QUANTITATIVE DETERMINATION OF ACIVICIN IN BULK DRUG AND PHARMACEUTICAL FORMULATIONS BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An ion-pair reversed-phase high-performance liquid chromatographic method for the determination of the purity of acivicin and the amount of drug in a sterile powder and two sterile solution formulations is described. The method displays good recoveries (98.4–100.4%) for all formulations and a linear range of 0.002–20 μg of drug injected. Estimates of assay precision were 1.3% for the bulk drug, 0.6% for sterile solution formulations and 1.6% for the sterile powder formulations.

INTRODUCTION

Acivicin (U-42,126, AT-125, NSC-163501) is a promising antineoplastic agent undergoing Phase II clinical studies under the sponsorship of the National Cancer Institute (NCI). It was originally isolated as a fermentation product of *Streptomyces sveceus*[1,2]. While the majority of the drug is presently produced via fermentation, several synthetic routes have been described[3–7].

Several methods have been reported for the determination of the purity of acivicin or its fermentation derived analogue, hydroxyacivicin (see Fig. 1). Microbiological assays and others employing circular dichroism or thin-layer chromato-
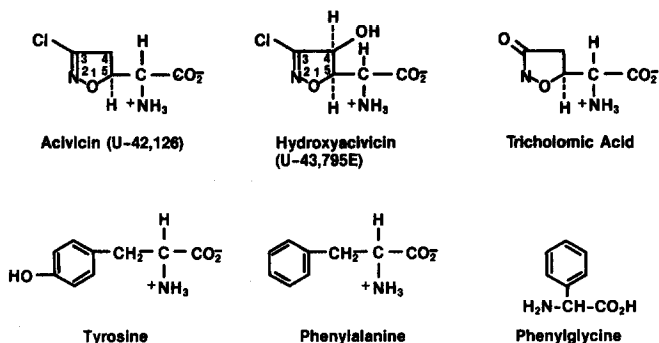

Fig. 1. Structures of acivicin, fermentation by-products and internal standard.

graphy[2,8] were found adequate for relatively pure samples of these compounds. Recently an enzyme inhibition assay[9] and a gas chromatography–mass spectrometry (GC–MS) assay[10] for the quantitation of acivicin levels in plasma have been described.

This report describes an ion-pair reversed-phase high-performance liquid chromatography (HPLC) assay for the determination of the purity of acivicin and the amount of drug in a sterile powder and two sterile solution formulations. The method is capable of separating acivicin from process impurities, degradation products and related compounds.

EXPERIMENTAL

Chemicals

All chemicals were analytical grade. Acetonitrile was obtained from Burdick & Jackson, Muskegon, MI, U.S.A. Ion-pair reagents were obtained from Aldrich, Milwaukee, WI, U.S.A. Acivicin was obtained from The Upjohn Company, Kalamazoo, MI, U.S.A. Hydroxyacivicin and tricholomic acid were kindly provided by D. G. Martin, Cancer and Viral Diseases Research, The Upjohn Company.

HPLC conditions

The chromatographic system consisted of an Altex 110 pump, a Waters Assoc. WISP® or Tracor Autosampler and an LDC UV Monitor III with a zinc lamp and 214-nm filter. Detection was performed at 214 nm. Waters Assoc. μBondapak C_{18} columns (30 cm × 0.39 cm I.D.) were employed. In addition a silica saturation column (30 cm × 0.39 cm I.D.), dry-packed with 50–200 mesh silica, 2 μm in-line filter and a Waters Assoc. Guard-Pak™ C_{18} guard column were employed. The flow-rate was normally set at 2.0 ml/min. Column pressure was normally 2500 p.s.i. A 20-μl injection volume was used. All calculations were based on peak area ratios.

The mobile phase consisted of 995 ml of deionized water, 5 ml of acetonitrile, 0.8 ml of perchloric acid, plus 1.88 g of hexanesulfonic acid and 0.62 g of dimethylcyclohexyl sulfate per liter of mobile phase. The pH of the mobile phase was adjusted to 2.6 ± 0.1 with sodium hydroxide. The mobile phase was filtered and degassed before use.

Spiking study

Spiked samples of the sterile solution formulation were produced by placing appropriate amounts of drug in a solution containing the correct proportions of the excipients. Spiked samples for the sterile powder formulation were prepared by dissolving the appropriate amount of acivicin in 2 ml of a solution containing the necessary amount of excipients, neutralizing to pH 7.0 with 0.1 N sodium hydroxide, freezing and lyophilizing the solution.

