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Note

Stability-indicating high-performance liquid chromatography assay for the anticancer drug bryostatin 1

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In 1968[1] we observed that extracts of the marine bryozoan *Bugula neritina* (Linnaeus) provided exceptional antineoplastic activity (100% life extension) against the U.S. National Cancer Institute's (NCI) murine P388 lymphocytic leukemia (PS system). Fourteen years later we reported[2] the isolation and X-ray crystal structure of bryostatin 1 the most prominent member of a new series[3] of macrocyclic lactones with remarkable biological properties. For example, against the PS leukemia bryostatin 1 has provided 52–96% life extension at 10–70 μg (injection dose per kg) levels and against the NCI murine M5 ovarian carcinoma tumor regression model a 20–65% curative response at 20–40 μg/kg. With the M531 murine ovarian sarcoma [intraperitoneal (i.p.) tumor implant with i.p. treatment] bryostatin 1 afforded 31–68% life extension at 5–40 μg/kg. More recently this potentially important substance has shown exceptional selectivity against human cancer cell lines (NCI representing leukemias, non-small cell lung cancer, melanoma and renal cancer).

Furthermore, bryostatin 1 has been found capable of producing powerful antitumor promoting[4,5], immunomodulating[6,7] and normal bone marrow cell growth stimulating[8] responses. Consequently, preclinical development of bryostatin 1 has been undertaken by the Cancer Research Campaign and the NCI. This work describes the development of a stability-indicating high-performance liquid chromatographic (HPLC) assay for the drug, prior to formulation for clinical trial.

EXPERIMENTAL

Materials

Before use, the HPLC-grade solvents (from BDH, Poole, U.K.) and the deionised and distilled water were filtered. The bryostatins were isolated as previously described[1]. Cholest-4-en-3-one (internal standard) was obtained from Aldrich (Gillingham, U.K.).

The HPLC system comprised three Waters 510 pumps, a 490 UV detector and

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a 710B WISP autosampler controlled by an 840 data station. The injection volume was 0.01 ml. A Waters μBondapak Phenyl column, 10 μm particle size, 8 mm I.D., was used under radial compression.

Method development

Assay development was restricted due to the small amount of bryostatin 1 available (8 mg). An initial isocratic mobile phase containing acetonitrile–distilled water (72:28, v/v) was selected with cholest-4-en-3-one as an internal standard. Detection was at the $\lambda_{max.}$ of bryostatin 1 (266 nm) and the mobile phase flow-rate was 1.5 ml/min.

Optimization was carried out using a "Snyder triangle" separation[9] with the three isocratic solvents (A) methanol–distilled water (85:15), (B) acetonitrile–distilled water (72:28), and (C) tetrahydrofuran–distilled water (56:44). A computer plot was used to predict the optimum mobile phase in terms of resolution between, and widths of, the two peaks. Resolution was greater than 2 for all predicted solvent mixtures and the optimum, in fact, turned out to be pure solvent A.

RESULTS AND DISCUSSION

Validation

Formulated bryostatin 1 solutions containing 0.1 mg/ml in absolute ethanol were prepared. HPLC vials were prepared containing an equal volume of internal standard, 0.4 mg/ml cholest-4-en-3-one in ethanol. The internal standard solution was stored at 4°C and renewed each week. Detection was at 266 nm, range 0.2 a.u.f.s.

The reproducibility of the chromatographic separation was good, both in terms of retention times and peak areas. The coefficient of variation for injection ($n = 6$) was 1.1% and between day variation ($n = 21$) was 3.1%. Calibration curves were linear over the concentration range 0.025 to 0.075 mg/ml with a correlation coefficient greater than 0.998. Using UV detection, the limit of detection of bryostatin 1 was 5 ng on column.

Degradation and related compounds

Bryostatin 1 was heated at 70°C in solutions containing 0.1 *M* hydrochloric acid, 0.1 *M* sodium hydroxide, 0.1 *M* hydrogen peroxide, or water. Samples were taken from 0–4 h and analysed by HPLC. After 4 h, the compound appeared to be stable in distilled water or hydrogen peroxide but was 100% degraded in alkali and 33% degraded in acid, presumably due to cleavage of ester groups (Fig. 1). Repeating the experiment in phosphate buffer (0.1 *M*, pH 9.3) gave a number of degradation peaks. Identification of the degradation products is ongoing. Bryostatin 2 (Fig. 1) in dimethyl sulphoxide, a desacetyl analogue of bryostatin 1 and potential degradation product, was resolved from bryostatin 1 (Fig. 2).

