Journal of Chromatography, 305 (1984) 496—501
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1940

Note

Quantitation of 1,3-bis(2-chloroethyl)-1-nitrosourea in plasma using high-performance liquid chromatography

R.L. YEAGER*,*

Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, N.I.H., Bethesda, MD 20205 (U.S.A.)

E.H. OLDFIELD

Surgical Neurology Branch, NINCDS, N.I.H., Bethesda, MD 20205 (U.S.A.)

and

D.C. CHATTERJI

Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, N.I.H., Bethesda, MD 20205 (U.S.A.)

(Received August 11th, 1983)

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is one of the more commonly used nitrosoureas [1—3], and has been shown to be effective in the treatment of certain brain tumors [4]. There is, however, a paucity of information about its pharmacokinetics and disposition in man, attributable partly to the lack of specific and sensitive assays for BCNU in biological samples. In order to study the pharmacokinetics of BCNU after intracarotid administration in patients, a simple and specific BCNU assay sensitive enough to quantitate the drug well into its terminal elimination phase was needed.

A spectrophotometric assay for BCNU was reported [5], but this method lacked sensitivity and specificity. Gas chromatography—mass spectrometry [6] and chemical ionization mass spectrometry [7, 8] assays reported for BCNU have the required sensitivity and specificity, but require sophisticated

*Present address: Merck, Sharp and Dohme Research Laboratories, Merck & Co., P.O. Box 2000, Rahway, NJ 07065, U.S.A.

0378-4347/84/$03.00

instrumentation, sample derivatization and/or labelled materials. A high-performance liquid chromatographic (HPLC) method reported by Krull et al. [9] called for a large sample volume, lacked adequate sensitivity and used phenytoin, a commonly co-administered drug to brain tumor patients as internal standard.

We present a simple, sensitive and selective assay for BCNU in plasma using reversed-phase HPLC. The assay is being used to study the pharmacokinetics of BCNU in humans and monkeys following intracarotid administration.

MATERIALS AND METHODS

Reagents

BCNU was obtained from Drug Development Branch, NCI, (Bethesda, MD, U.S.A.). Propyl paraben (PP) used as internal standard was purchased from Sigma (St. Louis, MO, U.S.A.). Glass-distilled and certified HPLC-grade acetonitrile was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetic acid (Fisher Scientific, Fairlawn, NJ, U.S.A.), diethyl ether (Mallinckrodt, Paris, KY, U.S.A.), and ethyl alcohol (USPHS, SSC, Perry Point, MD, U.S.A.) were all reagent grade. HPLC solvents were filtered using a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and degassed by applying vacuum.

Apparatus

The chromatographic system consisted of a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 3500B solvent pumping system, equipped with a Schoeffel (Kratos Instruments, Westwood, NJ, U.S.A.) Spectroflow 773 variable-wavelength ultraviolet detector set at 230 nm. The analytical column was an Ultrasphere ODS reversed-phase column, 25 cm × 4.6 mm I.D., 5 μm particle size, (Altex, Berkeley, CA, U.S.A.), and was preceded by a 7 cm × 2 mm guard column packed with Co:Pell ODS, 30—38 μm particle size, (Whatman, Clifton, NJ, U.S.A.). The mobile phase was 35% (v/v) acetonitrile and 0.1% glacial acetic acid in water. The flow-rate was 1.2 ml/min and the backpressure was 28 MPa.

Procedure

Blank control plasma was acidified by adding 4% (v/v) glacial acetic acid to a final pH of approximately 4. Blood samples were cooled on an ice-bath as they were drawn, they were centrifuged for 5 min at 2000 *g* while still cool. Plasma was removed and acidified as above, mixed and stored on ice until frozen.

Plasma standards were made fresh in acidified plasma and samples were thawed. To 0.5 ml plasma, treated as above, were added 1.68 μg of PP [in 5% (v/v) ethyl alcohol in water] as internal standard. The mixture was vortexed for 5 sec and then 4.0 ml of 1.25% (v/v) absolute ethyl alcohol in diethyl ether were added. The samples were mixed on an automatic shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min and centrifuged at 2000 *g* for 10 min. The ethyl alcohol—diethyl ether layer (top) was transferred to clean vials and evaporated under a gentle stream of air at a temperature of 35°C to a dry residue. The samples were then stored in the referigerator until assayed. This

residue was reconstituted with HPLC mobile phase, vortexed for 10 sec and injected into the HPLC system.

BCNU was quantitated by comparison of peak height ratios of drug to internal standard by means of a calibration curve. Calibration curves were analyzed by a weighted least-squares linear regression (weight = $1/c^2$) [10]. Weighted regression analysis was utilized due to the wide range (two orders of magnitude) of sample concentrations. Unweighted regressions were found to be biased toward the higher concentrations with large errors associated with the lower concentrations. The recoveries were determined by comparing absolute peak heights of BCNU and PP with those of aqueous standards injected into the chromatograph each day.

