for at least 5 min. The samples are centrifuged and 0.3—0.4 ml of the clear supernatant is transferred into a Waters plastic low-volume insert (part No. 72030) held in a standard 4-ml glass vial (Waters part No. 72711) by a compression spring (part No. 72708). Each vial is sealed with a cap containing a self-sealing septum (part No. 73010) and the vial is tapped to dislodge any air bubbles trapped at the bottom of the low-volume insert. The autoinjector (WISP 710B) is programmed to inject 100 μl out of a total volume of 0.5 ml for HPLC analysis. The concentration of I in the unknowns was determined as before. Typical chromatograms are shown in Fig. 3.

RESULTS AND DISCUSSION

Coumermycin A_1, compound I, is a lipophilic, amphoteric compound (MW = 1110) which is extractable into organic solvents at pH 6.5. A reversed-phase

HPLC assay was developed for the determination of coumermycin A_1, I, and its three known homologues. Sodium lauryl sulfate improves the peak shape and the retention characteristics of I. Novobiocin, compound II, an analogous compound containing a coumarin moiety in the molecule (Fig. 1), was chosen as the internal standard for the analysis of I in plasma due to compatible extraction and chromatographic behavior under the conditions of this assay.

The UV spectrum of I in the HPLC mobile phase showed absorbance maxima at less than 210 nm, at 280 nm and at 330 nm. The UV spectrum of II in the HPLC mobile phase showed absorbance maxima at less than 210 nm and at 325 nm (Fig. 4). Reversed-phase HPLC analysis of the residue of methyl-*tert.*-butyl ether—2-propanol (97.5:2.5) extracts of plasma buffered to pH 6.5 showed that endogenous constituents in the control plasma precluded quantitation of I at wavelengths shorter than 290—300 nm. However, the absorbance at 330 nm is sufficient for UV quantitation of 5 ng of I injected into the HPLC system.

Chromatographic resolution was made even more stringent due to the presence of biologically active homologues of coumermycin in the bulk drug. These homologues (designated as B, C and D) have similar UV absorbance spectra, as indicated in Fig. 4, which are UV spectra of their peaks seen in the chromatogram and taken on the fly at a flow-rate of 1.4 ml/min using a diode array HPLC detector (HP 1040A, Hewlett-Packard). The homogeneity of each peak seen in the chromatogram of bulk material (Fig. 4) is demonstrated in Fig. 5 which represents an isoabsorbance contour plot of a chromatogram of a solution of the bulk material containing added novobiocin (internal standard). The symmetrical nature of the contour plot for I seen at a retention time of 8.0 min indicates homogeneity [9]. The presence of unresolved components

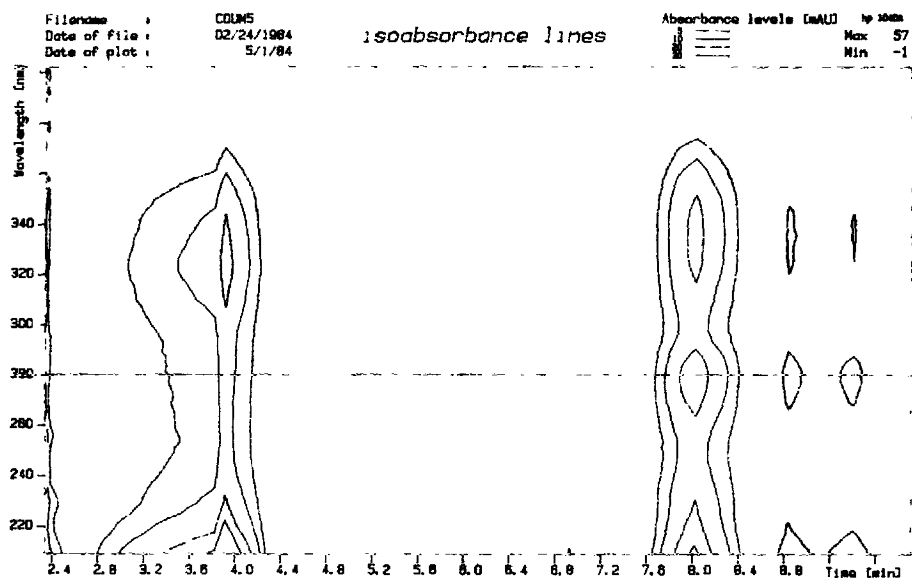


Fig. 5. Computer-generated contour plot of the isoabsorbance lines in the UV spectra of coumermycin A_1 (I), its known homologues, and novobiocin (II) (internal standard) taken on the fly using an HP 1040A diode array UV detector.

such as isomers or homologues would tend to distort the contour plot. Only two homologues (B and C) of coumermycin are seen in the contour plot as weak areas at retention times of 8.8 and 9.4 min, respectively. The concentration of homologue D is probably too low for the detection in the isoabsorbance spectral mode. Coumermycin and its known homologues have also been characterized by pyrolytic gas–liquid chromatography and UV spectroscopy [10].

