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Improvement in assay sensitivity for plasma dolastatin-10 using capillary electrophoresis at elevated temperatures☆

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Abstract

The very potent antimitotic and anticancer agent, dolastatin-10 (DOL-10), currently undergoing testing in a phase II clinical trial, has been quantitated previously in human plasma by high-performance capillary electrophoresis (HPCE). This method provides a lower limit of detection of 25 ng/ml DOL-10 from extracted patient samples. Without changes in preconcentration techniques, we report a significant improvement in the sensitivity of this method using elevated temperatures with conventional UV absorbance detection and liquid–liquid extraction which lowers the detection limit to 3 ng/ml of the drug. An elevated separation temperature of 50°C was critical in achieving this 8× improvement in the detection limit. Partial validation of the method at this temperature gave excellent linearity (0–100 ng/ml; $y=0.018x+0.085$, $r=0.993$), limit of quantitation (5 ng/ml), and good overall recovery of the drug (>85%). We have applied this improved method towards the in vivo quantitation of DOL-10 in mice and in a patient receiving the drug in a phase I clinical study. From these analyses we conclude that this method is suitable for clinical studies where plasma levels of DOL-10 are ≥5 ng/ml. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Dolastatin-10 (DOL-10), one of several analogues of the dolastatin series of compounds isolated and fully characterized from the sea hare *Dolabella auricularia* [1,2], is a novel linear, hydrophobic pentapeptide which has been shown in vitro to be a very potent antimitotic and antineoplastic agent with typical IC_{50} values of 0.1–1 n*M* [3,4]. DOL-10 was chosen by the National Cancer Institute for further evaluation as a candidate antitumor drug for patients with advanced refractory solid tumors. Preclinical evaluation in mice performed using tritiated DOL-10 showed that the drug is highly protein-bound (>80%), is rapidly metabolized with a relatively low AUC (area under the concentration–time curve) following bolus iv injection of a 240 μg/kg dose ($t_{1/2}$=5.6 h), and is cleared from the bloodstream through the bile by way of metabolites [5]. Urinary excretion of DOL-10 and metabolites accounted for <2% for each. The highest plasma DOL-10 con-

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centration achieved at the above dose was approximately 32 ng/ml but remained in the 1–8 ng/ml range for 8 h. DOL-10 was observed to be stable in mouse plasma for at least 24 h at 37°C. In vitro analysis with a rat liver homogenate showed extensive conversion of the drug to a minimum of three polar metabolites.

In a human phase I clinical trial, plasma levels of DOL-10 were shown to be in the low ng/ml to pg/ml concentration range which is much lower than detection limits of routine analytical methods [6]. These levels were not unexpected since the dose of drug administered intravenously was 65–300 μg/m^2 coupled with the rapid metabolism of the drug. Consequently, the highly sensitive and specific on-line LC–MS interfaced by electrospray ionization was used to analyze the parent DOL-10 and metabolites [6]. This assay provided a lower limit of quantitation of 5 pg/ml DOL-10 from extracted plasma samples using the analogue DOL-15 as the internal standard. An *N*-demethylated species was identified as the only metabolite to be generated in patients receiving this drug. However, using the same activated S9 rat liver preparation as was used in the preclinical studies, it was shown that two metabolites were generated in vitro, a major *N*-demethylated species and a minor hydroxylated species. Presently, the activity and toxicity of the *N*-demethyl DOL-10 compound have not been evaluated in vivo. An investigation of the pharmacokinetics of DOL-10 in humans revealed a rapid distribution and elimination of parent drug after bolus intravenous administration with a concomitant rapid appearance of the *N*-demethylated metabolite [7]. The maximum tolerated dose was documented as 300 μg/m^2 with dose-limiting grade 4 neutropenia [7]. It was earlier reported that one of the metabolites generated in vitro using the S9 rat liver microsomal preparation was a dihydroxylated species as shown by electron impact-MS studies [5]. However, these studies were preliminary for the structural elucidation of metabolites and were not considered as conclusive evidence without verification by additional chromatographic and mass spectral investigations. The LC–MS results on the metabolism of DOL-10 did not show any dihydroxylated species being produced from both in vitro and in vivo studies [6]. The phase II clinical trial has recently begun for DOL-10 at M.D. Anderson Cancer Center.

A validated high-performance capillary electrophoresis (HPCE) assay has recently been reported for DOL-10 [8]. This method provides a limit of detection of 25 ng/ml DOL-10 from extracted human plasma samples. By additional enhancement of sensitivity, this HPCE method could find use for the evaluation of pharmacologic parameters of DOL-10. Also, from the HPCE assay preliminary evidence revealed that three uncharacterized metabolites were produced from the activated rat liver preparation. In this communication we report an enhancement of the detection limit to 3 ng/ml DOL-10 in human plasma by modifications of the separation procedure. This detection improvement will allow the routine evaluation of clinical samples where plasma levels of drug are ≥5 ng/ml. In addition, it will facilitate the characterization of metabolites where more than one was produced from activated rat liver preparations.

