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Development and validation of a high-performance liquid chromatographic assay using solid-phase extraction for the novel antitumor agent pancratistatin in human plasma

Parveen Khan[a,*], Sayara Abbas[a], Bob Petit[b], Robert Caffrey[a], Victoria Megram[a], Alan McGown[a]

[a]*Paterson Institute for Cancer Research, Section of Drug Development and Imaging, Wilmslow Road, Withington, Manchester M20 4BX, UK*

[b]*Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, AZ, USA*

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Abstract

The stability of the experimental anti-tumour agent pancratistatin in human plasma has been investigated. A solid-phase extraction technique and an HPLC assay with external standards have been developed and validated. Extraction was performed using C_{18} cartridges and HPLC, analysis was performed on a 15 cm Hypersil BDS column using isocratic elution with 13% acetonitrile and aqueous solution of 1% (w/v) acetic acid. The lower limit of quantification for pancratistatin in 5% DMF–95% water was found to be 0.58 ng/ml (±10.58%) and 2.3 ng/ml (±9.2%) following extraction from human plasma. Mean recovery of 89.4% (±4.73%) was obtained over the concentration range 0.0023–9.45 μg/ml for a five day validation study. Pancratistatin was stable at room temperature in light or dark for at least 15 days, in the refrigerator at 4°C for at least 16 days and in the freezer at −20°C or −80°C for at least 28 days. Under all conditions monitored, % recovery of pancratistatin from human plasma was greater than 95% and no evidence of degradation had occurred. There also was no loss of pancratistatin after three cycles of freezing and thawing.

Keywords: Pancratistatin

1. Introduction

Numerous chemotherapeutic agents have their origins in terrestrial plant or marine organism sources [1,2]. Pancratistatin is one such potential chemotherapeutic agent. It is an isocarbostyril alkaloid (Fig. 1) isolated from the bulbs of the Hawaiian *pancratium littorale*, a plant in the Amaryllis family [3–6]. Pancratistatin was one of several amaryllidacea isoquinoline alkaloids which showed activity in cell culture against several viruses, including the RNA containing flaviviruses (Japanese encephalitis, yellow fever, and dengue virus).

The study presented here describes the development and validation of a sensitive and selective

*Corresponding author. Tel.: +44-161-446-3035; fax: +44-161-446-3109.

E-mail address: pkhan@picr.man.ac.uk (P. Khan)

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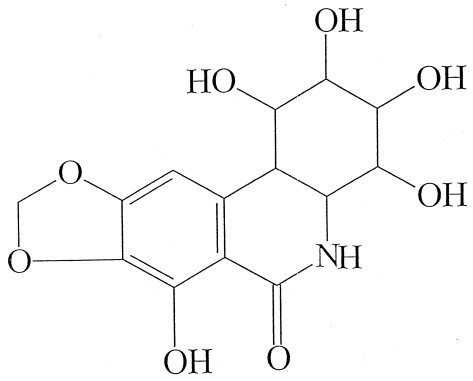

Fig. 1. Structure of pancratistatin.

solid-phase extraction and high-performance liquid chromatography assay for the quantitation of pancratistatin in human plasma. The stability profile of pancratistatin in human plasma under a variety of conditions was also investigated in order to further the compounds pre-clinical evaluation.

2. Experimental

2.1. Chemicals and reagents

Pancratistatin (MW 325.27) following isolation, is an off-white crystalline powder. A 19.8 μg/ml solution of pancratistatin in 5% DMF (dimethylformamide)–95% doubly distilled water was used to develop the HPLC assay. DMF was used due to the poor aqueous solubility of pancratistatin. HPLC grade acetonitrile and methanol were obtained from Rathburns Chemical Company (Walkerburn, UK). Dimethylformamide was obtained from Sigma Chemical Co. (Poole, UK). Control human plasma was obtained from the blood bank at Christie Hospital. Doubly distilled water was used throughout the study and was obtained in house from an all-glass distillation apparatus. C_{18} solid-phase extraction cartridges (500 mg) were obtained from Anachem International (Luton, UK).