The stability of BCNU in frozen plasma (pH adjusted to 4) was determined by freezing 0.5 ml of plasma standards II, V and VII (0.256, 2.07 and 34.7 μg/ml, respectively). With each curve done over the following month, one of each standard was thawed and assayed. Similarly, the stability of BCNU in refrigerated evaporated plasma extract was measured at the same three concentrations. Plasma standards were extracted as above and refrigerated. With each curve run over the next month, one of each standard was reconstituted and assayed.

RESULTS AND DISCUSSION

Ethyl alcohol added to diethyl ether increased the recovery of BCNU. However, above a certain concentration interferences also increased. The best solvent for extraction was 1.25% ethyl alcohol in diethyl ether.

Fig. 1 shows representative chromatograms from plasma extracts. Fig. 1A is a chromatogram of blank control plasma. Fig. 1B is of a spiked plasma standard, 0.256 μg/ml with internal standard (PP). Fig. 1C is a patient plasma sample drawn 5 min into a 45-min, 6 mg/kg BCNU infusion. The concentration of this peripheral vein sample was found to be 0.380 μg/ml.

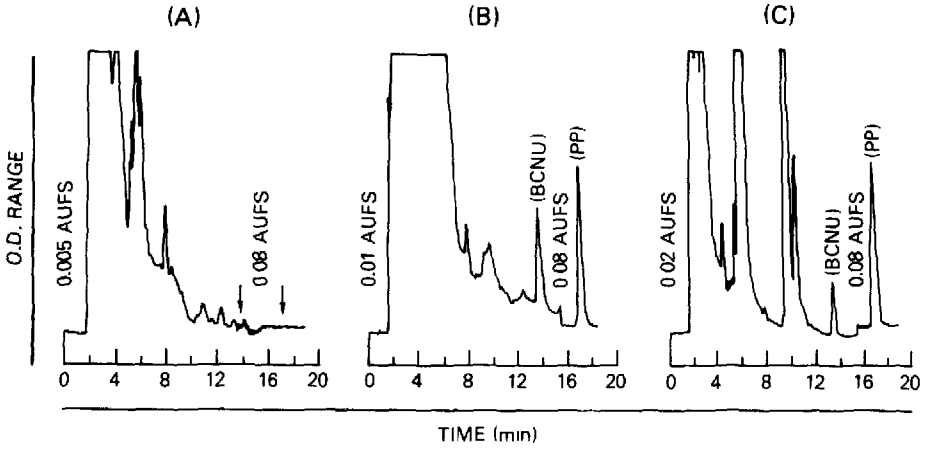

Fig. 1. Representative chromatograms. (A) Plasma blank; (B) spiked plasma standard containing 0.256 μg/ml BCNU and internal standard, PP; (C) representative patient sample following BCNU infusion (assayed and found to contain 0.380 μg/ml BCNU) with internal standard.

Preliminary studies indicated that the half-life of BCNU degradation in plasma at 37°C and pH 7.4 was about 0.25 h [7]. Since nitrosoureas are known to be more stable around pH 4 [11], the pH of the same plasma was adjusted to 4. The half-life increased to 5.3 h at 37°C and pH 4. For this reason, and due to the lack of interferences from adjustment of pH, acetic acid was used to adjust all samples prior to assay to reduce degradation.

The in vitro stability of BCNU at pH 4 in frozen plasma and in refrigerated evaporated plasma extracts was reasonably good. However, the frozen plasma sample chromatograms had more noise. Also after being frozen at pH 4 for more than two or three days the thawed plasma samples were gelled rather than completely liquid. Although these cosmetic changes did not interfere with the assay, sample storage as the refrigerated extract for more than two days is preferred. Samples stored in this manner showed no appreciable loss of BCNU for over a month, but analysis as soon as possible is recommended. Patient samples without pH adjustment were frozen for five days and assayed. BCNU was approximately 70% intact compared to the same samples adjusted to pH 4.

TABLE I

INTER-DAY VARIABILITY

Standard Concentration (μg/ml)	I 0 101	II 0 256	III 0.592	IV 1.01	V 2.07	VI 10 5	VII 34.7	VIII 75 2
Observed concentration (μg/ml)	0 110	0 244	0 448	1.22	2.05	12 0	43 2	70 1
	0.0970	0.286	0 631	0.928	1 82	12.3	37.4	71.4
	0.128	0 189	0 724	1.05	1.77	13.7	42.7	59.1
	0.0992	0.266	0.633	0.923	2.32	11.3	36 3	65 1
	0 0991	0.272	0 556	1.05	2.12	10.5	36 6	71.1
Mean	0 107	0.251	0.598	1 03	2.02	12.0	39 2	67.4
S.D.	0.0130	0.0380	0.103	0.121	0.225	1 19	3 42	5 27
C V. (%)	12.2	15.1	17 2	11 7	11 2	9.99	8 70	7.83

Mean C.V. = 11.7%.