Analysis of Bulk Drug

Samples and standards were prepared by accurately preparing a 0.1 mg/ml solution of the appropriate material in internal standard solution (0.1 mg/ml phenylglycine in mobile phase).

Analysis of sterile solution formulation (10 mg/ml) and sterile powder formulation (25 mg/vial)

Standards were prepared by accurately preparing a 0.2 mg/ml solution of standard in internal standard solution (0.2 mg/ml phenylglycine in mobile phase). The standard was then diluted 1:1 with deionized water. Sterile solution samples were prepared by accurately transferring 1.0 ml of the formulation to a 10.0-ml volumetric flask and filling to volume with mobile phase. This was mixed thoroughly and 1.0 ml of this solution was transferred to a 10.0-ml volumetric flask, 5.0 ml of internal standard solution (0.2 mg/ml phenylglycine in mobile phase) were added and the flask was filled to volume with mobile phase. Sterile powder samples were prepared by reconstituting the freeze dried cake in 5.0 ml of deionized water. The sample was sonicated until the contents dissolved. Exactly 1.0 ml was accurately transferred to a 5.0-ml volumetric flask and the flask was filled to volume with mobile phase and mixed thoroughly. Exactly 1.0 ml of this solution was transferred to a 10.0-ml volumetric flask, 5.0 ml of internal standard solution (0.2 mg/ml phenylglycine in mobile phase) were added, and the flask was filled to volume with mobile phase.

RESULTS AND DISCUSSION

Shown in Fig. 1 are the structures of some of the species separated on this system. Hydroxyacivicin is a fermentation by-product of the production of acivicin, while tricholomic acid is a degradation product. Phenylglycine is the internal standard of the assay and tyrosine and phenylalanine are possible contaminants from the fermentation broth.

Because of the zwitterionic form of acivicin and its analogues it was necessary to use reversed-phase, rather than normal-phase separation conditions. Trimethyl-, octyl-, and octadecylsilane bonded phase packings were evaluated, with the octadecyl packings providing the best peak shape and retention behavior. The effects of varying the different components of the mobile phase were as expected. Fig. 2 shows the effects of varying the amount of acetonitrile in the mobile phase. As expected, in-
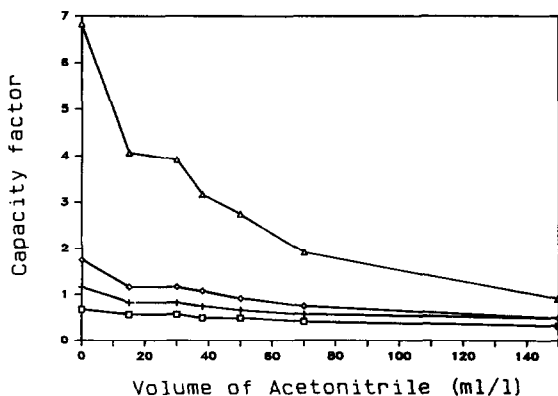