2.2. Chromatographic system

HPLC analysis of pancratistatin was carried out using a Gilson 306 solvent delivery system, a Gilson 117 UV-Vis detector and an ASPEC XL auto injector. The system was controlled using Gilson 715 software, which was also used for data capture and processing. A wavelength of 280 nm was used for detection. The reversed-phase chromatography column used was 150 mm×4.6 mm I.D. Hypersil BDS column (5 μ). Pancratistatin was eluted under isocratic conditions of 13% acetonitrile–87% acetic acid (1% w/v) and chromatography was performed at a flow of 1 ml/min.

2.3. Sample preparation

A solid-phase extraction technique for pancratistatin from human plasma was developed. Several solid-phase extraction cartridges were evaluated, including phenyl, cyanopropyl, C_{18}, C_8, C_2 and aminopropyl. Reproducible extraction was best obtained using a 500 mg C_{18} cartridge under the following conditions at room temperature:

1. Condition the cartridge with 1 ml of acetonitrile.
2. Condition the cartridge with 1 ml of water.
3. Load 0.5 ml of plasma containing pancratistatin.
4. Allow plasma proteins to elute to waste (Set push volume on ASPEC to 1 ml/min).
5. Wash off any plasma components with 2×1 ml 100% water.
6. Elute the retained drug with 1 ml of 30% acetonitrile–70% water.
7. Centrifuge samples in a microcentrifuge (13 000×g) for 10 min.
8. An aliquot of 200 μL was injected for analysis.

2.4. Method validation

2.4.1. Standard curve

A 37.8 μg/ml stock solution of pancratistatin in 5%DMF–95% water was made up and stored at 4°C. A response curve was generated from this stock over the range 37.8 μg/ml–0.000577 μg/ml for dilution with 5% DMF–water. Each concentration was analysed in triplicate and the mean of each concentration was used to plot the calibration curve. A linear regression analysis was applied to the concentration vs. area data.

2.4.2. Plasma standard curves

Calibration samples were prepared by adding 0.5 ml of the following concentrations of pancratistatin 37.8, 18.9, 9.45, 4.73, 2.36, 1.18, 0.59, 0.295, 0.148, 0.074, 0.037, 0.018, 0.009, 0.0046, 0.0023, 0.0012, 0.000577 μg/ml of pancratistatin in 5% DMF–95% water to 0.5 ml aliquots of drug free pooled human plasma. Each sample was extracted using the method described in Section 2.3. Extracted samples were centrifuged for 10 min and the supernatant analysed using the analytical assay described in Section 2.2. Each concentration was analysed in triplicate. To construct the calibration curves each mean area was plotted versus the nominal concentration of the standard and a linear regression analysis was carried out on the data. This procedure was repeated on five consecutive days.

2.4.3. Within day precision and accuracy

The within day precision and accuracy of this method was determined by the extraction and analysis of pancratistatin samples from pooled human plasma at four concentrations (9.45, 0.59, 0.037 and 0.0023 μg/ml) on the same day. Each concentration was analysed in triplicate.

2.4.4. Between-day precision and accuracy

The between-day precision and accuracy of this method were determined by the extraction and analysis of pancratistatin samples from pooled human plasma at thirteen concentrations: 9.45, 4.73, 2.36, 1.18, 0.59, 0.29, 0.147, 0.07, 0.037, 0.018, 0.009, 0.0046 and 0.0023 μg/ml on five different days. Each concentration was analysed in triplicate

2.4.5. Determination of percentage recovery

Following preparation of samples (2.4 Method Validation, Plasma Standard Curves). Each sample was analysed in triplicate. The mean area obtained at each concentration was expressed as a ratio of the mean area of the equivalent concentration obtained from the non-extracted calibration curve.

2.5. Stability study in human plasma

The stability of pancratistatin was studied in human plasma at a concentration of 9.45 μg/ml. Stability to light–dark was monitored at room temperature whilst stability at −20°C, −80°C and 4°C was tested in the dark. Samples kept at room temperature in light-dark conditions were monitored over a period of 15 days. Samples kept at −20°C and −80°C were monitored over 28 days and those kept at 4°C were monitored for 16 days. Each sample was extracted and analysed as described above.