Assay validation

In the original assay the sensitivity limit was 2.5 μg/ml, calibration curves for I were linear from 2.5 to 150 μg of I per ml of dog plasma using a 0.1-ml specimen. The correlation coefficient (r) for a typical curve ($y = 0.0124x - 0.0062$) was 0.9976 and the average percentage deviation of the calibration points from the line was 8.8. Intra- and inter-assay validation data over the linear concentration range of I are summarized in Table II, and yielded mean intra- and inter-assay coefficients of variation of 8.2% and 8.8%, respectively.

In the modified assay used for higher sensitivity, the calibration curves for I were linear from 0.05 to 2.5 μg of I per ml of human plasma using a 1.0-ml specimen. The correlation coefficient (r) for a typical curve ($y = 0.5496x +$

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COUMERMYCIN A_1 IN 0.1 ml OF DOG PLASMA

Number of samples	Amount added (μg/ml)	Amount found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Intra-assay variability			
3	150.00	148.60 ± 11.01	7.41
3	125.00	123.40 ± 2.59	2.10
3	87.50	91.53 ± 1.88	2.05
3	50.00	53.15 ± 1.07	2.02
3	37.50	37.69 ± 0.57	1.52
3	25.00	22.91 ± 1.69	7.36
3	12.50	11.34 ± 0.95	8.34
3	5.00	5.33 ± 0.45	8.37
3	2.50	2.96 ± 1.02	34.60
		Average =	8.20
Inter-assay variability			
3	150.00	161.80 ± 17.31	10.70
4	125.00	117.32 ± 10.49	8.94
3	87.50	88.60 ± 2.95	3.33
4	50.00	53.87 ± 2.35	4.36
4	37.50	37.32 ± 3.89	10.43
4	25.00	26.52 ± 2.82	10.62
4	12.50	11.61 ± 0.83	7.12
4	5.00	5.07 ± 0.24	4.82
3	2.50	2.58 ± 0.48	18.90
		Average =	8.80

TABLE III

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COUMERMYCIN A_1 IN 1.0 ml OF HUMAN PLASMA

Number of samples	Amount added (μg/ml)	Amount found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Intra-assay variability			
4	2.50	2.42 ± 0.120	4.75
4	1.00	1.03 ± 0.080	7.81
4	0.75	0.78 ± 0.040	4.85
4	0.50	0.53 ± 0.030	6.51
4	0.25	0.27 ± 0.010	4.82
4	0.10	0.11 ± 0.020	15.41
3	0.05	0.04 ± 0.004	7.81
		Average =	7.42
Inter-assay variability			
4	2.50	2.49 ± 0.090	3.64
4	1.00	0.97 ± 0.080	8.44
4	0.75	0.78 ± 0.010	1.57
4	0.50	0.53 ± 0.020	3.85
4	0.25	0.26 ± 0.005	1.81
4	0.10	0.10 ± 0.009	9.05
4	0.05	0.046 ± 0.004	9.33
		Average =	5.38

TABLE IV

PLASMA CONCENTRATIONS OF COUMERMYCIN A_1 (I) AND TWO HOMOLOGUES (PRESENT IN THE BULK DRUG) DETERMINED IN THREE DOGS FOLLOWING A 12 mg/kg INTRAVENOUS ADMINISTRATION OF THE DOSING SOLUTION