2. Experimental

2.1. Reagents and chemicals

The reagents and chemicals used for these studies are the same as previously described [8]. Working solutions of DOL-10 and the internal standard DOL-15 were 500 ng/ml each in methanol and were prepared from 100 μg/ml stock solutions. Male mice (CD2F1) were supplied by the National Institutes of Health (Bethesda, MD, USA). Mouse plasma was purchased from Pel-Freeze Biologicals (Rogers, AR, USA).

2.2. Extraction procedure

All plasma samples were extracted by liquid–liquid extraction using butyl chloride as previously described [8]. Briefly, 1 ml of sample was shaken once with 5 ml of butyl chloride at high speed for 1 h using a rocker shaker. The extractant was dried under nitrogen at room temperature and reconstituted in 50 μL of sample buffer (below) for analysis.

2.3. Capillary electrophoresis

The HPCE assay of DOL-10 was performed using a Beckman P/ACE System 2100 equipped with P/ACE 2000 Series software and coupled to an IBM

PS/2 computer. All parameters and conditions were the same as detailed earlier [8] which included a bare fused-silica capillary (75 μm×57 cm), a 50 m*M* NaH_2PO_4 (pH 2.9) separation buffer, sample reconstituted in 50 μL of 5 m*M* NaH_2PO_4 (pH 2.9)/ methanol (9:1) following the extraction procedure, and UV detection at 200 nm. Exceptions in the conditions from those described in the previous work [8] include the following: the capillary was maintained at 50°C during the separations, the separation potential was held constant at 12.5 kV producing approximately 70 μA of current across the capillary under these conditions, and the sample was pressure-injected (0.5 p.s.i., 3447 Pa) for 12 s onto the capillary before the separation.

2.4. Linearity

The linearity of the method was assessed at concentrations of DOL-10 ranging from 0 to 100 ng/ml ($n=7$; DOL-15 internal standard at 50 ng/ml) as addressed previously [8]. The results were analyzed using least-square linear regression analysis. Constructed curves for both extracted and neat, standard samples represent averages from duplicate tests.

2.5. Percentage recovery

The extraction efficiency was assessed using spiked human plasma at DOL-10 concentrations of 5, 10, 25, 50, 75, and 100 ng/ml. The assays were performed in duplicate at each level as addressed previously [8].

2.6. Limits of detection and quantitation

The limit of detection for DOL-10 was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1 for a blank plasma sample. The limit of quantitation was determined to be that level of drug with a coefficient of variation (C.V.) and a percentage deviation of the nominal concentration of less than 20%.

2.7. Application of method to murine model

DOL-10 was administered to 14 mice in groups of four or five by three routes: intravenous (tail i.v.), intraperitoneal (i.p.), and oral at a single dose of 600 μg/kg using a 150 μg/ml solution of DOL-10 in normal saline–ethanol (98:2). Untreated mouse plasma pool was used as a control. Each mouse was sacrificed by decapitation 15 min after injection and blood was collected immediately in a 2 ml Eppendorf tube containing heparin. Approximately 30 s before sacrifice, the mice were anesthetized with metofane in a closed dessicator. Plasma was obtained by centrifugation at 16 000 *g* for 10 min using an Eppendorf Centrifuge (Model 5415 C) and pooled for each route of drug administration (approximately 1.5–2 ml collected). A 1 ml aliquot of plasma from each pooled sample was added to a tube containing 50 ng of DOL-15 internal standard. Extraction of the plasma with butyl chloride was performed with subsequent HPCE analysis as described above.

2.8. Application of method to human patient

Blood from a selected patient in the phase I study was collected in a tube containing heparin and was immediately centrifuged to yield plasma which was stored frozen until analysis. The patient received a DOL-10 dose of 65 $\mu g/m^2$ as a short bolus iv injection and the blood was drawn immediately at the end of the drug infusion. A pre-dose plasma sample served as the control for this study. A 1 ml aliquot of plasma was added to a tube containing 50 ng of DOL-15 internal standard. Extraction of the plasma with butyl chloride was performed followed by HPCE analysis as described above.

3. Results

The effects of separation temperature on DOL-10 and DOL-15 peak shapes are illustrated in Fig. 1. The pronounced effects are depicted for three widely differing temperatures (15°C, 30°C, and 50°C). Actual experiments were performed in increments of 5°C for the range 15°C to 50°C with the peak shapes and heights improving during the trend of increasing temperature. This range of temperatures was selected due to the programmability limitations for the HPCE instrument.

Fig. 2 illustrates electropherograms generated from extracted human plasma samples in which the plasma is (A) devoid of DOL-10, (B) supplemented