Fig. 2. Effect of the amount of acetonitrile in the mobile phase on the retention of acivicin and related compounds. Data points: □ = tricholomic acid; + = hydroxyacivicin; ◇ = acivicin; △ = phenylglycine.
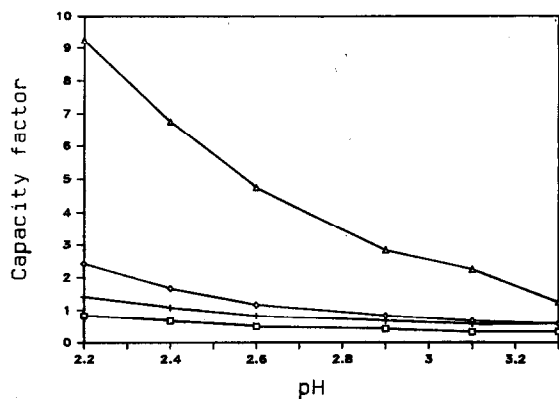
Fig. 3. Effect of pH on the retention of acivicin and related compounds. Symbols as in Fig. 2.
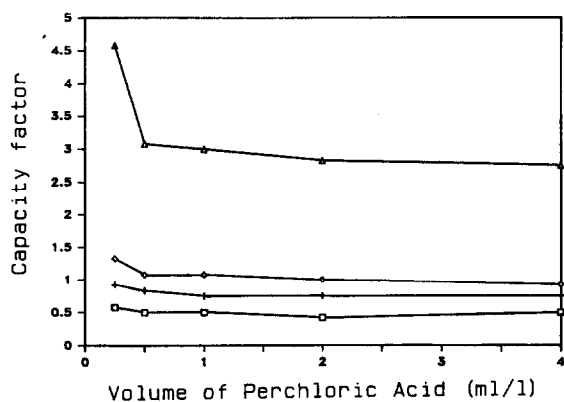
Fig. 4. Effect of perchloric acid concentration at a controlled pH of 2.6 on the retention of acivicin and related compounds. Symbols as in Fig. 2.
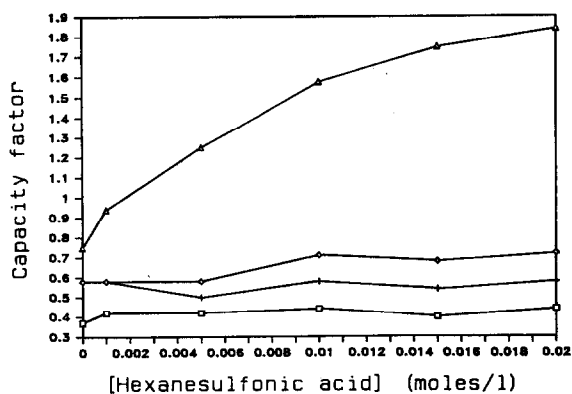
Fig. 5. Effect of the concentration of hexanesulfonic acid on the retention of acivicin and related compounds. Symbols as in Fig. 2.

creasing the concentration of the organic modifier decreases the capacity factors of all compounds. Methanol, 2-propanol and tetrahydrofuran were also tried as modifiers but were rejected because they gave increased background and/or gave retention behavior very similar to that observed with acetonitrile. The effect of the mobile phase pH on retention behavior is shown in Fig. 3. As expected, increasing the pH causes decreasing capacity factors. At a mobile phase pH of *ca*. 3, acivicin and hydroxyacivicin begin to coelute. Fig. 4 shows the effects of the perchloric acid concentration (at constant pH) on these capacity factors. Over the limited range studied increasing ionic strength leads to decreasing capacity factors.

In order to obtain sufficient separation and resolution, it was necessary to add an ion-pair reagent to the mobile phase. Various sulfonic acid ion-pair reagents were evaluated and hexanesulfonic acid was found to give the best separation. The most pronounced effect of changing the ion-pair reagent was on the internal standard, phenylglycine, which showed a rapidly increasing capacity factor when the size of the ion-pair reagent was increased. The capacity factors of acivicin and its analogues showed little change as the ion-pair reagent was varied.

Fig. 5 shows the effects of the hexanesulfonic acid concentration on the capacity factors of the compounds. In the range covered, increasing concentrations of hexanesulfonic acid produced increasing capacity factors.

In this system, the concentration of the dimethylcyclohexyl sulfate was kept constant at a relatively low level of 0.0025 *M*. This ion-pairing reagent was first used during assay development when it was discovered that it separated a hitherto poorly resolved impurity seen in some samples of synthetically produced drug. Shown in Fig. 6 is an example of this improvement in chromatography. Availability considerations mitigated against the total substitution of this ion-pairing reagent for the hexanesulfonic acid. The effects of this ion-pairing reagent are most pronounced with older columns, where the use of hexanesulfonic acid alone (in concentrations up to 0.05 *M*) will not adequately resolve this peak from the acivicin peak.

Examples of the chromatography obtained for a reference standard solution and a sterile powder sample preparation are shown in Fig. 7. No interferences are seen. Chromatograms obtained for the sterile solution formulations are shown in Fig. 8. Again, no interferences are seen in either case. Note that the preservative
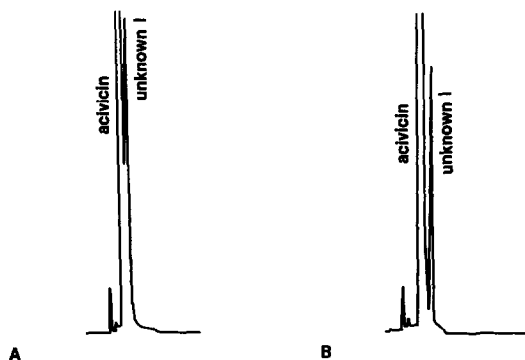

