ELSEVIER

Journal of Chromatography B, 724 (1999) 157–162

JOURNAL OF CHROMATOGRAPHY B

Quantitative high-performance liquid chromatography-based detection method for calphostin C, a naturally occurring perylenequinone with potent antileukemic activity

Chun-Lin Chen[a], Hao Chen[a], De-Min Zhu[b], Fatih M. Uckun[a,b,c,*]

[a]*Department of Pharmaceutical Sciences, Hughes Institute, 2665 Long Lake Road, Roseville, MN 55113, USA*
[b]*Department of Immunology, Hughes Institute, 2665 Long Lake Road, Roseville, MN 55113, USA*
[c]*Drug Discovery Program, Hughes Institute, 2665 Long Lake Road, Roseville, MN 55113, USA*

Received 17 July 1998; received in revised form 19 October 1998; accepted 3 December 1998

Abstract

Calphostin C is a potent inhibitor of protein kinase C and can induce Ca^{2+}-dependent apoptosis in human ALL cells. Further development of calphostin C will require detailed pharmacodynamic studies in preclinical animal models. Therefore, we established a sensitive and accurate high-performance liquid chromatography (HPLC)-based quantitative detection method for the measurement of calphostin C levels in plasma. Extraction of calphostin C from plasma was performed by precipitation of plasma protein using acetonitrile and an aliquot of extracted supernatant was injected onto a Hewlett-Packard HPLC system constituting a 250×4 mm LiChrospher 100, RP-18 (5 μm) in conjunction with a 4×4 mm LiChrospher 100, RP-18 guard column (5 μm). The eluted compounds were detected by diode array detection set at a wavelength of 479 nm. Acetonitrile–water containing 0.1% trifluoroacetic acid and 0.1% triethylamine (70:30, v/v) was used as the mobile phase. The average extraction recovery from plasma was 97.3%. Good linearity ($r>0.999$) was observed throughout the concentration range of 0.05–40 μ*M* for calphostin C in 50 μl of plasma. Intra- and inter-assay variabilities were less than 6% in plasma. The lowest detection limit of calphostin C in 50 μl plasma was 0.02 μ*M* at a signal-to-noise ratio of ~3. The availability of this assay will now permit detailed pharmacodynamic and pharmacokinetic studies of calphostin C in vivo.

Keywords: Calphostin C; Perylenequinone

1. Introduction

Recurrence of leukemia continues to be a major obstacle to a successful outcome of multiagent chemotherapy in the treatment of acute lymphoblastic leukemia (ALL) patients especially those who have relapsed despite intensive multiagent chemotherapy [1–6]. Consequently, the identification and development of new potent anti-ALL drugs have focal points for translational leukemia research.

Calphostin C, a naturally occurring perylenequinone antibiotic (structure see Fig. 1A), has been shown to have a pleiotropic biological profile [6–14]. A number of studies have demonstrated that this natural product can inhibit protein kinase C (PKC) [7,8,15–17] and induce apoptotic cell death [18–22]. We have demonstrated that calphostin C can induce

*Corresponding author. Tel.: +1-651-697-9228; fax: +1-651-697-1042; e-mail: Fatih@mercury.ih.org

