ELSEVIER

Journal of Chromatography B, 652 (1994) 109–113

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Determination of plasma novobiocin levels by a reversed-phase high-performance liquid chromatographic assay

Tian-Ling Chen*, M. John Kennedy, Vanessa M. Dunlap, O. Michael Colvin

Division of Pharmacology and Experimental Therapeutics, The Johns Hopkins Oncology Center, Room 1-121, 600 North Wolfe Street, Baltimore, MD 21287 USA

(First received August 11th, 1993; revised manuscript received November 2nd, 1993)

Abstract

A simple and specific reversed-phase high-performance liquid chromatographic (HPLC) assay for the determination of novobiocin levels in human plasma has been developed. The sample preparation was performed by deproteinization with methanol. Prednisone was used as an internal standard. Both novobiocin and prednisone were separated on a C_8 column with a gradient elution of acidic water (pH 3.0)–methanol. The recovery of novobiocin from plasma was nearly complete. The linear range was 5–1000 μM in 0.5 ml of plasma with a minimum limit of determination of 2.25 fmol of novobiocin at 254 nm. The method has been implemented and validated in an ongoing clinical trial.

1. Introduction

Novobiocin (Fig. 1), a coumermycin antibiotic, has been in limited use in the treatment of infectious diseases due to its adverse reactions and the rapid emergence of bacterial resistance [1]. Recently, increased interest in this antibiotic in the field of oncology has focused on the modulation of the cytotoxicity of various anticancer drugs to tumor cells, especially alkylating agents [2–8]. Novobiocin inhibits bacterial gyrase and its eukaryotic counterpart, topoisomerase II, a nuclear enzyme implicated in DNA replication, transcription, and repair [9,10]. Alkylating agents are lethal to cells via a mechanism involving the formation of DNA monoadducts and subsequent inter- or intrastrand crosslinks [2]. The decrease in the amount of DNA interstrand crosslinks associated with elevated topoisomerase II activities has been reported as one of the mechanisms by which tumor cells are resistant to alkylating agents [5]. Since novobiocin is a potent topoisomerase II inhibitor, it is rational to assume that the combination of novobiocin with alkylating agents would increase the formation of DNA monoadducts by inhibition of DNA repair, thereby resulting in enhanced alkylating agent effectiveness. Both *in vitro* and *in vivo* experiments have demonstrated that novobiocin potentiates DNA interstrand crosslink formation as well as alkylating agent cytotoxicity in various cell lines and murine models [2,3,5,6,11]. These promising preclinical data have prompted the investigation

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(93)E0379-5

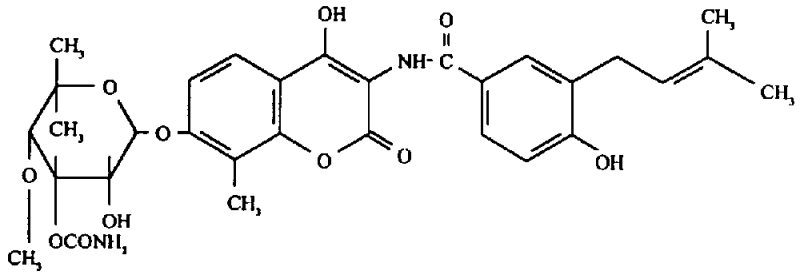

Fig. 1. Structure of novobiocin.

of combined regimens of novobiocin with alkylating agents in patients with refractory cancer [1,12].

There are several techniques available for the determination of the presence of novobiocin. However, microbiological and chemical methods are not sensitive and accurate enough for quantitative analysis [13]. A gas–liquid chromatographic method provides excellent precision but requires derivatization of the parent drug [13]. Several HPLC assays have been developed for formulation analysis [13–15] rather than for routine drug monitoring. One HPLC assay for residue analysis has been reported but involves somewhat complicated procedures [16]. We have developed a simple, sensitive, and specific reversed-phase HPLC assay for the routine quantification of novobiocin in plasma using prednisone as an internal standard [14]. This assay has been implemented and validated in an ongoing phase I clinical trial at the The Johns Hopkins Oncology Center. Since isonovobiocin and degradation products of novobiocin are microbiologically inactive [13,17,18], differentiating these compounds from novobiocin has not been taken into consideration in this study.

