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High-performance liquid chromatographic assay for the novel antitumor drug, bryostatin-1, incorporating a serum extraction technique

Parveen Khan[a], Alan T. McGown[a,*], Martin J. Dawson[a], Gordon Jayson[b], Joseph A. Prendiville[a], George R. Pettit[c], Derek Crowther[b]

[a]*CRC Department of Experimental Chemotherapy, Paterson Institute for Cancer Research, Christie CRC Cancer Centre, Wilmslow Road, Manchester M20 9BX, UK*

[b]*CRC Department of Medical Oncology, Wilmslow Road, Manchester, M20 9BX, UK*

[c]*Arizona State University, Tempe, AZ, USA*

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Abstract

An HPLC assay incorporating a solid-phase extraction technique has been devised for bryostatin-1. Quantitation of bryostatin was found to be linear over the concentration range 0.012–25 μg/ml (0.2–25 ng on column) and was found to have a limit of detection of 0.2 ng on column, with a correlation coefficient of 0.9999. Following extraction of bryostatin over a range of concentrations from horse serum (0.012–25 μg/ml) and human serum (0.01–0.32 μg/ml) using a 100-mg C_{18} solid-phase extraction cartridge, extraction efficiencies consistently greater than 90% were obtained for extraction from horse serum and varied between 57 and 85% from human serum. However, on extending this work to blood samples from patients undergoing therapy with bryostatin-1, the drug was not detectable even at the maximum dose given, demonstrating the rapid loss of this agent from peripheral circulation. © 1998 Elsevier Science B.V.

Keywords: Bryostatin-1

1. Introduction

The bryostatins are natural products isolated from a marine bryozoa, *Bugula neritina*. The structure has been elucidated to be a macrocyclic lactone [1], and is thought to represent a novel class of potent activators of protein kinase C [2].

Bryostatin has been shown to elicit a number of potent biological effects including antitumor activity, and stimulator of normal haematopoietic cells [3]. Bryostatin-1, first entered a clinical trial in Manchester, under the sponsorship of the CRC Phase I/II committee [6]. A full pharmacokinetically guided clinical trial was not possible due to the lack of a sensitive assay for bryostatin-1 in blood. Carr et al. [4] devised an indirect biological assay, which exploited the sensitivity of human platelets to bryostatin-1 induced aggregation. This assay could detect plasma bryostatin-1 levels in the nanomolar range (5 n*M*). Zhang et al. have described the use of a radiolabelled [^{3}H-C_{26}]bryostatin-1 to measure the distribution in mice [5].

*Corresponding author.

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The work described here involved the development of a HPLC assay incorporating a serum extraction technique, to enable the identification and quantification of bryostatin from serum samples at subnanogram levels. The procedure devised was used in an attempt to estimate the concentration of bryostatin-1 in patient samples obtained from phase I clinical trials [6].

2. Experimental

2.1. Chemicals and reagents

A 0.1 mg/ml solution of bryostatin, the clinically used ethanolic formulation which was used to develop the HPLC assay, HPLC grade acetonitrile and methanol were obtained from Sigma Chemical (Poole, Dorset, UK). Horse serum was obtained from Gibco Life Technologies (Paisley, Scotland). Human serum (expired) was obtained from the Christie hospital blood bank. Doubly distilled water was used throughout the study and was obtained in-house from an all-glass distillation apparatus, with alkaline permanganate present.

2.2. Chromatographic system

HPLC analysis of bryostatin-1 in ethanol was carried out using a Gilson 306 solvent delivery system, a Gilson 118 UV–Vis detector and an ASPEC XL autoinjector. The entire system was controlled using Gilson 715 controller software, this included the data capture and processing function. A wavelength of 266 nm was used for the duration of the work.

Analysis was carried out using a 5 μ*M* (150 mm×4.6 mm I.D.) Hypersil BDS column (Shandon, Runcorn, Cheshire, UK) under isocratic conditions of acetonitrile–water (80:20, v/v). A solid-phase technique for extraction of bryostatin-1 from serum was developed.

2.3. Sample preparation

Several solid-phase extraction cartridges were evaluated including C_8, C_{18}, C_2 and CN.

Reproducible extraction was performed using the following method:

1. Condition the 100-mg SPE cartridge (Varian Bond Elute, C_{18}) with 1 ml of acetonitrile, followed by 1 ml of a acetonitrile–water (50:50, v/v).
2. Condition with 1 ml of water.
3. Load 0.5 ml of serum containing bryostatin on to the cartridge.
4. Wash with 1 ml of water to remove soluble serum components.
5. Elute the retained drug with 1 ml acetonitrile.

The solid-phase extraction method developed was used to extract a range of concentrations of bryostatin, 0.012–0.32 μg/ml, from expired human plasma, and a linearity was obtained. The procedure was repeated for horse serum samples.

Extraction efficiencies consistently greater than 90% (±2%) were obtained for extraction of bryostatin from horse serum samples.