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00562-3

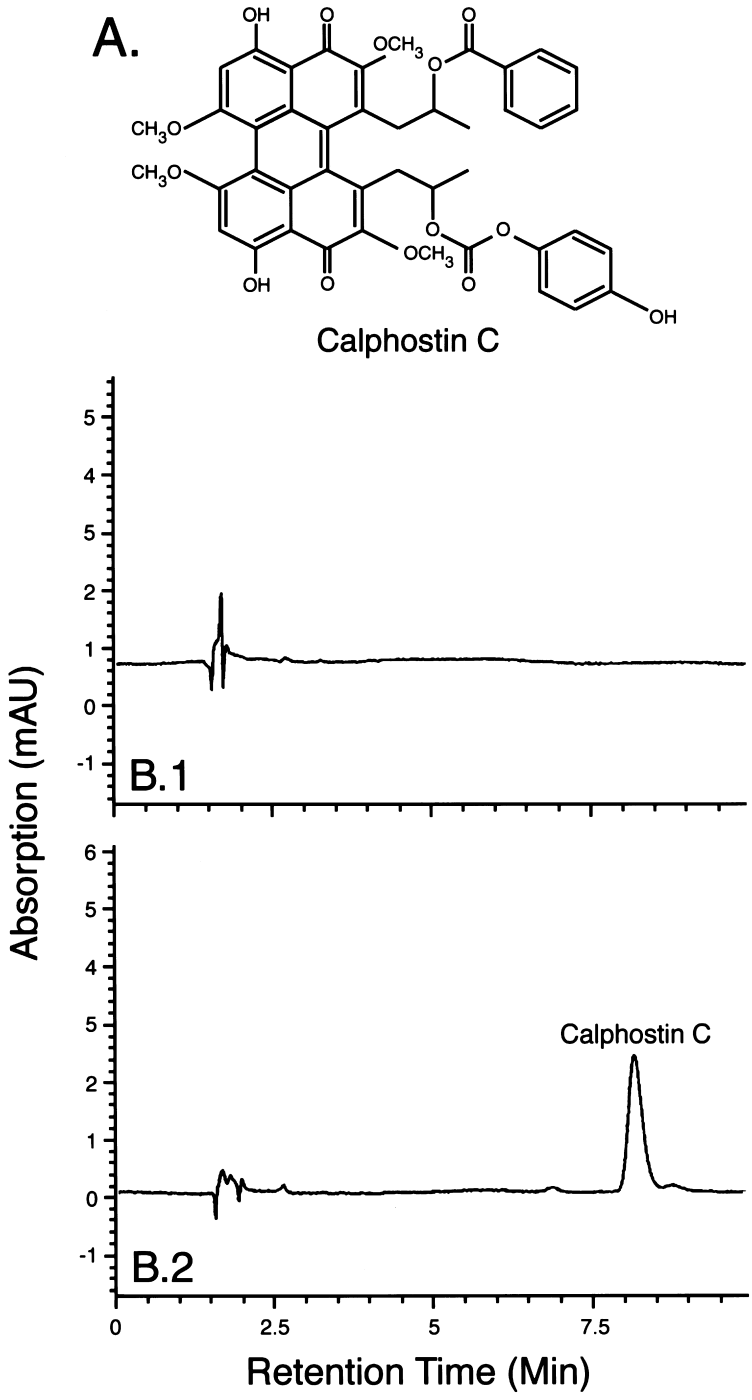

Fig. 1. (A) Chemical structure of calphostin C (M_r=790.6). Representative chromatograms from blank plasma (B.1) and a plasma sample spiked with calphostin C (B.2).

dose-dependent apoptosis in human ALL cell lines [23]. In biochemical studies, calphostin C was found to induce rapid calcium mobilization from intracellular stores of ALL cell lines and its cytotoxicity against ALL cell lines was well correlated with the magnitude of this calcium signal [23]. Calphostin C was also capable of inducing calcium mobilization and apoptosis in freshly obtained primary leukemic cells from children with ALL regardless of their immunophenotype or NCI risk classification and it is equally potent against leukemic cells from children in bone marrow relapse [23]. Further development of calphostin C will require detailed pharmacodynamic studies in preclinical animal models. To our knowledge, there are no quantitative high-performance liquid chromatography (HPLC)-based analytical methods for detecting calphostin C in biological fluids. Therefore, we set out to establish a sensitive and accurate detection method for calphostin C. Here, we first describe a HPLC-based quantitative detection method which will permit the measurement of calphostin C levels in plasma for pharmacokinetic studies.