Time (h)	Concentration (μg/ml)											
	Dog A				Dog B				Dog C			
	I	B	C	Total	I	B	C	Total	I	B	C	Total
0	NM*	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
0.05	106	22.6	22 1	151	117	21.2	20.6	159	102	17.4	16.3	136
0 08	95	19 3	17 0	131	121	21 6	20 8	163	99	16 9	16.6	133
0 17	96	19.1	16.2	131	119	21 4	20.6	161	85	14 4	13.8	113
0 33	117	23.3	19.0	159	107	19 6	20.0	147	88	15.1	14.3	117
0 50	76	14.9	11.9	103	87	16.3	16.1	119	72	13.0	12.6	99
0 75	51	9.8	8 4	69	70	14.2	14.5	99	66	11.4	11.2	89
1.0	50	9.9	7.5	67	64	13.0	14.0	91	56	10.0	10.1	76
1.5	51	10.3	7.7	69	53	10.4	12.1	76	43	7.4	8.3	59
2.0	24	4.8	3.8	33	48	9.6	11.6	69	36	6.6	7.1	49
3.0	NS**	—	—	NS	32	6.6	8.3	47	25	4.8	5.3	36
4.0	NS	—	—	NS	29	6.0	7.3	42	24	4.5	5.0	34
5.0	15	2.5	2 6	20	28	4.9	7.1	40	24	4.7	4.8	34
7 0	17	3.1	2.3	22	15	3 9	4.1	23	14	3 0	3 0	20
24.0	5 2	NM	NM	5.2	5 4	NM	NM	5.4	6 2	NM	NM	6.2

*NM = Not measurable < 2.5 μg/ml
**NS = No sample.

TABLE V

PHARMACOKINETIC PARAMETERS OF INTRAVENOUSLY ADMINISTERED COUMERMYCIN IN THE DOG

Dose: 12 mg/kg of body weight.

Dog	Weight (kg)	Amount of drug administered (mg)	$AUC_{0-24\,h}$		$AUC_{0-\infty}$		Elimination rate (β)		Elimination half-life ($t_{1/2}$)***	
			I	Total*	I	Total	I	Total	I	Total
A	16.3	195.6	402	520	487	585	0.061	0.079	11.3	8.7
B	14.8	177.6	461	652	532	707	0.076	0.098	9.1	7.1
C	12.8	153.6	409	514	509	591	0.062	0.082	11.2	8.5
Mean	14 6	175.6					0.067	0.086	10.3**	8.1**
Mean ± S.D.			424 ± 32	562 ± 78	509 ± 22	628 ± 69				

**Total* = *total active coumermycins*, i.e. I plus *homologues* B and C.

**Harmonic mean $t_{1/2}$ = 0.693/mean β.

***Recent studies with multiple oral doses and plasma sampling extended to 96 h indicate a longer $t_{1/2\beta}$ of 24 h. The mean value reported herein of 10.3 h appears to be an underestimate.

0.0041) was 0.9987 and the average percentage deviation of the calibration points from the line was 5.2. Intra- and inter-assay validation data over the linear concentration range of I are summarized in Table III. The mean intra- and inter-assay coefficients of variation are 7.4% and 5.4%, respectively.

Percentage recovery and sensitivity limits

The apparent overall recovery of I from plasma is ⩾ 100% (a biological matrix effect increases the peak height of the compound by 5% to 10%). The sensitivity limit of the assay is 2.5 μg of I per ml of plasma using a 0.1-ml plasma sample, and may be increased (if needed) to 0.05 μg per ml of plasma using a 1-ml specimen. The assay is specific for I and its known homologues by virtue of chromatographic separation, and peaks due to endogenous impurities are fully resolved from that of the parent drug for accurate quantitation.

Stability of I

Solutions of I were stable for two to three weeks when stored at 5°C in their respective solvents. Compound I was stable for up to 6 h in fresh control human plasma at ambient temperature, so that the usual sample plasma collection technique can be used without any special precautions.