2. Experimental

2.1. Reagents

Novobiocin, prednisone, and trifluoroacetic acid were purchased from Sigma (Sigma Chemical Company, St. Louis, MO, USA). All organic solvents were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Instrumentation and chromatographic conditions

A Spectra Physics HPLC system (Thermo Separation Products, Fremont, CA, USA) equipped with a P4000, quaternary gradient pump with a four-solvent helium degassing kit, an AS3000 variable-loop autosampler (100-μl loop installed), and a UV2000 dual-wavelength UV-Vis programmable detector with a 10-mm flow cell was used. Separations were performed on a C_8 column, 150 × 4.6 mm I.D., packed with 5-μm particle-sized APEX octyl EC (Jones Chromatography, Lakewood, CO, USA) with a CN Guard-Pak precolumn insert (Waters Chromatography, Marlborough, MA, USA). The precolumn was changed frequently to prolong the column life. The mobile phase consisted of solvent A, Milli-Q water (Millipore, Marlborough, MA, USA) acidified to pH 3.0 with trifluoroacetic acid, and solvent B, methanol. A gradient elution was run linearly from 45% B to 80% B in 20 min at a flow-rate of 1.5 ml/min. Detection was performed at 254 nm. Chromatographic data were acquired by a PE Nelson 900 series intelligent interface (Perkin-Elmer, Morrisville, NC, USA). The converted digital data were then transmitted to an IBM compatible personal computer, Dell 486 SX (Dell, Austin, TX, USA), and were analyzed using a PE Nelson 2600 chromatographic data system (Perkin-Elmer).

2.3. Preparation of standards

Stock solutions were prepared by weighing and diluting appropriate amounts of novobiocin

and prednisone in methanol to make up 1 m*M* and 5 m*M* solutions, respectively. These solutions were stored at 4°C and were stable for several months [19].

2.4. Plasma sample preparation

To each 0.5-ml aliquot (1 ml for patients receiving < 2 g/day) of plasma, 10 μl of 5 m*M* of prednisone solution and 5 ml of methanol were added. The mixture was vortex-mixed for 10 s and centrifuged at 2000 *g* for 10 min. The supernatant was transferred to an autovial and 15 μl were injected directly onto the column.

2.5. Quantification

Calibration curves were obtained daily by plotting the area ratios of novobiocin to prednisone against the concentration of novobiocin over the range 0–500 μM. The area ratios of unknown samples were compared with the calibration curve in plasma.

2.6. Method validation

The recovery of novobiocin from plasma was determined by comparing peak areas following injections of plasma spiked with novobiocin in quintuplicate with those by direct column injection at three concentrations: 10 μM, 100 μM, and 500 μM. The intra-day precision was completed by injections of quintuplicate plasma samples spiked with novobiocin at 10 μM, 100 μM, and 500 μM on the same day. The inter-day precision was determined by injecting 50 μM and 200 μM samples daily for five days. The linear response of the detector was tested by making triplicate injections of a series of plasma standards ranging from 5 to 1000 μM.

2.7. Pharmacological application

The method described above has been applied to an ongoing phase I clinical trial at The Johns Hopkins Oncology Center. Patients with resected breast cancer received high-dose alkylator chemotherapy with novobiocin, doses ranged from 0.25 g to 2.5 g every 12 h for a total of fourteen doses. Plasma novobiocin levels were measured before the first dose (pre-dose sample), before the fourth dose, and 1–7 h following the fourth dose of novobiocin.

3. Results and discussion

The UV absorption of novobiocin in methanol shows maxima at 242–254 nm and 302–314 nm (data not shown). By HPLC, we found that, quantitatively, there is no significant difference in the absorption at wavelengths between 242 and 254 nm. Since prednisone is suitable for monitoring at 254 nm, the wavelength of 254 nm was chosen for the determination of novobiocin in this study. Chemically, novobiocin is a weak acid with pK_a values of 4.3 and 9.1 [19]. The separation of novobiocin can be influenced by the pH of the mobile phase, the column selected, and the method of elution (isocratic *vs.* gradient). Fig. 2 shows representative chromatograms of novobiocin from patient plasma samples. At pH 3.0 and with gradient elution, novobiocin and prednisone were well separated on a C_8 column. Retention times of prednisone and novobiocin were 5.5 and 18.1 min, respectively. Both peaks were symmetrical and had good base line resolution with minimum tailing. Therefore, the HPLC conditions utilized were suited for the adequate separation and retention of novobiocin.