TABLE II

INTRA-DAY VARIABILITY

Standard Concentration (μg/ml)	II 0.256	V 2.07	VII 34.7
Peak height ratios	0.0430	0.371	4.45
	0.0440	0.344	3.81
	0.0402	0.388	4.83
	0.0428	0.347	4.54
	0.0473	0.359	3.98
Mean	0 0435	0.362	4.32
S.D.	0.00256	0.0181	0.419
C.V. (%)	5.90	5.01	9.70

Mean C.V. = 6 87%.

Table I shows the inter-day variability of the assay over a two-week period (n = 5) as demonstrated by observed concentrations. Observed concentrations were calculated by entering individual peak height ratios into the equation of the line for the entire standard curve. The coefficients of variation (C.V.) were consistent over the whole range of the assay and their mean was 11.7%.

Table II reports the intra-day variability of the assay at three concentrations (0.256, 2.07 and 34.7 μg/ml) as demonstrated by a comparison of peak height ratios (n = 5); again the coefficients of variation were consistent over the whole range of the assay, and the mean found to be 6.87%.

The slope of the calibration curve was 0.203 ± 0.020 $(\mu g/ml)^{-1}$ (mean ± S.D.). The mean squared correlation coefficient (r^2) was 0.97 and the mean standard error for the slope was less than 10%.

The mean recoveries of BCNU ranged from 47.6 to 73.4% (58.9 ± 9.74%, mean ± S.D.). The retention times were 13.8 min and 16.9 min for BCNU and PP, respectively. The mean signal-to-noise ratio (S/N) for STD I (0.101 μg/ml) was 11.0. Using a S/N ratio limit of 3.0 the assay sensitivity is at least 0.050 μg/ml.

The assay presented here has been shown to be selective and sufficiently sensitive for use in pharmacokinetic analyses. Fig. 2 is a representative concentration versus time profile from a patient receiving BCNU, 220 mg/m² over 45 min. The reliability and reproducibility are demonstrated by low coefficients of variation for both intra-day and inter-day variability.

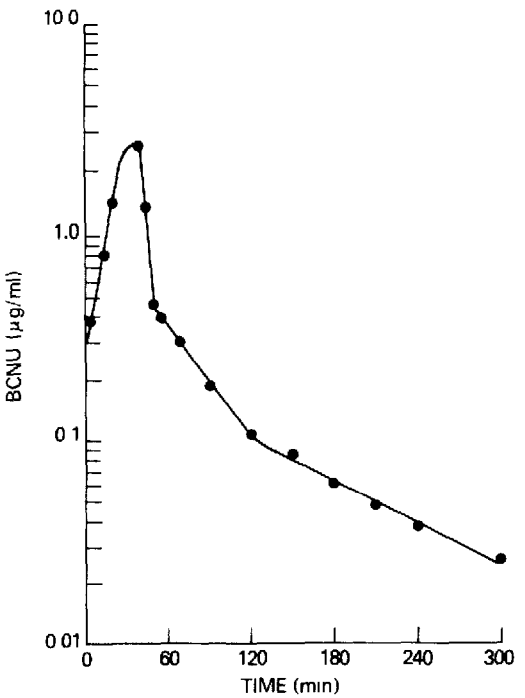

Fig. 2. Representative patient BCNU concentration versus time profile following 387-mg infusion over 45 min. Samples at 180 min and following were assayed by doubling sample volumes.

ACKNOWLEDGEMENT

The authors wish to acknowledge Mrs. Audrey Pellegrino for her assistance in preparation of the manuscript.

REFERENCES

1 V.T. Devita, J.D. Denham, J.D. Davidson and V.T. Oliverio, Clin. Pharmacol. Ther., 8 (1976) 566.
2 T.H. Wasserman, M. Slavik and S.K. Carter, Cancer (Amsterdam), 36 (1975) 1258.
3 V.A. Levin and C.B. Wilson, Cancer Treat. Rep., 60 (1976) 719.
4 M.O. Walker and J. Hilton, Cancer Treat. Rep., 60 (1976) 725.
5 T.L. Loo and R.L. Dion, J. Pharm. Sci., 54 (1965) 809.
6 R.G. Smith, S.C. Blackstock, L.K. Cheung and T.L. Loo, Anal. Chem., 53 (1981) 1205.
7 V.A. Levin, W. Hoffman and R.J. Weinkam, Cancer Treat. Rep., 62 (1978) 1305
8 R.J Weinkam, J.H.C. Wen, E.E. Furst and V.A. Levin, Clin. Chem., 24 (1978) 45.
9 I.S. Krull, J. Strauss, F Hochberg and N.T. Zervas, J. Anal. Toxicol., 5 (1981) 42.
10 A.I. Nichols and C.C. Peck, General Weighted Least Squares Non-linear Regression Program, Version 1, Users Manual, Uniformed Services University of Health Sciences, Technical Report No. 50, Bethesda, MD, May, 1981.
11 D.C. Chatterji, R.F. Greene and J.F. Gallelli, J. Pharm. Sci., 67 (1978) 1527.