2. Experimental

2.1. Chemicals and drugs

All the reagents used in this study were HPLC grade. Deionized distilled water was used throughout the work (US Filter, US Filter Corporation, Cowell, MA, USA). Methanol, acetonitrile, triethylamine (TEA), and trifluoroacetic acid (TFA) were obtained from Fisher (Fair Lawn, NJ, USA). Calphostin C ($C_{44}H_{38}O_{14}$, molecular mass, M_r 790.6, purity 99% by thin-layer chromatography, catalog No. EI-198) was purchased from Biomol Research Labs. (Plymouth Meeting, PA, USA). Micro-osmotic pump (0.5 μl/h, seven days) was purchased from Alza (Palo Alto, CA, USA).

2.2. Standard solutions

Stock solutions of calphostin C were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1.26 m*M*. The stock solutions were then diluted with methanol–water (50:50, v/v) to yield appropriate working solutions for the preparation of standards to calibrate. All solutions were stored at −20°C until use.

2.3. Apparatus and chromatographic conditions

The HPLC system (Palo Alto, CA, USA) consisted of a Hewlett-Packard (HP) series 1100 in conjunction with a quaternary pump, an autosampler, an auto electronic degasser, an automatic thermostatic column compartment, diode array detector and a computer with a Chemstation software program for analysis of the data. A 250×4 mm LiChrospher 100, RP-18 (5 μm analytical column and a 4×4 mm Lichrospher 100, RP-18 (5 μm) guard column were provided by Hewlett-Packard. Acetonitrile–water containing 0.1% TFA and 0.1% TEA (70:30, v/v) was used as the mobile phase. The mobile phase was degassed automatically by the electronic degasser system. The column was equilibrated and eluted under isocratic conditions utilizing a flow-rate of 1.0 ml/min at ambient temperature. The detection wavelength was set at 479 nm. Peak width, response-time and slit were set at >0.03 min, 0.05 s and 8 nm, respectively.

2.4. Extraction procedures

For determination of calphostin C levels in plasma, 100 μl of acetonitrile was added to 50 μl of plasma containing calphostin C, and the mixture was vortexed for 30 s. Following centrifugation in a microcentrifuge at 7000 *g* for 10 min, the supernatant (~125 μl) was transferred into HPLC injection vials. A 100-μl aliquot of this solution was injected for HPLC analysis.

2.5. Extraction recovery

Replicate ($n=5$) plasma samples (50 μl per sample) were spiked with known amounts of calphostin C to yield final concentrations of 1.5 and 15 μ*M* of calphostin C. Using the extraction procedures and analytical methods described above, the calphostin C concentrations were determined and the extraction recovery (ER) was calculated using the formula: $\%ER=\{\text{Peak area[calphostin C]}_{\text{extracted}}/\text{Peak area[calphostin C]}_{\text{unextracted}}\}\times 100$.

2.6. Calibration curve

A calibration curve was generated to confirm the linear relationship between the absolute peak area and the concentration of calphostin C in the tested samples. Calphostin C was added to plasma to yield final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 10, 20 and 40 μ*M*. Fifty μl of these spiked plasma samples with known amounts of calphostin C was extracted as described above, and the calibration curves were generated by plotting the absolute peak area against the tested drug concentrations. Linear regression analysis of the calibration curve was performed by using the CA-Cricket graph III computer program, version 1.0 [24].

The standard samples of calphostin C (0.15, 1.5 and 15 μ*M*) were used as calibrators. The calphostin C contents of these standards were calculated by interpolating the peak area with the calibration curve.

2.7. Intra-assay and inter-assay accuracy and precision

To evaluate the intra-assay accuracy and precision, calphostin C was added to drug-free plasma ($n=5$) at concentrations of 0.15, 1.5 and 15 μ*M*. The standard samples were prepared and analyzed within a single day. The concentrations were calculated using the calibration curve described above. The ratio of the calculated concentration over the known concentration spiked was calculated to evaluate the intra-assay accuracy of the method, and the coefficient of variation (C.V.) was used as an index of the intra-assay precision. The inter-assay accuracy and precision were determined similarly using five independent experiments. The inter-assay accuracy was calculated as the ratio of the calculated concentration over the known concentration and the inter-assay precision was estimated by determining the C.V. from five independent experiments.