Calibration curves obtained by plotting peak-area ratios of novobiocin to prednisone *vs.* novobiocin concentrations showed good linearity with correlation coefficients >0.99. Typical linear regressions were $y = 76.96x + 1.06$ (0.5 ml plasma) and $y = 51.79x - 1.35$ (1 ml plasma), respectively.

The recoveries of novobiocin from plasma were 97%, 104%, and 98% at concentrations of 10 μM, 100 μM, and 500 μM, respectively. These results were similar to those reported by Moats and Leskinen [16]. Intra-day and inter-day precision data are shown in Table 1.

The detector response was linear over the range of 5–1000 μM in 0.5 ml plasma with a

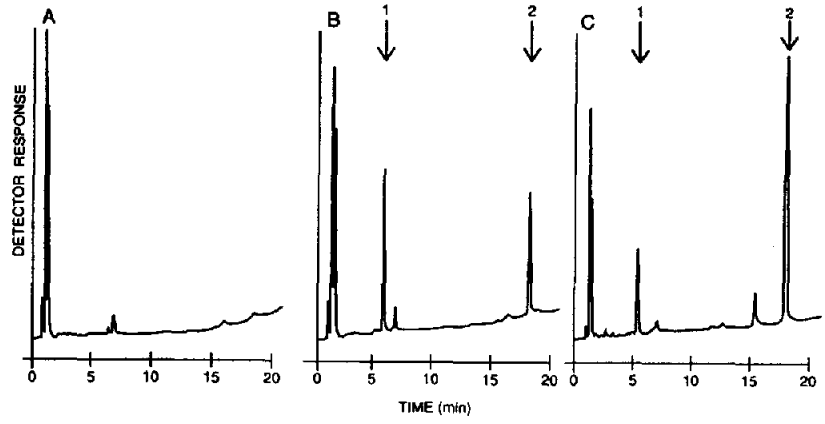

Fig. 2. Chromatograms of (A) patient pre-dose plasma sample after methanol deproteinization; (B) patient pre-dose plasma sample spiked with prednisone and novobiocin; (C) patient plasma sample 7 h after the fourth dose of novobiocin at 1.5 g. Peaks: 1 = prednisone; 2 = novobiocin.

linear regression equation of y (peak area) = $573x$, and a correlation coefficient of 0.9972.

The limit of the determination, defined as the minimum drug concentration corresponding to two times the signal-to-noise ratio, was 5 μM in 0.5 ml plasma and 2.25 fmol by direct column injection. The sensitivity of the determination of the plasma sample can be further improved by increasing the sample size and reducing the plasma:methanol ratio to 1:3.

To date, the method described here has been successfully utilized to analyze over 100 samples from patients after administration of 0.5–5 g/day oral doses of novobiocin in an ongoing phase I clinical trial at The Johns Hopkins Oncology Center. The range of novobiocin concentration was found to be 5–700 μM, indicating that the calibration range for the assay was adequate. Fig. 3 shows typical novobiocin plasma concentration–time profiles in patients receiving the fourth oral doses of 0.5 g and 2.5 g, respectively.

Table 1
Precision data of the HPLC assay for novobiocin

Concentration (μM)	Peak area (mean ± S.D.) (μV s)	Coefficient of variation (%)
Intra-day[a] *(n = 5)*		
10	6219 ± 236	3.8
100	62 846 ± 651	1.04
500	357 588 ± 5060	1.42
Inter-day[b] *(n = 5)*		
50	2 706 175 ± 77 397	2.86
200	11 427 764 ± 325 434	2.85

[a] 0.5 ml of plasma were spiked at the indicated concentrations.
[b] 1 ml of plasma was spiked at the indicate concentrations.