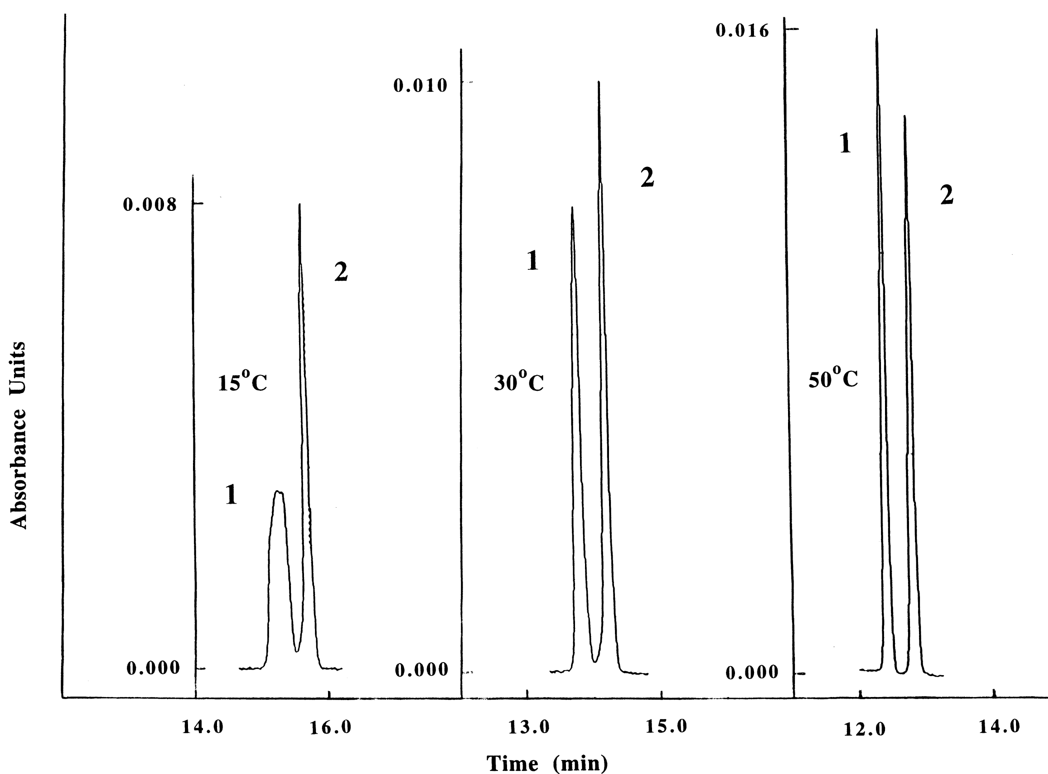

Fig. 1. HPCE electropherograms demonstrating the temperature effects (15–50°C) on peak shapes for DOL-10 (peak 1 at 1250 ng/ml) and DOL-15 (peak 2 at 1250 ng/ml). Samples were pressure-injected for 12 s onto the capillary before separation. The separation conditions are described in the experimental section of the text.

with a high level of DOL-10, and (C) supplemented with a low level of DOL-10. The average migration times for DOL-10 and DOL-15 were 12.0 min and 12.5 min, respectively. No interfering peaks were detected in the range of the migration times of DOL-10 and DOL-15.

The HPCE method was evaluated for linearity, recovery, and detection limit of drug from plasma samples. Quantitation of DOL-10 concentrations was determined from an external constructed linearity curve. The linearity was evaluated from 0 to 100 ng DOL-10 (n=7) using 1 ml of plasma. Regression analysis gave a line with the equation $y=0.018x+0.085$, where y is the peak–area ratio (DOL-10/DOL-15) and x is the concentration of DOL-10 (in ng/ml). The coefficient of correlation (r) was 0.993 ($r^2=0.985$). For comparison, the neat samples gave a line of $y=0.025x+0.015$ ($r=0.991$ and $r^2=0.982$). The extraction recovery of DOL-10 averaged 84.9% over all 6 levels. Excluding the lowest level of drug that was analyzed (5 ng/ml at 61.8% recovery), the average recovery for the remaining 5 levels was 89.5%. The detection limit for this assay was determined to be 3 ng/ml DOL-10 in human plasma at a signal-to-noise ratio of 3:1. No endogenous components interfered with this determination from a blank sample. The lower limit of quantitation for this assay was established at 5.0 ng/ml DOL-10, with a C.V. of 16.2% ($n=7$) and a percentage deviation of the nominal concentration of 12% (mean conc.= 5.7±0.9 ng/ml).

The improved HPCE assay was applied to mice given the drug by three different routes and with a human patient enrolled in a phase I study. Fig. 3 shows the electropherograms that were generated from the plasmas of mice and a patient administered