Fig. 6. Chromatograms of bulk drug (B) with and (A) without dimethylcyclohexylsulfate in the mobile phase.
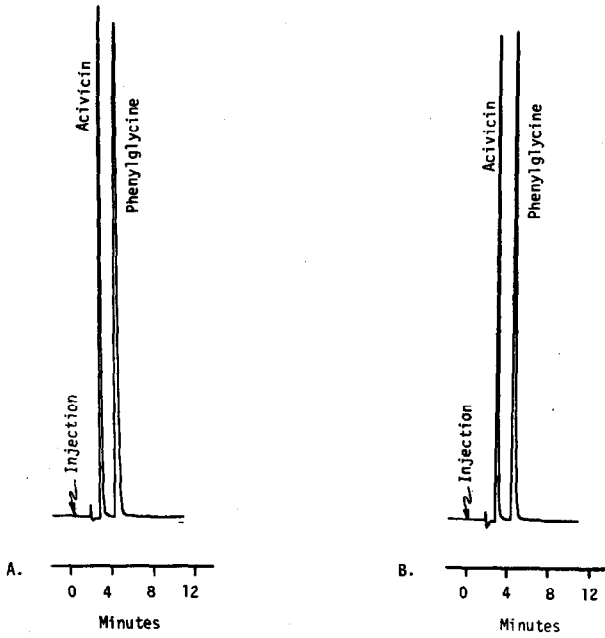
Fig. 7. Chromatograms of a reference preparation (A) and a sterile powder sample preparation (B).
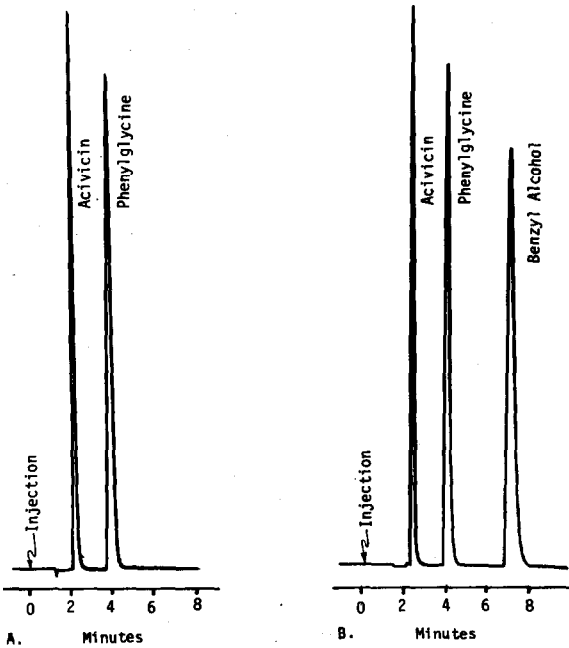

Fig. 8. Chromatograms of preparations of the sterile solutions without (A) and with (B) the preservative.

TABLE I

RECOVERY OF ACIVICIN BULK DRUG FROM SAMPLE PREPARATIONS AT 50–150% OF THE RECOMMENDED CONCENTRATION

Amount added (mg/10 ml)	Amount recovered (mg/10 ml)	% of theory
0.479	0.482	100.6
0.736	0.734	99.7
	0.735	99.9
1.005	1.009	100.4
	1.008	100.3
1.241	1.237	99.7
	1.237	99.7
1.550	1.549	99.9
	1.549	99.9
	Mean	100.0
	R.S.D.	0.3%

(benzyl alcohol) in the sterile solution formulation is well resolved from any peaks of interest.

The linear range of the detector response was established to be 0.002–20 μg of drug injected on column. Over this range, linear regression analysis gives a line with a slope of $1.1 \cdot 10^9$ peak area response units per milligram of drug injected with a correlation coefficient of 1.000. A plot of peak height response *versus* amount of drug injected is not linear as it displays a negative deviation with high sample loads (linear

TABLE II

RECOVERY OF ACIVICIN FROM A PROTOTYPE STERILE SOLUTION FORMULATION WITHOUT PRESERVATIVES

Concentration of label, 10 mg/ml.