Extraction efficiencies between 57 and 85% (±9%) were obtained for extraction of bryostatin from human serum.

3. Results

From Fig. 1, it can be seen that bryostatin elutes at a retention time of 3.6 min with a coefficient of variation value of 0.81% (n=3) for peak area

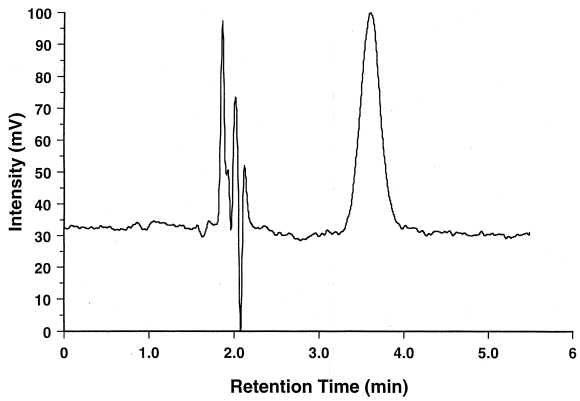

Fig. 1. Chromatogram of the standard bryostatin-1 (in ethanol) peak at a concentration of 0.19 μg/ml.

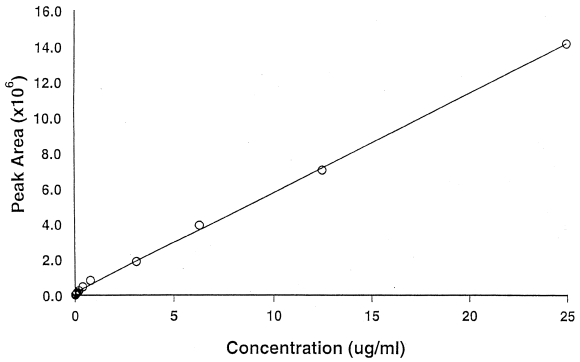

Fig. 2. Linearity of response of bryostatin-1 extracted from horse serum samples.

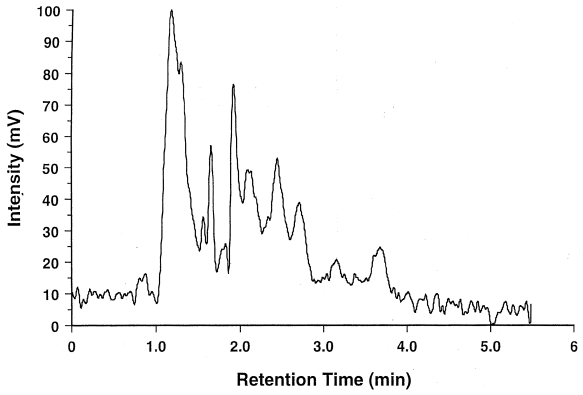

Fig. 3. Chromatogram of bryostatin-1 extracted from horse serum at its limit of detection = 0.012 μg/ml.

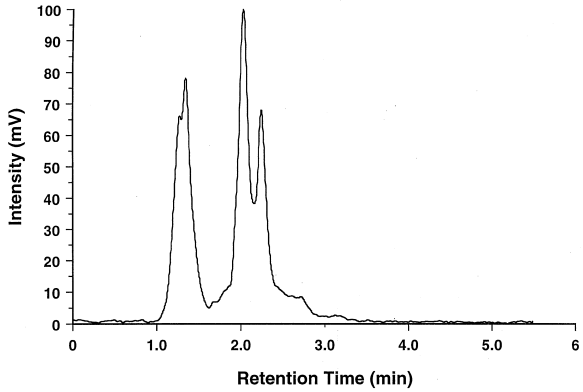

Fig. 4. Chromatogram of a patient sample, a 24-h infusion of bryostatin-1 in ethanol at 40 μg/m^2 was administered.

reproducibility. These data were used to establish linearity of response (Fig. 2) and a limit of detection of 0.012 μg/ml.

Fig. 3 depicts the chromatogram obtained when bryostatin-1 is extracted from a horse serum sample using a C_{18} solid-phase extraction cartridge at the limit of detection in serum of 0.012 μg/ml. Analysis of patient sample (Table 1), following a 24-h infusion of bryostatin at a concentration of 40 μg/m^2, with analysis carried out at several time points between 0 and 24 h, showed no detectable drug (bryostatin) (Fig. 4). The procedure was applied to a

Table 1
Table of further patient treatments

Patient No.	Type of drug administration	Dose (μg/m^2)	Total dose (μg)	Sampling time (h)
1	IV Bolus	20	41	0, 0.5, 1.0, 2.0, 4.0, 6.0 8.0+24 h
2	IV Bolus	50	86.5	0, 0.5, 1.0, 2.0, 4.0+ 6 h
3	IV Bolus	50	94	T=0, 0.5, 1.0, 2.0, 4.0 8.0+24.0 h
4	IV Bolus	35	56	0.2, 4, 6 h
5	IV Bolus	65	96.2	0, 0.5, 1.0, 2.0, 4.0+ 6.0 h
6	24-h infusion	50	92	0, 0.5, 1.0+2.0 h
7	24-h infusion	50	100	0, 0.5, 1.0, 2.0, 4.0, 6.0 h

further six patient samples, again with no drug being detected even when bryostatin was given as a bolus injection (65 $\mu g/m^2$) at the maximum dose used.