The percentage recovery of each sample was calculated using a fresh standard of the same concentration. In addition, plasma containing 9.45 μg/ml of pancratistatin was frozen to −80°C and was subjected to a three times freeze/thaw cycle where plasma was allowed a defrost period of 2 h prior to

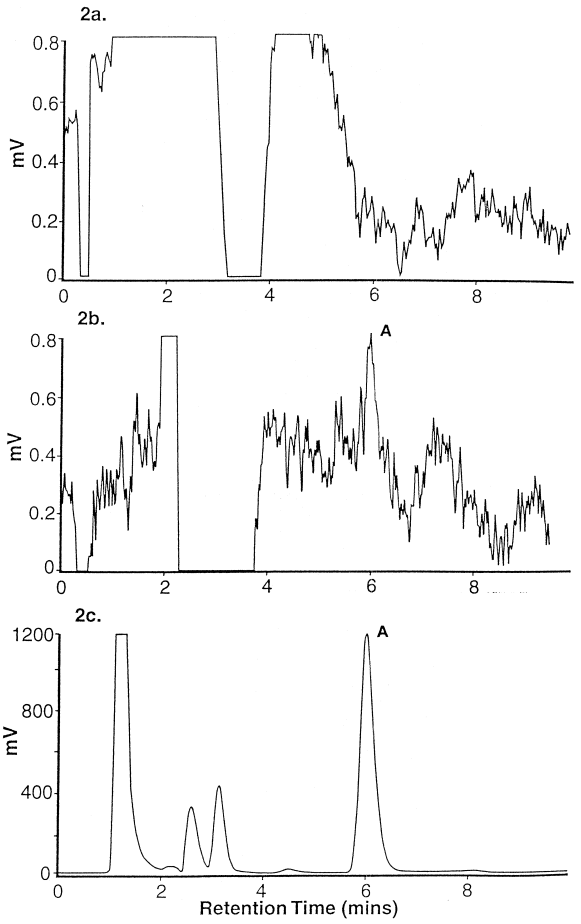

Fig. 2. (a) Chromatogram of extracted drug free human plasma. (b) Chromatogram of extracted pancratistatin standard (peak A) isolated from human plasma at the LLQ (0.0023 μg/ml). (c) Chromatogram of extracted pancratistatin standard (peak A) isolated from human plasma at 9.45 μg/ml.

Table 1
Percentage recovery data for pancratistatin extracted from human plasma over a 5 day validation study

μg/ml	9.45	4.75	2.36	1.18	0.59	0.29	0.148	0.074	0.037	0.019	0.009	0.005	0.0023
Mean	87.4	96.6	89.3	87.1	83.7	81.9	92.4	90.8	91.2	93.3	84.8	91.4	92.6
SD	3.0	8.1	6.2	6.6	4.4	5.6	5.1	6.1	10.3	2.1	8.0	8.0	13.1
%RSD	2.1	5.7	7.0	4.7	3.1	3.9	3.6	4.3	7.2	1.5	5.6	5.6	9.3

extraction and analysis. The un-extracted plasma was returned to the freezer, and the procedure repeated a further two times.

3. Results

From Fig. 2 it can be seen that the blank (extracted drug free plasma) is clear, Fig. 2 shows pancratistatin (LLQ 0.0023 μg/ml) at a retention time of 6.0 min (peak A). Chromatogram 2c illustrates elution of pancratistatin (9.45 μg/ml) (peak A) following isolation from human plasma.

The linearity of response for stock pancratistatin in 5% DMF–95% doubly distilled water was constructed from which the lower limit of quantification of pancratistatin was determined to be 0.58 ng/ml. Slope=5.04, intercept=0.13 and correlation coefficient of 1.00 were obtained for this curve. The LLQ was determined to be 2.3 ng/ml when extracted from human plasma, a value confirmed in a 5 day validation study where pancratistatin was extracted from human plasma over the range 9.45–0.0023 μg/ml. Simple least squares linear regression analysis was performed on the peak area vs. concentration data from each day of the validation study, the mean slope=4.51×10^6 intercept=0.08 and correlation coefficient of 0.999.

Percentage extraction recoveries obtained over the concentration range 9.45–0.0023 μg/ml over the 5 day validation study can be seen in Table 1. Inter- and intra-day precision and accuracy data obtained using the developed method can be seen in Tables 2 and 3.