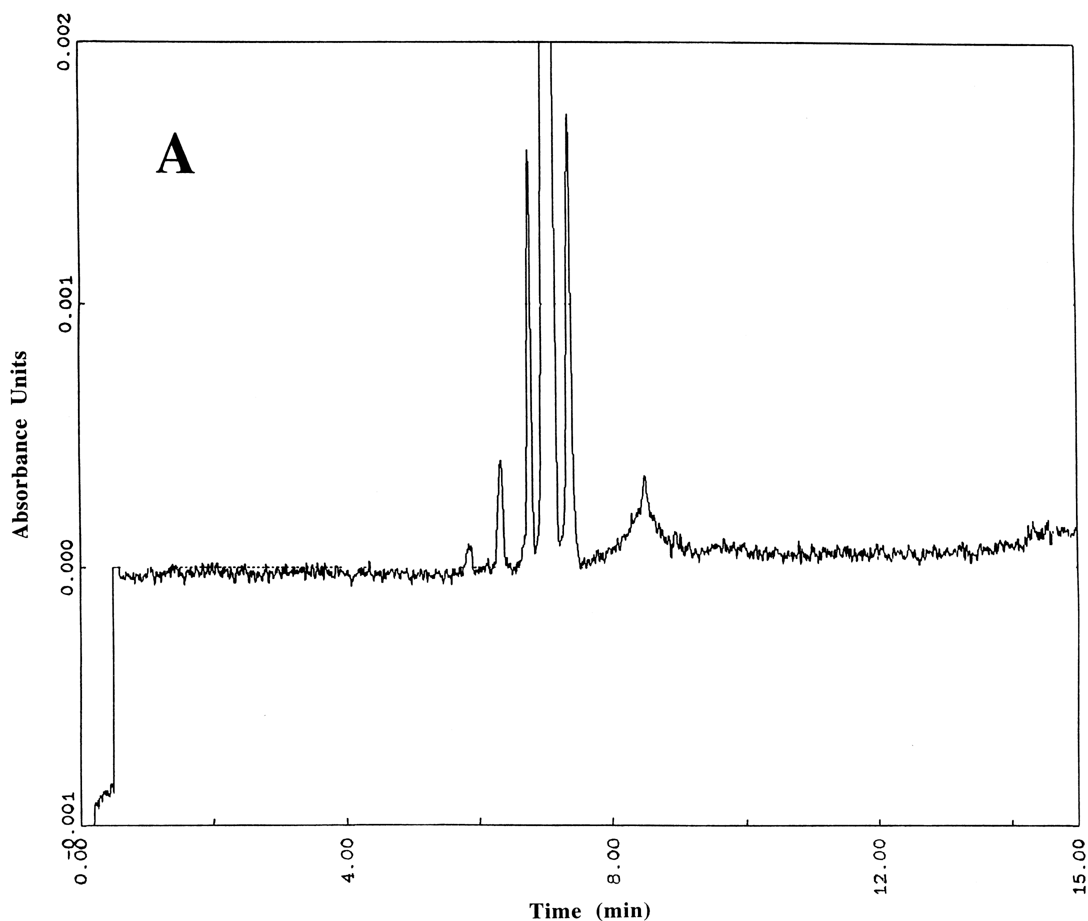

Fig. 2. HPCE electropherograms generated from drug-spiked human plasma of (A) blank sample, (B) sample supplemented with DOL-10 (peak 1 at 100 ng/ml) and DOL-15 (internal standard, peak 2 at 50 ng/ml), and (C) sample supplemented with DOL-10 (peak 1 at 5 ng/ml) and DOL-15 (peak 2 at 50 ng/ml). The separation conditions are described in the experimental section of the text.

DOL-10 by a rapid iv injection. The calculated concentrations of DOL-10 in these plasmas were 32 ng/ml and 44 ng/ml for the mice and patient, respectively. The DOL-10 plasma concentration in the mice administered the drug by ip route (same dosage as with iv route) was 26 ng/ml. No drug was detected in the plasma of mice which were given DOL-10 by the oral route. Also, no interfering peaks were observed for control plasma samples from the mice or the patient. It is noteworthy that in the murine studies no discernable DOL-10 peaks were observed in the electropherograms when these same plasma samples were subjected to capillary separation at 25°C while using the assay parameters as described in the original HPCE/DOL-10 paper [8].

4. Discussion

We have greatly improved on the detection limit of plasma DOL-10 by HPCE analysis. This achievement was made primarily by the use of elevated separation temperatures, specifically 50°C, coupled with a larger sample injection volume (a 12 s low-pressure injection is approximately 2.9% of the entire capillary volume or 73 nL of sample volume