4. Discussion

From the results it can be seen that although a limit of detection at the subnanogram level (0.012 $\mu g/ml$) has been achieved in both horse serum and human serum, and that an extraction efficiency of 98% has been demonstrated when recovering a drug from spiked horse serum samples and 57–85% when recovering a drug from spiked human serum samples, no drug was detectable in the samples obtained from patients undergoing the clinical trial.

This may be due to the very low doses of bryostatin used ($\leq$65 $\mu g/m^2$) coupled with the lipophilic nature of bryostatin-1 which would result in its sequestration into body compartments other than the blood [5]. Therefore our method, although capable of detecting subnanogram levels, is not sensitive enough to detect the small quantities of drug present in blood, even at the highest achievable clinical dose (65 $\mu g/m^2$).

The maximum theoretical level of bryostatin which would be achieved in patient plasma (assuming 100% availability and an average body surface area of 2 m^2 and 5 litres of blood), would be 0.026 $\mu g/ml$ in the patient plasma. However, this is not likely to be attained due to the lipophilic nature of this drug. This would be in agreement with the study by Zhang et al. described below.

Zhang et al. [5] used [C_{26}-^{3}H]-labelled bryostatin-1 to carry out phamacokinetic, tissue distribution, metabolism and elimination studies in mice. Here a relatively high (40 $\mu g/kg$) dose of drug was given as a rapid bolus injection. It was found that in the initial 30 min following the dosing of mice, bryostatin-1 was widely distributed in many organs but concentrated in the lung, liver, gastrointestinal tract and fatty tissue.

The plasma disappearance curve for bryostatin-1 shows that it is lost rapidly from the plasma and is undetectable 12 h after treatment. The maximum concentrations observed (i.v.=92.9 ng/ml and i.p.= 13.5 $\mu g/ml$) are above the limits of detection described in this work. However, in the patient samples no bryostatin was detected. This may reflect both species differences and the slower rates of infusion of drug into patients (up to 24 h), which would reduce the peak concentration attained. The HPLC analyses described in this study have a limit of detection equivalent to that of the radiolabelled drug described by Zhang et al. [5] whilst avoiding the use of radioisotopes.

5. Conclusion

A HPLC assay incorporating a serum extraction technique, which enables detection of analyte down to subnanogram levels and which would compare favourably with the expected level of drug in patient plasma from clinical trials (assumming 100% availability) has been developed. However, on application of the procedure to patient samples, it was found that detection of the drug was not possible by light absorption.

The conclusion reached from this study is that the serum concentration of bryostatin-1 achieved at drug doses up to the maximum tolerated dose is less than 12 ng/ml.

The extraction–analysis systems developed in this study will be applicable to more sensitive assays of the drug currently under development.

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Appendix A

List of ammendments

(1) Bryostatin-1 is active at very low levels. The agent is also highly lipophilic and will be bound by many tissues and proteins, therefore the maximum possible concentration achievable on a 50 $\mu g/m^2$ (an

adult is 2 m^2) would be (approx.) 100 μg in 5 dm^2=20 ng/ml. This would fall within our limits of detection (12 ng/ml).

Our inability to detect the drug is almost certainly due to its lipophilic nature which will result in rapid elimination from the blood. This point has been emphasised in Section 4 (paragraph 3) calculated on the maximum dose use (65 $\mu g/m^2$).

(2) We have demonstrated also that bryostatin can be added and recovered from human serum in vitro at a level of 12 ng/ml, which is comparable to that expected to be achieved in patients after the bolus injection, calculated as 20 ng/ml (above). Human serum is a very complex matrix compared to horse serum and this is reflected in the lower recoveries obtained from human serum (57–85%) as described in Section 2.3, paragraph 2.

Appendix B

Regression data for calibration graph (Fig. 1)

$y = A*X + B$

parameter	value	±SE	±95.0%CI	t ratio
A	562500.6	6039.508	13662.32	93.13684
B	147430.8	52491.01	118742.9	2.808686

$y = A*X + B$

Sum of squares $SS = chi^2 = 2.027e11$
Degrees of freedom $df = 9$
Estim. of variance $s^2 = SS/df = 2.253e10$
Correlation, data vs. model $r = 0.999482$
$(Correlation)^2$ $r^2 = 0.998964$

No estimate of the measurement errors (data point weighting) was specified, so no quantitative Goodness-of-Fit Index can be calculated.

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