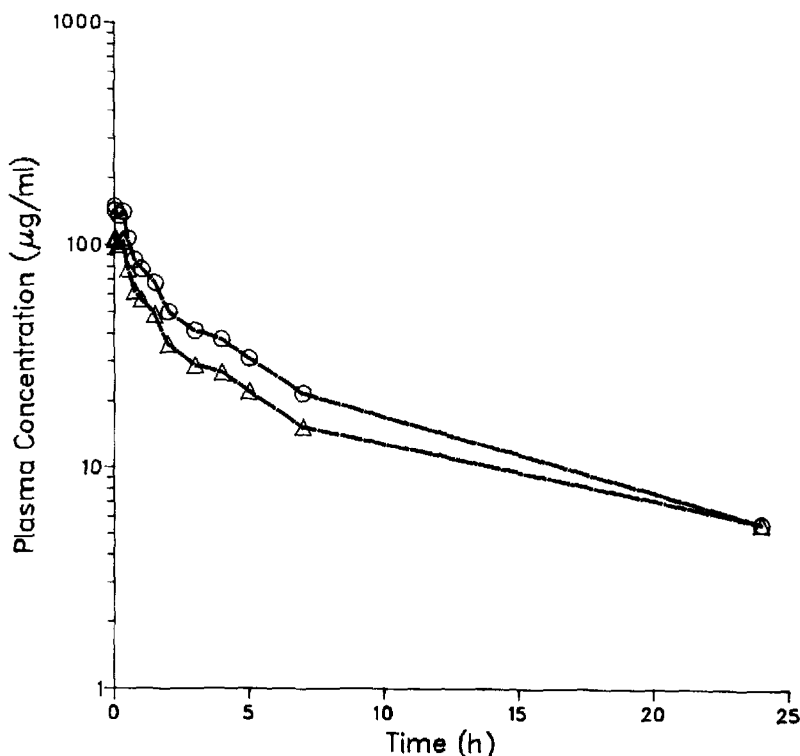


Fig. 6. Mean plasma concentration—time profiles of coumermycin A_1 (I) and total coumermycins (i.e. I and homologues B and C) in three dogs following a 12 mg/kg intravenous administration of a solution of the bulk drug. (○) Total coumermycins; (△) mean coumermycin A_1.

Application of the method to biological specimens

Plasma concentrations of I were determined by HPLC in three dogs following an intravenous dose of 12 mg of I · 2Na per kg of body weight (Table IV), calculated to contain 46% I and 18% homologues B and C, i.e. 64% total active coumermycins against the pure (99%) Sigma standard. Homologue D was not present in this sample lot of bulk drug. The homologue content varies from lot to lot, hence it is important to assay each lot prior to dosing to determine the exact composition of the material. The pharmacokinetic parameters are summarized in Table V. The mean plasma concentration—time profile of I in the three dogs (Fig. 6) shows a triphasic decline with an apparent half-life of elimination ($t_{½\beta}$) of about 10 h. The concentration of the homologues B and C (Fig. 2) in the plasma was determined against the Sigma standard. Their plasma concentration—time profiles appeared to parallel that of the parent compound I, and their concentration appears to reflect what was initially present in vitro in the administered dosage form, rather than to any biotransformation in vivo of I to either of the components detected. This is reflected in the mean plasma concentration—time profile of the total active coumermycins; i.e. I and homologues B and C versus time (h) seen in Fig. 6 which is also triphasic and parallels that for I; i.e., coumermycin A_1 per se. It is also indicated in the ratio of the area under the curve (AUC) values (Total/I) ranging from 1.2 to 1.4 for either $AUC_{0-24\,h}$ or $AUC_{0-\infty}$ which are constant for the three dogs studied. The biotransformation of coumermycin A_1 has not yet been elucidated using radiolabelled drug.

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REFERENCES

1 E. Grunberg and M. Bennett, Antimicrob. Agents Chemother., (1965) 786.
2 J. Berger, A.J. Schocher, A.D. Batcho, B. Pecherer, O. Keller, J. Maricq, A.E. Karr, B.P. Vaterlaus, A. Furlenmeier and H. Spiegelberg, Antimicrob. Agents Chemother., (1965) 778.
3 J.C. Godfrey and K.E. Price, in D. Perlman (Editor), Structure—Activity Relationships Among the Semi-Synthetic Antibiotics, Academic Press, New York, 1977, pp. 653—717.
4 J.C. Godfrey, Adv. Appl. Microbiol., 27 (1981) 125—136.
5 H.C. Neu, N.-X. Chin and P. Labthavikul, Antimicrob. Agents Chemother., 25 (1984) 687.
6 J. Unowsky, W. de Lorenzo, L. Gulow and R. Cleeland, Proceedings 23rd Interscience Conference Antimicrobial Agents and Chemotherapy, Las Vegas, NV, October 24, 1983, Abstract No. 541.
7 S.A. Kaplan, J. Pharm. Sci., 59 (1970) 309.
8 J. Unowsky and M. Douglas, unpublished data on file, Hoffmann-La Roche, Nutley, NJ, 1968.
9 A.F. Fell, B.J. Clark and H.P. Scott, J. Pharm. Biomed. Anal., 1 (1983) 557.
10 J. Scannell, Antimicrob. Agents Chemother., (1967) 470.