Journal of Chromatography, 568 (1991) 385–392
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5944

High-performance liquid chromatographic determination of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil and its metabolite (*E*)-5-(2-bromovinyl)uracil in serum

DAISY B. WHIGAN* and ALLEN I. COHEN

Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Pharmaceutical Research Institute, 1 Squibb Drive, New Brunswick, NJ 08901 (USA)

(First received December 6th, 1990; revised manuscript received April 19th, 1991)

ABSTRACT

A high-performance liquid chromatographic method was developed to assay 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil and its metabolite (*E*)-5-(2-bromovinyl)uracil in serum. The chloro analogue of the parent drug is used as internal standard. Human serum samples were assayed to establish the pharmacokinetic parameters. Acetonitrile, used as a protein precipitant, was evaporated to dryness and the residue, containing the analytes and internal reference, was dissolved in mobile phase prior to chromatographic analysis. The minimum quantifiable level was 0.02 μg of each analyte per ml of serum.

INTRODUCTION

1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU, I, Fig. 1) is a synthetic nucleoside analogue [1] that is currently being evaluated for clinical use as a potent and selective inhibitor of the replication of HSV-1 [2,3], varicella-zoster virus [4–6] and Epstein-Barr virus [7].

[^{14}C]BV-araU, administered orally to rats, was found circulating in blood as the parent compound, and it was recovered unchanged in urine [8]. The only metabolite identified was (*E*)-5-(2-bromovinyl)uracil (BV-uracil, II, Fig. 1), which is devoid of antiviral activity.

High-performance liquid chromatographic (HPLC) analysis of BV-araU has been applied [9] but the methodology has not been reported. Several investigators have reported the HPLC bioanalysis of a similar nucleoside, (*E*)-5-(2-bromovinyl)-2-deoxyuridine (BVdUrd) [10–12]. Two of these references [10,11] also reported the measurement of BV-uracil as a metabolite of BVdUrd. However, these methods lack the sensitivity required to quantitate the lower concentrations of BV-uracil formed from BV-araU.

The HPLC assay method described here was developed to support human clinical studies of BV-araU. Both parent drug and metabolite are measured in serum extracts prepared by protein precipitation with acetonitrile. The chroma-

0378-4347/91/$03.50

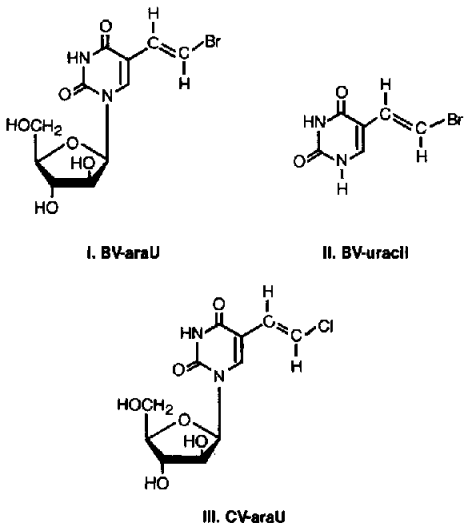

Fig. 1. Structures of BV-araU (I), BV-uracil (II), and the internal standard (III).

tographic system which employs an alkyl phenyl analytical column is selective for all the analytes. With the incorporation of an internal standard, CV-araU (III, Fig. 1), the extractive precipitation and the chromatographic system resulted in a rugged, selective, and reproducible measurement of the analytes.

EXPERIMENTAL

Materials

BV-araU, BV-uracil, and CV-araU were obtained from Yamasa Shoyu (Choshi, Japan). Double-distilled water or purified water, obtained by passing it through a Milli-Q reagent water system (Millipore, Bedford, MA, USA), was used. Ammonium acetate, HPLC grade, was obtained from Fisher Scientific (Fairlawn, NJ, USA). Glacial acetic acid, HPLC grade, was obtained from J. T. Baker (Phillipsburg, NJ, USA). HPLC-grade solvents were used.

Instrumentation

The isocratic HPLC system consisted of the following components: a Kratos Spectroflow Model 400 pump and a Kratos Spectroflow Model 783 variable-wavelength detector (ABI Instruments, Ramsey, NJ, USA), and a Perkin-Elmer ISS-100 autosampler equipped with a 50-μl loop (Perkin-Elmer, Norwalk, CT, USA).The autosampler was equipped with a refrigerated tray maintained at 15°C by circulating a mixture of ethylene glycol–water (20:80, v/v) using a Neslab Instruments Model RTE-4 recirculating bath (Portsmouth, NH, USA). Chromatograms were recorded on a 10-mV strip chart recorder (Kipp and Zonen, Model BD-40, Delft, Netherlands).