Tables 4–6 illustrate the percentage recover data and associated standard deviation and relative standard deviation data obtained for 9.33 μg/ml of pancratistatin monitored in human plasma under various light and temperature conditions. Table 7 represents data obtained from a 3× freeze-thaw cycles at −80°C.

4. Discussion and conclusions

A solid-phase extraction and HPLC assay has been developed and validated over 5 days for the isolation from plasma of the novel anti-tumor agent, pancratistatin. Mean recovery over 5 days and thirteen concentrations was 89.41% (±2.12–9.25%) and a

Table 2
Inter-day precision and accuracy data for pancratistatin isolated from human plasma

μg/ml	9.45	4.75	2.36	1.18	0.59	0.29	0.15	0.074	0.037	0.019	0.009	0.005	0.0023
Mean	9.3	5.0	2.4	1.1	0.6	0.3	0.2	0.1	0.04	0.02	0.009	0.004	0.002
SD	0.3	0.4	0.1	0.1	0.03	0.02	0.01	0.005	0.004	0.000	0.001	0.0003	0.0003
%RSD	3.6	8.4	3.9	7.6	4.9	6.8	5.1	6.6	10.8	2.3	10.6	8.1	13.1
% Accuracy	98.6	105.9	99.5	92.4	92.7	93.1	101.9	101.4	99.2	103.2	93.5	84.8	82.6

Table 3
Intra-day precision and accuracy data from pancratistatin isolated from human plasma

Actual concentrations	9.45	0.59	0.037	0.0023
Mean measured conc.	9.40	0.58	0.03	0.002
SD	0.21	0.006	0.0014	0.00009
%RSD	2.3	1.1	4.6	3.9
% Accuracy	99.5	98.3	86.1	95.7

Table 4
% Recovery data for stablility of pancratistatin in human plasma up to 15 days monitored at ambient temperature in the light (A) and dark (B)

Day	Conc (A) μg/ml	Conc (B) μg/ml
0	9.3	9.3
1	9.3	9.4
2	8.8	9.6
3	8.9	9.4
4	9.1	9.1
7	9.3	9.3
8	9.4	9.6
14	9.5	9.3
15	9.6	9.4
Mean concentration	9.3	9.4
SD	0.3	0.1
%RSD	2.9	1.5
Mean % recovery	99.3	100.6
SD	2.9	2.1
%RSD	2.9	2.0

Table 5
% Recovery data for stability of pancratistatin in human plasma up to 28 days monitored at −20°C (A) and −80°C (B)

Day	Conc (A) μg/ml	Conc (B) μg/ml
0	9.3	9.3
1	9.2	8.8
2	9.1	9.1
3	9.0	9.0
7	9.0	9.0
14	9.5	9.3
21	9.3	9.4
28	9.2	8.8
Mean conc.	9.2	9.1
SD	0.2	0.3
%RSD	1.8	2.8
Mean % rec.	98.7	97.8
SD	1.6	2.8
%RSD	1.6	2.8

LLQ of 0.0023 μg/ml in plasma was established. Inter- and intra-day precision and accuracy data obtained from the 5 day validation study was very odd, as can be seen in Tables 3 and 4.

It was found that pancratistatin was stable in human plasma for at least 28 days when stored at temperatures of −20°C or −80°C for 16 days when stored at 4°C and at least 15 days when pancratistatin was monitored at room temperature in light or dark. The mean percentage recovery of drug for the stability study was 99% (±0.93%). Pancratistatin

Table 6
% Recovery data for stability of pancratistatin in human plasma up to 16 days monitored at 4°C

Day	Conc μg/ml
0	9.3
1	9.1
2	9.7
3	8.8
4	8.9
7	9.5
8	9.6
14	9.0
15	9.4
16	8.9
Mean conc.	9.2
SD	0.3
%RSD	3.4
Mean % rec.	98.7
SD	3.1
%RSD	3.2

Table 7
Data from 3×freeze-thaw cycles

Day	Concentration μg/ml
0	9.45
1	9.17
2	9.37
3	9.26
Mean conc.	9.31
SD	0.12
%RSD	1.32
Mean % rec.	98.04
SD	1.08
%RSD	1.09

was also stable following a three times freeze-thaw cycle. The mean percentage recovery was 98% (±1.09%).

The technique can be applied to the analysis of patient samples and is sensitive to 2.3 ng/ml.

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