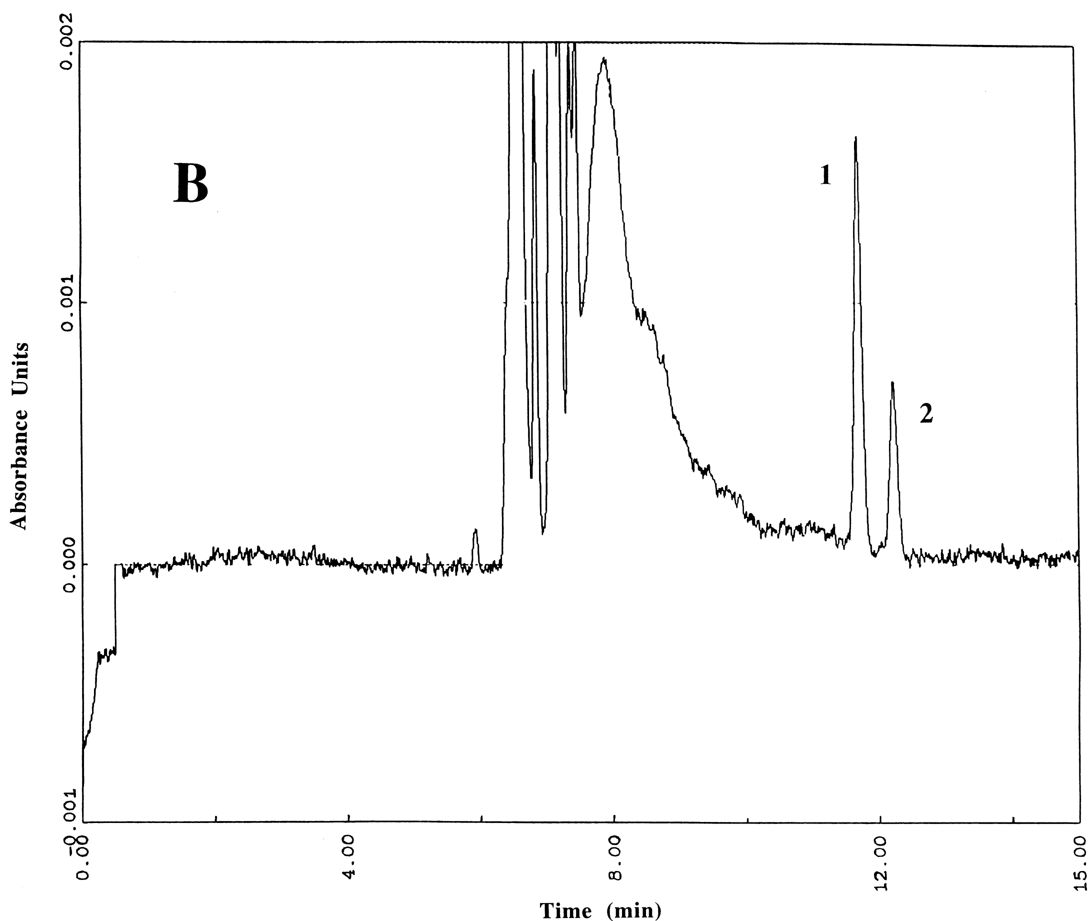

Fig. 2. (*continued*)

for a 75 μm×57 cm capillary [9]). At this elevated temperature, greater injection volumes resulted in gradually poorer baseline resolution for the two dolastatin compounds. With the high separation temperature of 50°C, the peak shape and peak heights of DOL-10 were dramatically improved from ambient temperatures, with DOL-15 exhibiting considerably less improvement in peak shape and height. The sharper and highly-resolved peaks translate into much improved peak area counts and peak heights resulting in lower limits of detection for the drug. The separation efficiency is improved for the closely-related DOL-10 and DOL-15 compounds (compare electropherograms from this report and the original HPCE assay for DOL-10 [8]). No noticeable selectivity difference was observed for the two dolastatin peptides as the separation temperature increased from 15°C to 50°C. This assay also gave more rapid analysis times (<15 min) and better baseline resolution for the dolastatin peptides in comparison to the results obtained at ambient temperature [8]. Although we were restricted to an upper limit of 50°C separation temperature on the Beckman instrument, it would be interesting to determine if higher temperatures would give even sharper peaks for DOL-10. Additionally, because of the very clean electropherograms generated using butyl chloride extractions, it is quite possible that even lower detection limits could be realized by using a different internal standard which would provide a greater electrophoretic separation from DOL-10. This would allow a larger volume of sample to be injected onto

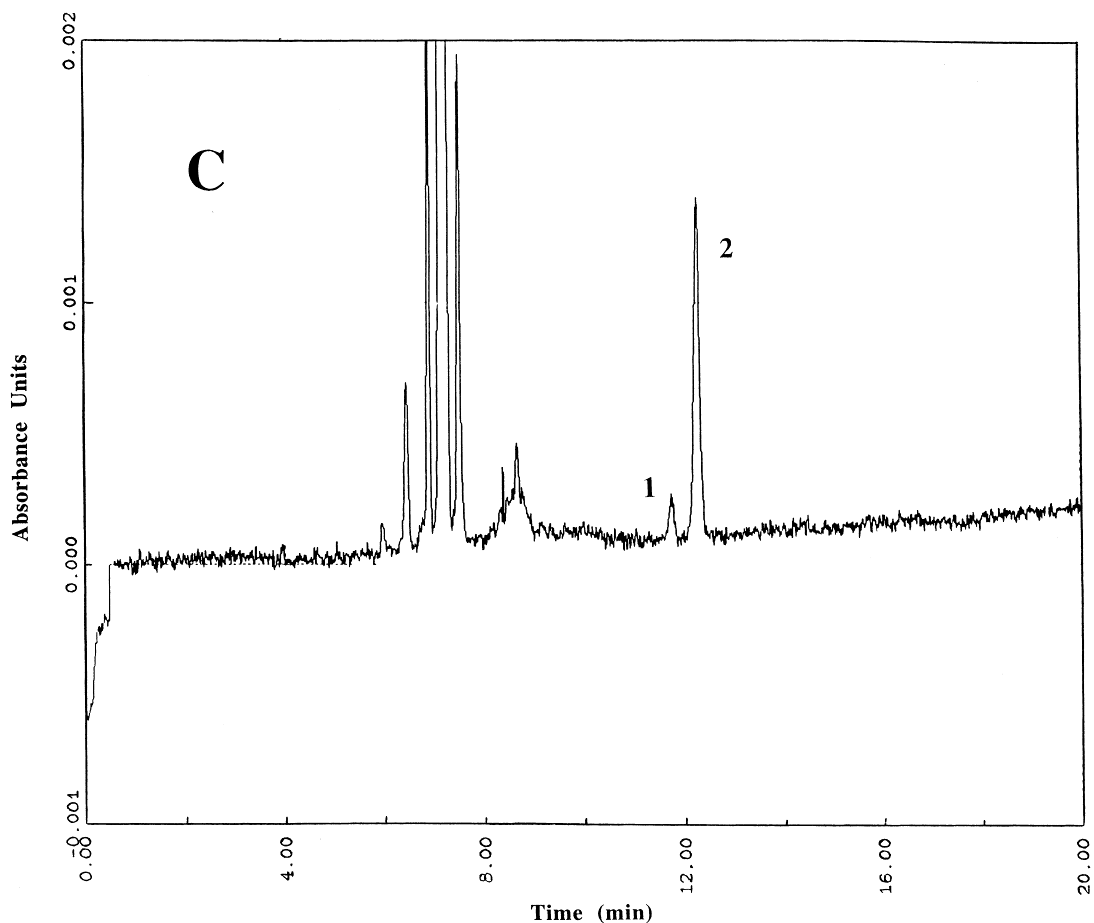

Fig. 2. (*continued*)