Chromatographic conditions

Isocratic separation was achieved using an alkyl phenyl column (25 cm × 4.6 mm I.D., 5-μm packing) supplied by ES Industries (Marlton, NJ, USA). A pre-column, dry-packed with 37–53 μm silica gel (Whatman, Clifton, NJ, USA), was inserted in front of the injector to protect the analytical column. The mobile phase consisting of an aqueous buffer–acetonitrile–methanol (80:15:5, v/v) was degassed by sonicating for 20 min. The flow-rate was maintained at 1.0 ml/min. The aqueous buffer was 0.05 *M* ammonium acetate adjusted to pH 5.0 with glacial acetic acid. The analytes were detected at 295 nm using a setting of 0.010 a.u.f.s.

Standard solutions

Note that BV-araU, BV-uracil, and CV-araU are light-sensitive. Care must be taken to protect these compounds from light.

Stock solutions of I, II, and III were prepared in methanol at concentrations of 500, 250, and 500 μg/ml, respectively. These solutions were stable for at least eight weeks, provided they were protected from light and stored in a refrigerator. A secondary internal reference standard solution, prepared at 10 μg/ml in water, was used to spike the samples prior to extraction. An unextracted working standard solution was prepared daily to contain 1.0 μg of I, 0.25 μg of II and 1.0 μg of III per ml of mobile phase.

Control serum samples were prepared to contain 1.0 μg of I and 0.25 μg of II per ml of serum. The control samples were stable for at least two months if stored in the freezer. For every ten unknown samples extracted, one control sample was also extracted to monitor the assay.

Sample preparation

An aliquot of the serum sample (0.50 or 0.25 ml) was transferred into a 150 mm × 20 mm glass disposable test tube and 100 μl, containing 1 μg, of the internal reference standard were added to the sample. While mixing, 5 ml of acetonitrile were added to the sample to precipitate the proteins. The sample was centrifuged and the acetonitrile layer was poured off into a 20-ml glass scintillation vial. The acetonitrile was evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was dissolved in 1.0 ml of mobile phase, filtered through a 0.45-μm nylon acrodisc disposable filter unit, and transferred into an autosampler vial for chromatography.

Measurement

Measurement was performed by comparing peak-height ratios from extracted samples to non-extracted working standard solutions. Peak heights may be measured manually or automatically using appropriate chromatographic software.

RESULTS AND DISCUSSION

Selectivity of the chromatography

In mobile phase BV-araU, BV-uracil, and CV-araU show two UV absorption maxima (250 and 295 nm). Detection at 295 nm is both selective and sensitive for all three analytes. The use of an alkyl phenyl column contributed to the selectivity of the system. The effects of methanol and acetonitrile as organic modifiers in the mobile phase were studied. By adding 10–40% acetonitrile and 20–50% methanol, the retention times of both BV-araU and BV-uracil are affected more by acetonitrile than by methanol. A mobile phase consisting of 20% methanol and 80% buffer did not resolve BV-araU from BV-uracil but as the amount of methanol was increased, the compounds were resolved from each other. The pH of the ammonium acetate buffer in the mobile phase has little effect on the capacity ratios of both analytes. A pH range of 4.0–6.0 gave similar capacity ratios for both BV-araU and BV-uracil. These findings are similar to those reported by Reeuwijk *et al.* [12] for BVdUrd, an analogous compound. A pH of 5.0 was used to avoid extreme conditions that might be deleterious to the analytical column. The combination of an appropriate mixture of organic modifiers with the aqueous buffer allowed selecting the retention times with baseline resolution of the analytes without significant interference from endogenous extracted serum components (Fig. 2).

Sample extraction

The amount of acetonitrile added was varied from 3 to 10 ml without affecting the extraction of as much as 8–12 μg/ml BV-araU. A volume of 5 ml of aceto-

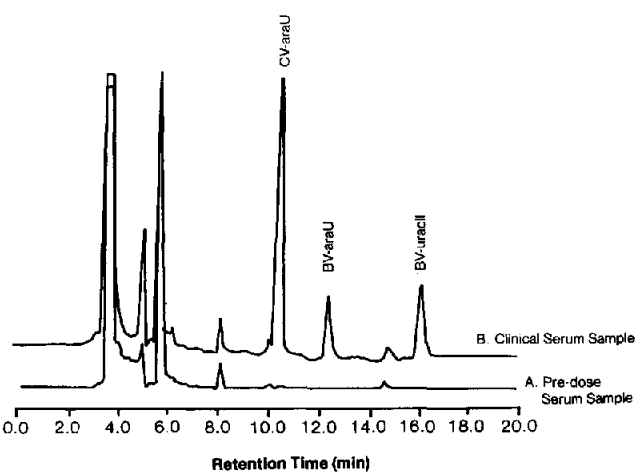

Fig. 2. Chromatograms showing selectivity of the assay. (A) Pre-dose serum sample; (B) clinical serum sample; CV-araU (1.0 μg added) at 10.5 min, BV-araU (0.53 μg/ml) at 12.5 min, and BV-uracil (0.50 μg/ml) at 16.0 min.

nitrile is recommended as a convenient amount to use in preparing the sample extract.