Solubility

The solubility of bryostatin 1 in ethanol–aqueous solutions was assessed using this analysis method. Briefly, a known concentration of bryostatin 1 in ethanol was stirred with increasing volumes of 0.9% (w/v) saline solution and aliquots of the supernatant taken for analysis against a standard in ethanol. While this method is

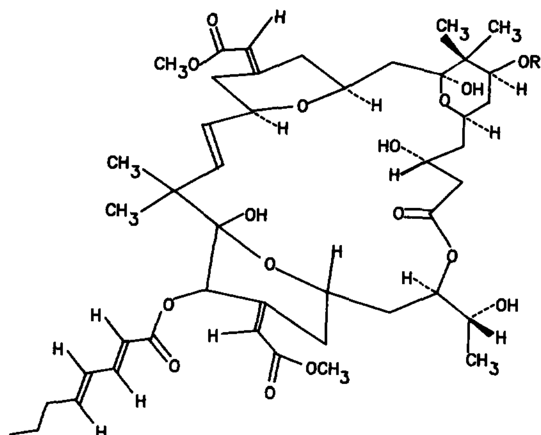

Fig. 1. Structure of bryostatin 1 (R = –CO–CH$_3$) and bryostatin 2 (R = H).

more efficient in the use of drug, however there is the possibility of supersaturation. The results are shown in Table I. Bryostatin 1 proved to be extremely insoluble in pure aqueous solvents.

Stability

The results of a pilot batch formulation, of a 0.1-mg/ml solution in absolute ethanol of the drug in glass ampoules, indicated no sign of degradation after three

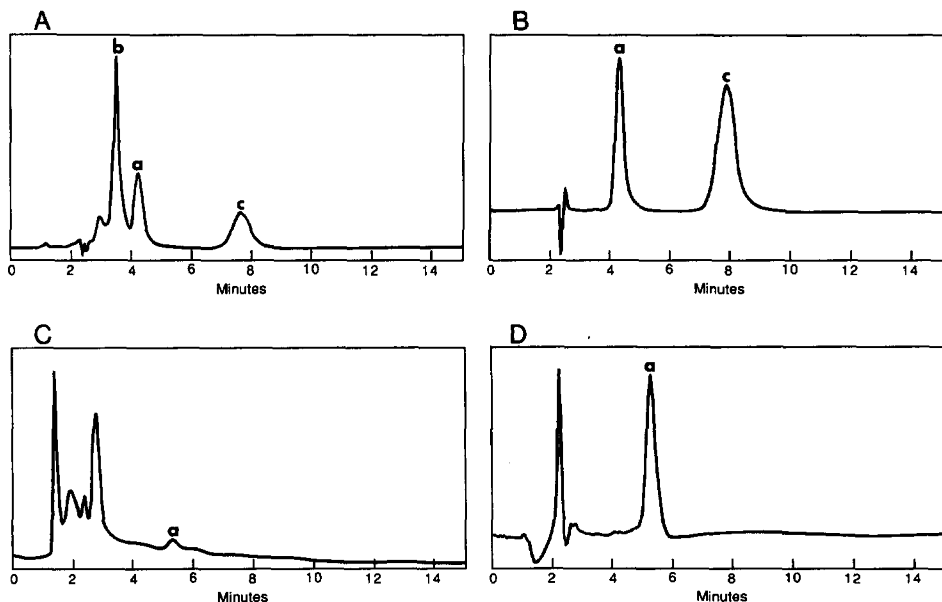

Fig. 2. (A) Chromatogram of bryostatin 1 (a), bryostatin 2 (b) and cholest-4-en-3-one (internal standard) (c). (B) Chromatogram of bryostatin 1 and internal standard. (C) Degradation of bryostatin 1 in 0.1 *M* phosphate, pH 9.3 (70°C, 1 h). (D) Degradation of bryostatin 1 in 0.1 *M* hydrochloric acid (70°C, 1.5 h).

TABLE I

SOLUBILITY OF BRYOSTATIN 1 IN ETHANOL–SALINE MIXTURES AT 20°C

Solution	*Solubility (mg/l)*
Water	0.77
0.9% (w/v) Saline	0.72
10% (v/v) Ethanol in 0.9% (w/v) saline	1.43
20% (v/v) Ethanol in 0.9% (w/v) saline	5.68
60% (v/v) Ethanol in 0.9% (w/v) saline	97.0
100% Ethanol	>4000

weeks at 50 or 20°C in daylight, or thirty weeks at −20°C. In addition a 0.06-mg/ml solution in 60% (w/v) ethanol–40% saline in a polypropylene syringe was found to be stable over a 24-h period at 20°C.

CONCLUSIONS

A stability-indicating assay for bryostatin 1 has been developed for use in formulation studies and as a basis for analysis of the drug in biological fluids.

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