the capillary without compromising the resolution of DOL-10. However, caution would need to be exercised when injecting large sample volumes as capillary overloading could lead to non-linear calibration curves. Many critics of capillary electrophoresis point to the fact that the HPCE analytical technique cannot achieve concentration detection limits comparable to HPLC using UV absorbance detection (mass detection limits for HPCE are generally much lower than those from HPLC). However, with improved on-line stacking techniques and other new developments such as the elevated temperature effects shown here, progress has indeed been made to achieve concentration detection limits comparable to HPLC (typically, 5–10 ng/ml with UV detection).

Increased temperatures are known to give greater efficiencies for separations, to a large extent for HPLC analyses of various peptide and protein compounds. Temperature effects in HPLC separations have been recently reviewed [10]. As examples, the large-molecular immunosuppressants such as the cyclic oligopeptide, cyclosporine [11,12], and the cyclic macrolides, FK-506 [13] and rapamycin [14–16], have been analyzed at temperatures between 40°C and 80°C with enhanced column separations and efficiencies. The high separation temperatures appear to favor one major conformation versus several possible conformations of these molecules at ambient temperatures thereby giving rise to sharp, gaussian-shaped peaks. Additionally, the higher temperatures could alter the solubility of the analytes causing enhanced chromatographic results. The

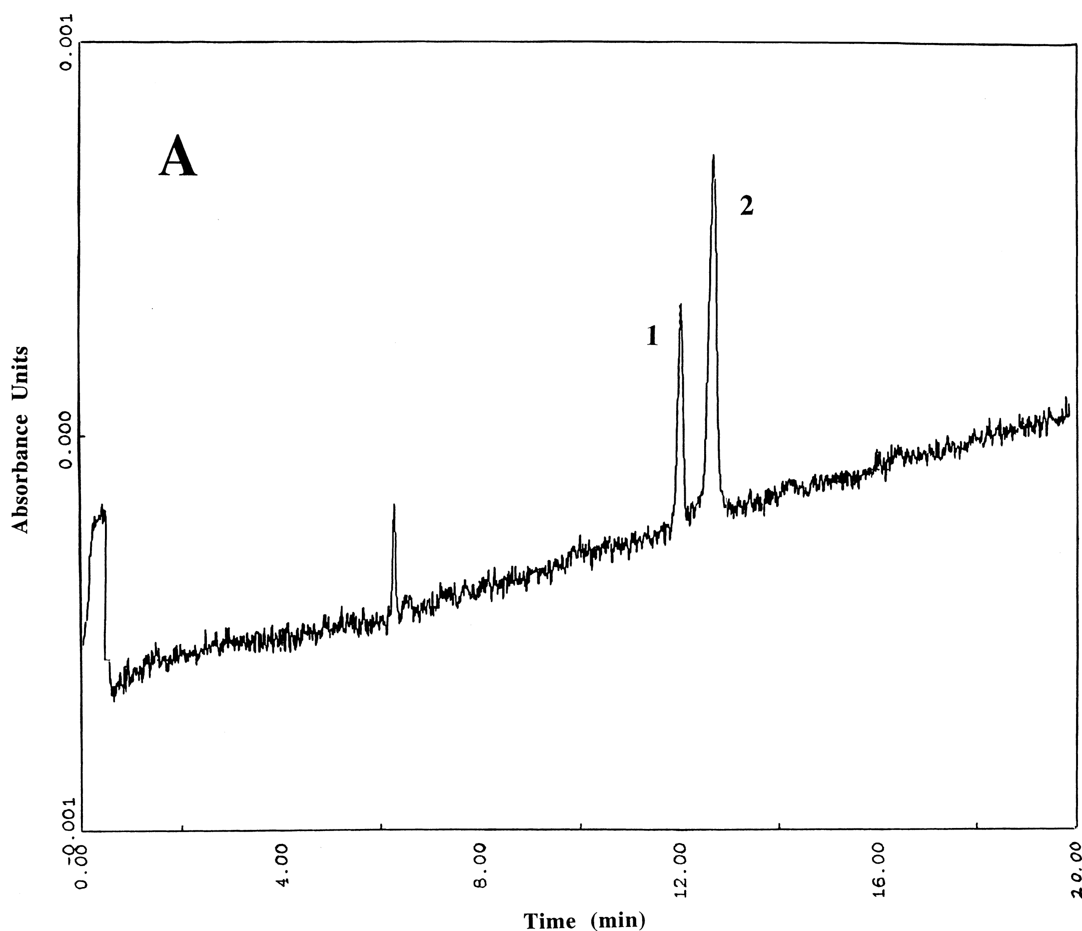

Fig. 3. HPCE electropherograms generated from the plasma of (A) mice and (B) patient following rapid iv injection of DOL-10 at dosages of 600 μg/kg and 65 μg/m^2, respectively. Calculated DOL-10 (peak 1) concentrations are 32 ng/ml and 44 ng/ml, respectively. DOL-15 (peak 2 at 50 ng/ml) is the internal standard. The separation conditions are described in the experimental section of the text.