Standard curves

The peak-height ratios of analyte to internal reference standard are directly proportional to the concentration of analyte. Following the recommended procedure, the standard curves are linear to at least 25 μg of BV-araU and 5 μg of BV-uracil per ml of serum. Linear regression results in the following equations: concentration of BV-araU (μg/ml) = 2.6435 (ratio) − 0.009 (r = 0.9999); concentration of BV-uracil (μg/ml) = 2.2714 (ratio) + 0.003 (r = 0.9999). If the sample contains higher concentrations of the analytes, 0.25 ml instead of 0.5 ml is assayed.

Sample recovery

The recovery of BV-araU, BV-uracil, and CV-araU from serum was determined by comparing peak heights from spiked serum assayed accordingly to unextracted solutions of the analytes injected directly onto the analytical column. Absolute recovery was 85–95% for each of the analytes.

Accuracy and precision

The accuracy and precision of the assay were determined by spiking both drug and metabolite into drug-free serum at various concentrations. Recovery, precision, and accuracy were constant over the whole concentration range studied. For BV-araU in the range 0.02–25.0 μg/ml of serum ($n \geqq 4$), the weighted overall mean recovery was 103 ± 8.9% and for BV-uracil over the concentration range 0.02–5.00 μg/ml of serum the weighted overall recovery was 103 ± 8.1%. The average recoveries are somewhat biased by high recoveries at the lowest concentrations (120 ± 20.8% for 0.02 μg/ml BV-araU and 115 ± 17.4% for 0.02 μg/ml BV-uracil). The high recoveries possibly due to interferences from the matrix do not contribute significantly at concentrations ≧ 0.05 μg/ml of serum where approximately 100% recoveries were obtained. The limit of detection for both I and II of 0.02 μg/ml was established as the lowest limit of quantitation.

The intra- and inter-day assay variations were assessed with six spiked serum samples prepared with varying amounts of I and II assayed four times each on three different days. Statistical evaluation of the results established good accuracy and precision of the method. The data in Table I show the accuracy of the method. For the three-day analyses, the overall mean recovery for BV-araU from 0.50 to 25.0 μg/ml was 99.4 ± 4.18% whereas for BV-uracil from 0.10 to 5.00 μg/ml the overall mean recovery was 99.9 ± 4.97%. The mean daily recovery ranged from 98.5 to 101% for each analyte. The coefficient of variation (C.V.) was typically 4–5% on each day. The variance components were estimated using a two-way analysis of variance model with input as a fixed factor and day as random factor. No between-day variation was observed for the determination of

TABLE I

ACCURACY FOR BV-araU AND BV-URACIL: DESCRIPTIVE STATISTICS BY INPUT LEVEL FOR PERCENTAGE RECOVERED

Input (μg/ml)	*n*	Recovery (mean ± S.D.) (%)	C.V. (%)
BV-araU			
0.50	12	101.7 ± 8.22	8.08
1.00	12	95.7 ± 3.55	3.70
2.00	12	97.4 ± 1.33	1.36
5.00	12	100.8 ± 1.03	1.02
10.0	12	100.9 ± 0.87	0.86
25.0	12	99.9 ± 0.14	0.14
Overall	72	99.4 ± 4.18	4.20
BV-uracil			
0.10	12	102.8 ± 10.6	10.3
0.25	12	96.8 ± 3.61	3.73
0.50	12	98.1 ± 1.93	1.96
1.00	12	101.3 ± 1.81	1.78
2.50	12	100.3 ± 1.51	1.50
5.00	12	99.9 ± 0.35	0.35
Overall	72	99.9 ± 4.97	4.98

either analyte. The total C.V. for BV-araU was 0.7% and for BV-uracil the total C.V. was 1.29%.

The analytes are quite stable during the analysis. Reconstituted sample extracts are stable for at least 24 h if the autosampler tray is maintained at 15°C. Likewise, the dry residues may be stored in the freezer for at least 48 h prior to reconstitution with mobile phase for chromatographic analysis. To make the processing of samples more productive, the samples are extracted and prepared during the day and the chromatographic analyses are run overnight.