Amount added (mg/ml)	Amount recovered (mg/ml)	Recovery (%)
4.292	4.162	97.0
	4.211	98.1
7.153	6.967	97.4
	7.004	97.9
8.941	8.780	98.2
	8.788	98.3
11.921	11.763	98.7
14.901	14.921	100.1
	14.851	99.7
	Mean	98.4
	R.S.D.	1.0%

TABLE III

RECOVERY OF ACIVICIN FROM A PROTOTYPE STERILE SOLUTION FORMULATION WITH PRESERVATIVE (BENZYL ALCOHOL)

Concentration of label, 10 mg/ml.

Amount added (mg/ml)	Amount recovered (mg/ml)	Recovery (%)
4.606	4.619	100.3
	4.573	99.3
7.687	7.740	100.8
	7.706	100.4
9.596	9.677	100.8
	9.393	97.9
11.995	12.182	101.6
	12.110	101.0
14.994	15.259	101.8
	15.020	10.2
	Mean	100.4
	R.S.D.	1.1%

range of *ca.* 0.02–2 μg injected). Such behavior is not unusual with ion-pair HPLC assays.

The recovery of acivicin from bulk drug, sterile solution and sterile powder sample preparations was evaluated over a range of *ca.* 50–150% of the recommended

TABLE IV

RECOVERY OF ACIVICIN FROM A STERILE POWDER FORMULATION

Concentration of label, 25 mg per vial.

Amount added (mg per vial)	Amount recovered (mg per vial)	Recovery (%)
0.00	0.0	—
11.87	11.66	98.2
	11.66	98.2
18.30	18.06	98.7
	18.08	98.8
25.33	25.22	99.6
	25.25	99.7
30.67	31.05	101.2
	31.02	101.1
38.13	38.11	99.9
	38.45	100.8
	Mean	99.6
	R.S.D.	1.1%

TABLE V

ESTIMATED PRECISION OF THE POTENCY ASSAY FOR ACIVICIN BULK DRUG AND ACIV-
ICIN IN STERILE SOLUTION AND STERILE POWDER FORMULATIONS

	Bulk drug	Sterile solution without preservative	Sterile solution with preservative	Sterile powder
R.S.D. Day 1	1.0%	0.5%	1.0%	1.3%
R.S.D. Day 2	0.3%	0.8%	0.1%	2.0%
R.S.D. Overall	1.3%	0.6%	0.7%	1.6%

assay concentration. These data are presented in Tables I–IV for the bulk drug, the
sterile solutions and the sterile powder, respectively. No interferences were seen from
the excipients in either of the sterile solution formulations or the sterile powder for-
mulation. Excellent recovery (98–100%) was seen in all cases.

The overall precision of the assays was estimated from the results of six rep-
licate sample preparations performed over a two day period. These data are summa-
rized in Table V for the bulk drug, sterile solution and sterile powder formulations.
Overall precision was 1.3% for the bulk drug, 0.6% for the sterile solution formu-
lations and 1.6% for the sterile powder formulation.

REFERENCES

1 L. J. Hanka and A. Dietz, Antimicrob. Agents Chemother., 3 (1973) 425.
2 L. J. Hanka, S. A. Gerpheide, P. R. Spieles, D. G. Martin, P. A. Belter, T. A. Coleman and H. F.
  Meyer, Antimicrob. Agents Chemother., 7 (1975) 807.
3 R. C. Kelly, I. Schletter, S. J. Stein and W. Wierenga, J. Am. Chem. Soc., 101 (1979) 1954.
4 A. A. Hagedorn, B. J. Miller and J. O. Nagy, Tetrahedron Lett., 21 (1980) 229–230.
5 J. E. Baldwin, L. L. Kruse and J. K. Cha, J. Am. Chem. Soc., 103 (1981) 942–943.
6 R. B. Silverman and M. W. Holladay, J. Am. Chem. Soc., 103 (1981) 7357–7358.
7 P. A. Wade, M. K. Pillay and S. M. Singh, Tetrahedron Lett., 23 (1982) 4563–4566.
8 D. G. Martin, C. G. Chidester, S. A. Mizsak, D. J. Duchamp, L. Baczynskyj, W. C. Krueger, R. J.
  Wnuk and P. A. Meulman, J. Antibiotics, 28 (1975) 91.
9 H. N. Jayaram, T. W. Kensler and B. Ardalan, Cancer Treatment Rep., 65 (1981) 491.
10 J. P. McGovren, M. G. Williams, R. H. Robins and B. L. Roach, Anal. Chem., 57 (1985) 2046.