linear pentapeptide structure of DOL-10 may adopt a major conformational structure at higher temperatures in similar nature to the cyclic undecapeptide structure of cyclosporine and/or be influenced by increased solubility in the separation buffer. Interestingly, the effects are not as dramatic for the linear depsipeptide DOL-15, a heptapeptide used as the internal standard in the present assay.

Very few reports have been published for HPCE analyses employing elevated separation temperatures. Some examples for small molecules include serum felbamate by micellar electrokinetic capillary chromatography (MECC) at 35°C [17], MECC analysis of non-steroidal anti-inflammatory agents at 50°C in tablet samples [18], serum tamoxifen/metabolites [19] and pyrazoloacridine metabolites [20] both at 40°C using non-aqueous buffer systems, and a temperature study (25–40°C) of nine β-blockers by MECC [21]. The influence of temperature on HPCE separations of macromolecules include the proteins myoglobin and α-lactalbumin (20–50°C) [22] and DNA fragments (60°C) [23].

The in vivo application of this HPCE method to the quantitation of DOL-10 in mice and a patient enrolled in the clinical phase I study was successful. Pooled blood samples from mice were necessary in order to have a minimum of 1 ml of plasma for the study. The concentrations of DOL-10 analyzed by

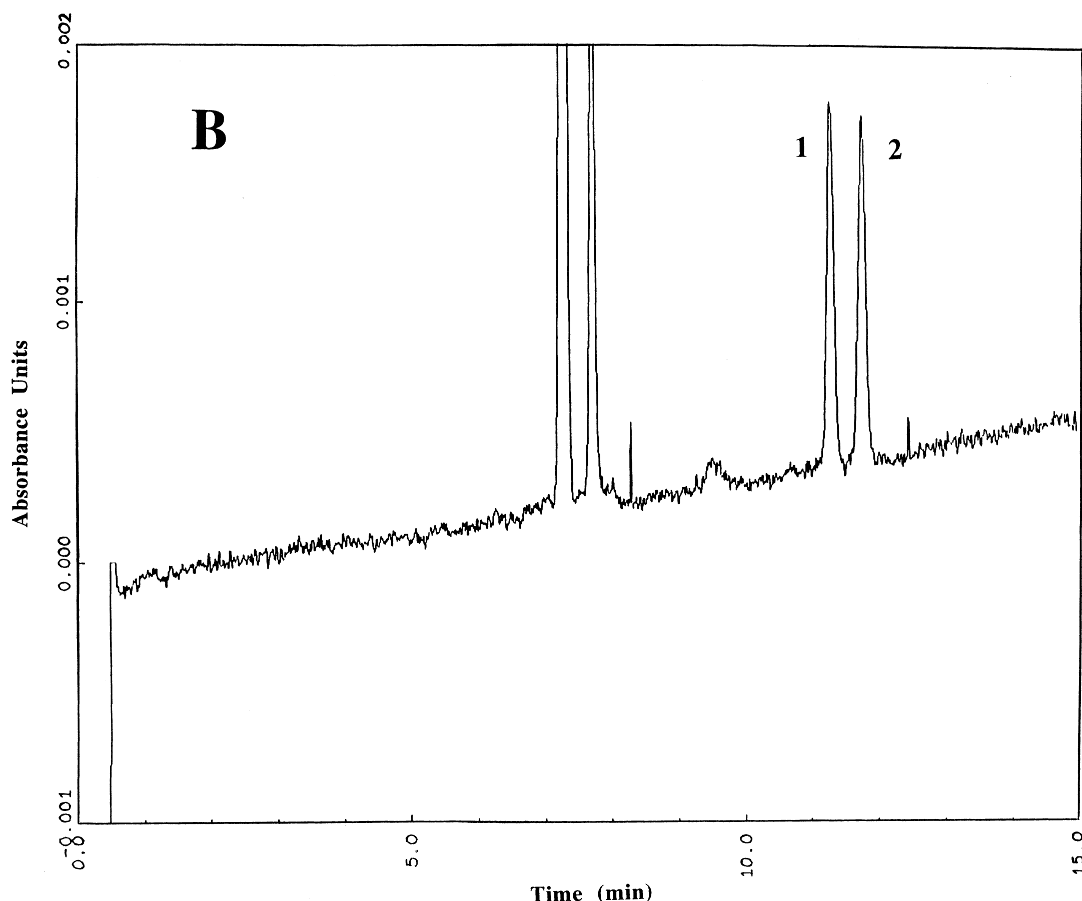

Fig. 3. (*continued*)