Stability

The stability of BV-araU and BV-uracil in serum was determined by spiking different amounts of each compound into drug-free serum. Three levels of spiked samples were subdivided into 0.5-ml aliquots before storage in the freezer. Another three levels were not subdivided. Consequently, these samples were thawed at the specified time of analysis and then frozen again until the next analysis time period. The results showed that repeated thawing and freezing of the samples do not affect the stability of either BV-araU or BV-uracil. Furthermore BV-araU and BV-uracil are stable in serum at room temperature for 24 h and in frozen serum for at least sixteen weeks.

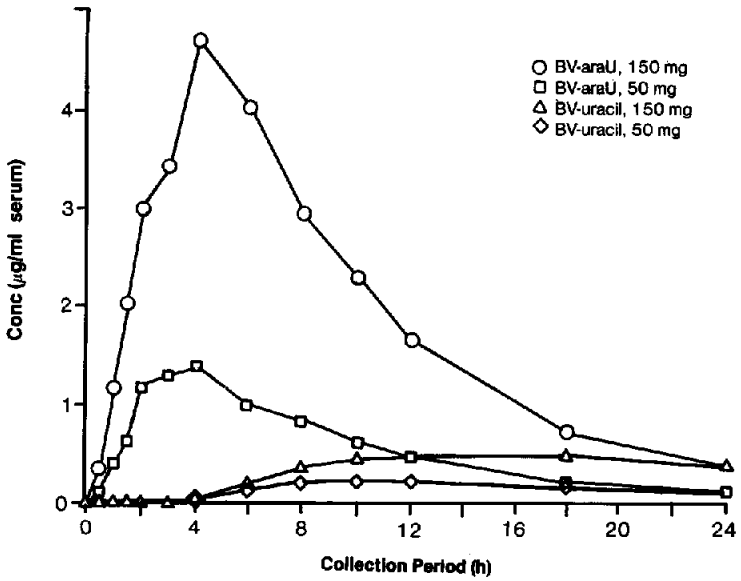

Fig. 3. Serum concentration–time profiles from an ascending single-dose study.

Application

The method has been successfully used to assay human serum samples from pharmacokinetic and clinical studies. A chromatogram of an actual clinical sample is shown in Fig. 2B. Typical concentration–time profiles for samples from an "ascending dose" study are shown in Fig. 3. The concentrations of BV-uracil observed from the administration of BV-araU are much lower compared to those observed with BVdUrd [10]. This difference in pharmacologic activity between the two congeners has been reported by Machida *et al.* [9].

The method has also been used to assay protein-free filtrate samples and mice whole blood samples. The method is quite rugged and can be used to assay hemolyzed and lipemic samples, as well as female serum samples.

ACKNOWLEDGEMENTS

The authors thank Dr. James Bergum for performing statistical analysis and the competent technical assistance of Mrs. Esther Puritz is gratefully acknowledged. The authors also thank Mr. Jose Alcantara for the preparation of the diagrams.

REFERENCES

1 S. Sakata, S. Shibuya, H. Machida, H. Yoshino, K. Hirota, S. Senda K. Ikeda and Y. Mizuno, *Nucleic Acids Res. Symp.*, Ser. 8 (1980) s39–s42.

2 H. Machida, S. Sakata, A. Kuninaka and H. Yoshino, *Antimicrob. Agents Chemother.*, 20 (1981) 47.
3 J. Reefschlager, P. Wutzler, K.-D. Thiel and G. Herrmann, *Antiviral Res.*, 6 (1986) 83.
4 S. Shigeta, T. Yokota, T. Iwabuchi, M. Baba, K. Konno, M. Ogata and E. De Clercq, *J. Infect. Dis.*, 147 (1983) 576.
5 H. Machida, *Biken J.*, 29 (1986) 1.
6 K. Soike, G. Baskin, C. Cantrell and P. Gerone, *Antiviral Res.*, 4 (1984) 245.
7 J.-C. Lin and H. Machida, *Antimicrob. Agents Chemother.*, 32 (1988) 1068.
8 M. Kumagai, S. Sakata, T. Yamaguchi, K. Ikeda and H. Machida, *J. Labelled Compd. Radiopharm.*, 27 (1988) 503.
9 H. Machida, S. Sakata and K. Saito, in R. Kono and A. Nakajima (Editors), *Herpes Viruses and Virus Chemotherapy*, Excerpta Medica, Amsterdam, 1985, p. 63.
10 Y. Robinson, N. Gerry and C. Vose, *J. Chromatogr.*, 338 (1985) 219.
11 N. Ayisi, R. Wall, E. de Clercq and S. Sacks, *J. Chromatogr.*, 375 (1986) 423.
12 H. J. E. M. Reeuwijk, H. Lingeman, U. R. Tjaden, E. A. de Bruijn, H. J. Keizer and J. van der Greef, *J. Chromatogr.*, 428 (1988) 93.