2.8. Animals

Female CD-1 mice (22–24 g) purchased from Charles River Labs. (Wilmington, MA, USA) were housed in a controlled environment (12-h light/12-h dark photoperiod, 22±1°C, 60±10% relative humidity), which is fully accredited by the USDA (United States Department of Agriculture, USA). Mice were allowed free access to pelleted food and tap water throughout the experiments. Animal studies were approved by the Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985, USA). These mice were used to obtain plasma.

2.9. In vitro quantification test of calphostin C released from a micro-osmotic pump

To test whether the micro-osmotic pump (0.5 μl/h, seven days) can consistently release calphostin C, we filled the micro-osmotic pump with 100 μl calphostin C (total 0.3 mg, dissolved in DMSO–PBS–2% Tween 80) according to the manufactor's specification, and then this micro-osmotic pump was placed into a 10-ml test tube prefilled with 7 ml of phosphate-buffered saline at 37°C. At 24 h intervals, the micro-osmotic pump filled with calphostin C was transferred to a new test tube filled with 7 ml of PBS. The concentration of calphostin C released from the micro-osmotic pump into PBS was determined by the HPLC-based quantitative method described.

3. Results and discussion

3.1. Chromatographic separations

Several combinations of acetonitrile, methanol and water (with 0.1% TFA and 0.1% TEA) were evaluated as possible mobile phases. It was determined that the mobile combination described herein is the most suitable for detecting calphostin C. It seems that TEA is an universal modifier for many compounds [25–29], it is also an important modifier for the system described hereby. Under the described chromatographic conditions, the retention times for calphostin C was 8.2 min. At the retention time, calphostin C was eluted without an interference peak from the blank plasma (Figs. 1B.1 and B.2).

Table 1
Extraction recovery of calphostin C from plasma (n=5)

Added (μM)	Peak area (mAU s)	Peak area extracted (mAU s)	Extraction recovery (%)
1.5	43.2±0.7	41.6±0.2	96.5±0.5
15	428.2±1.5	420.1±1.2	98.1±0.3
Overall			97.3±0.9

3.2. Extraction recovery

Extraction of calphostin C from plasma using chloroform failed due to high variation in the peak area. Extraction of calphostin C from plasma by using TCA to precipitate plasma protein also failed due to low extraction recovery of calphostin C from plasma. However, using two volumes of acetonitrile to precipitate plasma protein and supernatant directly following centrifugation for HPLC analysis have been found to be satisfactory. Under the above described extraction procedures, extraction recovery was average 97.3±0.9% (Table 1).

3.3. Sensitivity of the HPLC method

The calibration curve for calphostin C demonstrated a good linear relationship between concentrations and absolute peak area. The calibration curve obtained from extraction of plasma containing known amounts of calphostin C was linear over the concentration ranges tested (0.05–40 μM) in 50 μl plasma. The calibration curve was found to be linear and could be described by the regression equations: $y=0.0366x+0.0411$ ($r>0.999$), in which y was the agent recovered in μM and x was absolute peak area. The lowest limit of detection of calphostin C was 0.02 μM in 50 μl plasma at a signal-to-noise ratio of ~3. This method was sensitive enough for future pharmacokinetic studies in animals.

3.4. Precision and accuracy of the HPLC method

The results obtained indicate that intra- and inter-assay C.V.s in plasma were less than 6%. The overall intra- and inter-assay accuracies of this method were 97.6±5.8% and 98.8±4.1%, respectively (Table 2). These results suggest that the procedures described above are satisfactory with respect to both accuracy and precision.

3.5. Determination of calphostin C released from the in vitro micro-osmotic pump

In order to maintain effective plasma concentrations of ≥2 μM in vivo, drug delivery via a micro-osmotic pump was evaluated. The results depicted in Fig. 2 demonstrated that a controlled release of calphostin C can be achieved with this micro-osmotic pump. Therefore, this micro-osmotic pump may be useful for delivering calphostin C in preclinical animal models such as the SCID mouse model of human ALL [30,31].