HPCE for both the iv and ip routes of drug administration were similar (32 ng/ml and 26 ng/ml, respectively). However, no drug was detected in the plasma from the mice which were administered the drug by the oral route. Assuming sufficient oral absorption of DOL-10, degradation of this relatively small peptide could occur by non-specific peptidases in the stomach and/or small intestine. It is reported from the preclinical pharmacology studies of DOL-10 that this peptide is stable to plasma proteases and peptidases from the mouse, dog, and human [5]. The DOL-10 concentration obtained by the HPCE analysis of the plasma from the human patient agrees favorably to the concentration obtained by the LC–MS analysis [6] (44 ng/ml and 37 ng/ml DOL-10 from HPCE and LC–MS, respectively). Based on preliminary LC–MS results from the phase I study [6], this HPCE assay would allow routine DOL-10 sampling up to 15–30 min and 1–2 h post-drug administration for the 65 $\mu g/m^2$ and 100 $\mu g/m^2$ drug doses, respectively. Higher dosages, up to the maximum tolerated dose level of 300 $\mu g/m^2$, would allow extended sampling times from the patients. Further sampling times for plasma DOL-10 would require more sensitive analytical methods such as LC–MS or CE–MS.

In conclusion, high separation temperatures may provide lower detection limits in the HPCE analyses of other oligopeptide and non-peptide drugs. In developing an optimal HPCE method for organic or bioorganic compounds, the effects of temperature on peak areas/heights and separations should be care-

fully investigated. Above or below ambient temperatures may prove beneficial in effecting ideal separations and sensitivities.

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References

[1] G.R. Pettit, Y. Kamano, Y. Fujii, C.L. Herald, M. Inoue, P. Brown, D. Gust, K. Kitahara, J.M. Schmidt, D.L. Doubek, C. Michel, J. Natl. Prod. 44 (1981) 482.
[2] G.R. Pettit, Y. Kamano, C.L. Herald, Y. Fujii, H. Kizu, M.R. Boyd, F.E. Boettner, D.L. Doubek, J.M. Schmidt, J.-C. Chapuis, C. Michel, Tetrahedron 49 (1993) 9151.
[3] G.R. Pettit, Y. Kamano, C.L. Herald, A.A. Tuinman, F.E. Boettner, H. Kizu, J.M. Schmidt, L. Baczynskyj, K.B. Tomer, R.J. Bontems, J. Am. Chem. Soc. 109 (1987) 6883.
[4] G.W. Aherne, A. Hardcastle, M. Valenti, A. Bryant, P. Rogers, G.R. Pettit, J.K. Srirangam, L.R. Kelland, Cancer Chemother. Pharmacol. 38 (1996) 225.
[5] R.A. Newman, A. Fuentes, J.M. Covey, J.A. Benvenuto, Drug Metab. Dispos. 22 (1994) 428.
[6] D.A. Garteiz, T. Madden, D.E. Beck, W.R. Huie, K.T. McManus, J.A. Abbruzzese, W. Chen, R.A. Newman, Cancer Chemother. Pharmacol. 41 (1998) 299.
[7] H.T. Tran, R.A. Newman, D.E. Beck, W.R. Huie, J.L. Abbruzzese, T. Madden, Proc. Am. Assoc. Cancer Res. 38 (1997) 306.
[8] W.R. Huie, R.A. Newman, T. Hutto, T. Madden, J. Chromatogr. B 693 (1997) 451.
[9] J. Harbaugh, M. Collette, H.E. Schwartz, Technical Bulletin TIBC-103. Palo Alto, CA, Beckman Instruments, 1990.
[10] B. Ooms, LC·GC 14 (1996) 306.
[11] R.J. Sawchuk, L.L. Cartier, Clin. Chem. 27 (1981) 1368.
[12] L.D. Bowers, S.E. Mathews, J. Chromatogr. 333 (1985) 231.
[13] A.M. Alak, Ther. Drug Monit. 19 (1997) 338.
[14] K.L. Napoli, B.D. Kahan, Clin. Chem. 37 (1991) 294.
[15] R.W. Yatscoff, D.F. LeGatt, N.M. Kneteman, Ther. Drug Monit. 15 (1993) 478.
[16] J.O. Svensson, C. Brattstrom, J. Sawe, Ther. Drug Monit. 19 (1997) 112.
[17] Z.K. Shihabi, K.S. Oles, Clin. Chem. 40 (1994) 1904.
[18] R. Weinberger, M. Albin, J. Liq. Chromatogr. 14 (1991) 953.
[19] J.M. Sanders, L.T. Burka, M.D. Shelby, R.R. Newbold, M.L. Cunningham, J. Chromatogr. B 695 (1997) 181.
[20] L.M. Benson, A.J. Tomlinson, J.M. Reid, D.L. Walker, M.M. Ames, S. Naylor, J. High Resolut. Chromatogr. 16 (1993) 324.
[21] P. Lukkari, A. Ennelin, H. Siren, M.-L. Riekkola, J. Liq. Chromatogr. 16 (1993) 2069.
[22] R.S. Rush, A.S. Cohen, B.L. Karger, Anal. Chem. 63 (1991) 1346.
[23] J.-Z. Zhang, Y. Fang, J.Y. Hou, H.J. Ren, R. Jiang, P. Roos, N.J. Dovichi, Anal. Chem. 67 (1995) 4589.