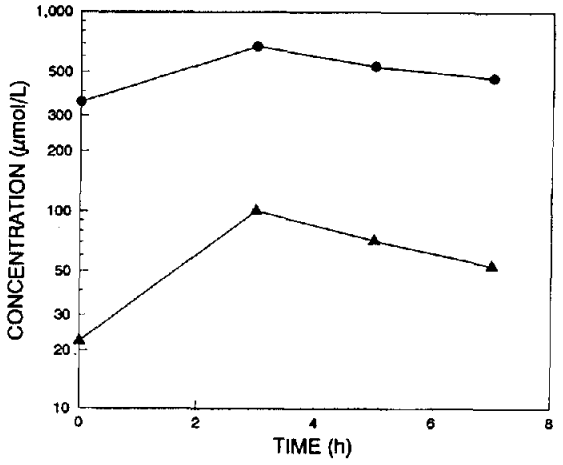

Fig. 3. Plasma concentration–time profiles of novobiocin following the fourth dose of novobiocin at 0.5 g (▲) and 2.5 g (●), respectively.

4. Conclusion

The new HPLC assay described in this study for the quantification of novobiocin has several advantages over previous methods. The sample preparation is rather simple with just a single step of methanol deproteinization. The run time is reduced to less than 20 min as a result of the shorter column employed. Since only 15 μl are used for each injection, the sample volume can be decreased proportionally (50 μl plasma in 0.5 ml of methanol) if necessary. The sensitivity, specificity, and simplicity of this HPLC procedure render it suitable for routine clinical drug monitoring.

5. Acknowledgements

We are grateful to Drs. John Hilton, Susan M. Ludemen, and Ellen Shulman-Roskes for many helpful suggestions, Dr. Hilton for his assistance in spectrophotometry, and Ms. Rosemary Clark for her secretarial assistance. This study was supported in part by Grant 5 PO1 CA15396 from National Institutes of Health, and ACS RD-353 and 1 RO3 CA 58340 to Dr. M.J. Kennedy.

6. References

[1] J.P. Eder, C.A. Wheeler, B.A. Teicher and L.E. Schnipper, *Cancer Res.*, 51 (1991) 510.
[2] J.B. Eder, B.A. Teicher, S.A. Holden, K.N.S. Cathcart and L.E. Schnipper, *J. Clin. Invest.*, 79 (1987) 1524.
[3] J.P. Eder, B.A. Teicher, S.A. Holden, K.N.S. Cathcart, L.E. Schnipper and E. Frei III, *Cancer Res.*, 49 (1989) 595.
[4] P.J. Smith and S.M. Bell, *Cancer Chemother. Pharmacol.*, 26 (1990) 257.
[5] K.B. Tan, M.R. Mattern, R.A. Boyce and P.S. Schein, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 7668.
[6] K.B. Tan, M.R. Mattern, R.A. Boyce and P.S. Schein, *Biochem. Pharmacol.*, 37 (1988) 4411.
[7] A. Lorico, G. Rappa and A.C. Sartorelli, *Int. J. Cancer*, 52 (1992) 903.
[8] G. Rappa, A. Lorico and A.C. Sartorelli, *Int. J. Cancer*, 51 (1992) 780.
[9] M. Gellert, *Annu. Rev. Biochem.*, 50 (1981) 879.
[10] K. Drlica and R.J. Franco, *Biochemistry*, 27 (1988) 2253.
[11] F.Y.F. Lee, D.J. Flannery and D.W. Siemann, *Cancer Res.*, 52 (1992) 3515.
[12] G.K. Ellis, J. Crowley, R.B. Livingston, J.W. Goodwin, L. Hutchins and A. Allen, *Cancer*, 12 (1991) 2969.
[13] K. Tsuji and J.H. Roberston, *J. Chromatogr.*, 94 (1974) 245.
[14] K. Tsuji, P.D. Rahn and M.P. Kane, *J. Chromatogr.*, 235 (1982) 205.
[15] R.E. Hornish, *J. Chromatogr.*, 236 (1982) 441.
[16] W.A. Moats and L. Leskinen, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 776.
[17] J.W. Hinman, E.L. Caron and H. Hoeksema, *J. Am. Chem. Soc.*, 79 (1957) 5321.
[18] L.V. Birlova and D.M. Trakhtenberg, *Antibiotiki (Moscow)*, 11 (1966) 395.
[19] Susan Budavari (Editor), *The Merck Index*, 11th ed., Merck & Co. Inc., Rahway, NJ, 1989, p. 1064.