In summary, we have developed a highly sensitive and accurate analytical HPLC method for quantitative detection of the potent anti-leukemic agent calphostin C in plasma. The availability of this assay will now permit detailed pharmacodynamic and pharmacokinetic studies of calphostin C in vivo.

Table 2
Intra-assay and inter-assay accuracy and precision of the determination of calphostin C in plasma

	Added (μM)	Found	Accuracy (%)	C.V.[a] (%)
Intra-assay (n=5)	0.15	0.156±0.003	104.3±2.1	2.0
	1.5	1.417±0.023	94.5±1.6	1.7
	15	14.12±0.775	94.2±5.2	5.5
Inter-assay (n=5)	0.15	0.156±0.002	104.0±1.2	1.2
	1.5	1.43±0.04	95.5±2.8	2.9
	15	14.67±0.29	97.8±2.0	2.0

[a] C.V.=Coefficient of variation. The data are presented as mean±SD.

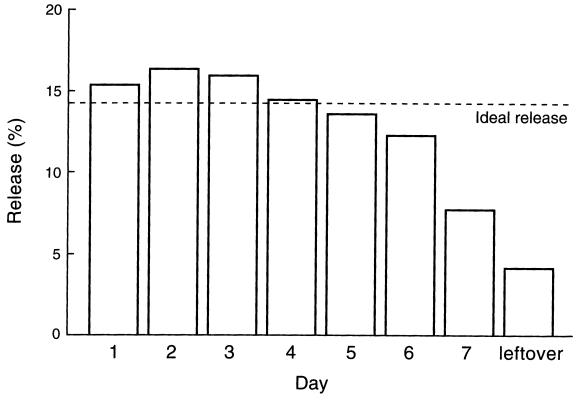

Fig. 2. Controlled release of calphostin C from a micro-osmotic pump.

References

[1] F.M. Uckun, J.B. Nachman, H.N. Sather, M.G. Sensel, K. Theil, P. Kraft, P.G. Steinherz, B.J. Lange, R. Hutchinson, G.H. Reaman, P.S. Gaynon, N.A. Heerema, Cancer 83 (1998) 2030.
[2] N.A. Heerema, H.N. Sather, M.G. Sensel, P. Kraft, J.B. Nachman, P.G. Steinherz, B.J. Lange, R.S. Hutchinson, G.H. Reaman, M.E. Trigg, D.C. Arthur, P.S. Gaynon, F.M. Uckun, J. Clin. Oncol. 16 (1998) 1270.
[3] F.M. Uckun, M.G. Sensel, H.N. Sather, P.S. Gaynon, D.C. Arthur, B.J. Lange, P.G. Steinherz, P. Kraft, R. Hutchinson, J.B. Nachman, G.H. Reaman, N.A. Heerema, J. Clinical Oncology 16 (1998) 527.
[4] F.M. Uckun, M.G. Sensel, K.G. Waddick, P.G. Steinherz, M.E. Trigg, N.A. Heerema, H.N. Sather, G.H. Reaman, P.S. Gaynon, Blood 91 (1998) 735.
[5] P.S. Gaynon, A. Desai, B.C. Bostrom, R. Hutchinson, J.B. Nachman, G.H. Reaman, H.N. Sather, P.G. Steinherz, M.E. Trigg, D.G. Tubergen, F.M. Uckun, Cancer 80 (1997) 1717.
[6] M.E. Trigg, P.S. Gaynon, F.M. Uckun, in: J.F. Holland, E. FryIII, R.C. BastJr., D.W. Kufe, D.L. Morton, R.R. Weichselbaum (Eds.), Cancer Medicine, Fourth Edition, 1996, p. 2945, Ch. 164.
[7] E. Kobayashi, H. Nakan, M. Morimoto, T. Tamaoki T, Biochem. Biophys. Res. Commun. 159 (1998) 548.
[8] E. Kobayashi, K. Ando, H. Nakano, T. Iida, H. Ohno, M. Morimoto, T. Tamaoki, J. Antibiotics 42 (1989) 1470.
[9] S. Gamou, N. Shimizu, J. Cell Physiol. 158 (1994) 151.
[10] M. Fabbri, S. Bannykn, W.E. Balch, J. Biol. Chem. 269 (1994) 26848.
[11] S. Gupta, K. Patel, H. Singh, S. Gollapudi, Cancer Lett. 30 (1994) 139.
[12] S. Svetlov, S. Nigam, Biochem. Biophys. Acta 1177 (1993) 75.
[13] S.C. Lee, S.D. Yang, J. Cell Biochem. 60 (1996) 121.
[14] H.K. Wang, J.X. Xie, J.J. Chang, K.M. Hwang, S.Y. Liu, L.M. Ballas, J.B. Jiang, K.H. Lee, J. Med. Chem. 35 (1992) 2717.
[15] R. Gopapakrishna, Z.H. Chen, U. Gundimeda, FEBS Lett. 314 (1992) 149.
[16] G.R. Dubyak, S.B. Kertesy, Arch. Biochem. Biophys 341 (1997) 129.
[17] S.A. Rotenberg, M.H. Huang, J. Zhu, L. Su, H. Riedel, Molecular Carciongenesis 12 (1995) 42.
[18] H. Ikemoto, E. Tani, T. Matsumoto, A. Nakano, J. Furuyama, J. Neurosurg. 83 (1995) 1008.
[19] S. Grant, A.J. Turner, T.M. Bartimole, P.A. Nelms, V.C. Joe, W.D. Jarvis, Oncol. Res. 6 (1994) 87.
[20] W.D. Jarvis, A.J. Turner, L.F. Povirk, R.S. Traylor, S. Grant, Cancer Res. 54 (1994) 1707.
[21] J.J. Zulueta, F.S. Yu, I.A. Hertig, V.J. Thannickal, P.M. Hassoun, Am. J. Respir. Cell Mol. Biol. 12 (1995) 41.
[22] J.A. Prezioso, J. Wang, M. Kim, L. Duty, D.J. Tweardy, E. Dorelik, Cytokine 7 (1995) 517.
[23] D.-M. Zhu, R.-K. Narla, W.-H. Fang, N.-C. Chia, F.M. Uckun, Clin. Cancer Res. 4 (1998) 2967.
[24] User Guide, WinNonlin, CA-Cricket Graph III, Computer Associates International Inc., Islandia, NY, USA.
[25] C.L. Chen, B.A. Lesseley, C.R. Clarke, J.D. Roder, S. Sangiah, K.D. Berlin, G.L. Garrison, B.J. Scherlag, R. Lazzara, E. Patterson, J. Chromatogr. B 583 (1992) 274.
[26] C.L. Chen, S. Sangiah, J.D. Roder, H. Chen, K.D. Berlin, G.L. Garrison, B.J. Scherlag, R. Lazzara, Anal. Lett. 26 (1993) 1125.
[27] C.L. Chen, S. Sangiah, J. D. Roder, H. Chen, K.D. Berlin, G.L. Garrison, B.J. Scherlag, R. Lazzara, Anal. Sci. 9 (1993) 429.
[28] C.L. Chen, S. Sangiah, J.D. Roder, H. Chen, K.D. Berlin, G.L. Garrison, B.J. Scherlag, R. Lazzara, J. Liq. Chromatogr. 17 (1994) 913.
[29] C.L. Chen, S. Sangiah, H. Chen, K.D. Berlin, G.L. Garrison, B.J. Scherlag, R. Lazzara, J. Liq. Chromatogr. 17 (1994) 3681.
[30] F.M. Uckun, W.E. Evans, C.J. Forsyth, K.G. Waddick, L. T-Ahlgren, L.M. Chelstrom, A. Burkhardt, J. Bolen, D.E. Myers, Science 267 (1995) 886.
[31] F.M. Uckun, C.F. Stewart, G. Reaman, L.M. Chelstrom, J. Jin, M. Chandan-Langlie, K. G. Waddick, J. White, W.E. Evans, Blood 85 (1995) 2817.