Journal of Chromatography, 375 (1986) 423—430
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam – Printed in The Netherlands

CHROMBIO 2914

Note

High-performance liquid chromatographic analysis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and its metabolites in serum, urine and herpes simplex virus type-1 infected cells

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(First received May 14th, 1985, revised manuscript received October 21st, 1985)

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVdU) is a nucleoside analogue of current interest because of its selective activity against herpes viruses. Several pharmacokinetic studies on BVdU have used a microbiological assay procedure [1, 2] This method, while sensitive and possessing a high capacity for processing samples, measures only the biologically active compounds in the test system. However, lack of selectivity, limited reproducibility and inability to

0378-4347/86/$03 50

detect biologically inactive metabolites are disadvantages. Furthermore, the length of time required for sample analysis makes this impractical. Biological activity assays have utilized herpes simplex or bovid herpes 1 and require incubation periods of at least 48 h. High-performance liquid chromatography (HPLC) with ultraviolet absorption photometric detection allows a sensitive and selective alternative assay with the additional advantages of high precision, shorter analysis time and ability to measure biologically inactive metabolites. An HPLC assay for the measurement of BVdU in human serum and urine is described which has been further adapted to the measurement of BVdU and its metabolites in herpes simplex virus type-1 infected Vero cells.

EXPERIMENTAL

Chemicals and reagents

(*E*)-5-(2-Bromovinyl)uracil (BVU), BVdU, BVdU monophosphate (BVdUMP), BVdU diphosphate (BVdUDP), BVdU triphosphate (BVdUTP) and (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVdU) were synthesized by P. Herdewyn (BVdU), R Busson, L. Colla and H. Vanderhaeghe (BVdU and IVdU) and T Fukui (BVdUMP, BVdUDP and BVdUTP) of the Rega Institute (Katholieke Universiteit Leuven, Leuven, Belgium), who prepared them by established procedures [3] Tetrabutylammonium bromide was purchased from Sigma (St. Louis, MO, U.S.A.). Sodium hydroxide, sodium dihydrogen phosphate, potassium hydroxide, dipotassium hydrogen phosphate and HPLC-grade methanol were purchased from Fisher Scientific (Fairlawn, NJ, U.S A)

Instrumentation

The analyses were carried out using an SP-8000B chromatograph fitted with an SP-8400 variable-wavelength ultraviolet detector (Spectra Physics, San Jose, CA, U S A.) Spherisorb ODS 2, particle size 5 μm (Phase Separations, Queensberry, U.K) was packed in the laboratory into 125 mm × 4 6 mm columns. A presaturation column (120 mm × 4.6 mm) packed with 20-μm Partisil was inserted into the solvent feed line between the pump and the sample injector

Cells and viruses

Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 I.U./ml), streptomycin (100 mg/ml), fungizone (2 5 μg/ml), glutamine (2 μM) and sodium bicarbonate (0 2%) Sera were purchased from British Drug Houses (Toronto, Canada) and other supplements were obtained from Flow Labs (McLean, VA, U S A). The McIntyre strain of HSV-1 was obtained from the American Type Culture Collection (Rockville, MD, U S A)

Preparation of serum and urine extracts

IVdU (0 2 ml, 301.3 μM in water) was added to samples (1 ml) of serum or urine containing 1 4—186 7 nmol of BVdU Ice-cold perchloric acid (0 1 ml, 4 *M* in water) was added to each serum or urine sample. The samples were vortexed for 1 min and left in an ice bath for 5 min All samples were then

centrifuged at 2000 *g* for 10 min at 4°C. The supernatants were then decanted, neutralized with a mixture of 2 *M* potassium hydroxide and 0 5 *M* dipotassium hydrogen phosphate, and recentrifuged at 2000 *g* for 10 min at 4°C to remove precipitated potassium perchlorate. The final supernatants were freeze-dried and redissolved in HPLC-grade water (0.4 ml) for assay. Urine samples were made up to a final volume of 8 ml with HPLC-grade water before HPLC analysis

Recovery and reproducibility studies

BVdU was added to blank serum and urine samples at seven concentrations ranging from 1.4 to 186.7 μM. The recoveries from serum and urine were determined by comparison of BVdU peak areas from extracted samples with those obtained from unextracted aqueous standards of the same nominal concentrations. The reproducibility of the assay on the same day was determined by comparison of the peak areas of four replicate analyses Standard solutions were run each working day to confirm the constancy of the operating system

Preparation of cell extracts

Cell extract preparation was similar to the procedure reported for acyclovir [4]. Briefly, Vero cells were mock-infected or infected with HSV-1 at a multiplicity of infection of 1 After 1 h of infection, the supernatant was removed and replaced by MEM containing 4% FBS and 30 1 μM BVdU and incubated for an additional 7 h at 32– 34°C under 5% carbon dioxide. The medium was removed and the cells washed twice with phosphate-buffered saline (PBS) The cells were then trypsinized, collected in cold PBS, and centrifuged at 800 *g* at 4°C for 10 min The cell pellet was resuspended in distilled water (2 ml). BVdU and its acid soluble metabolites were extracted by adding ice-cold aqueous perchloric acid (0 2 ml, 4 *M*) to the cell suspension, vortexing the mixture for 1 min and letting it stand in an ice bath for 5 min. Samples were then centrifuged at 2000 *g* at 4°C for 10 min. The supernatant was decanted and mixed with an aqueous IVdU solution (0.2 ml, 301 3 μM) as an internal standard This extract was neutralized with a mixture of 2 *M* potassium hydroxide and 0 5 *M* dipotassium hydrogen phosphate, centrifuged at 2000 *g* at 4°C for 10 min and the final supernatant was decanted and freeze-dried. The freeze-dried samples were redissolved in HPLC-grade water (0 4 ml) before analysis

Analysis of acid-soluble extracts of serum, urine and HSV-1-infected cells

The HPLC method was modified from that of Hoffman and Liao [5]. The BVdU concentration was calibrated using IVdU as an internal standard. Four or seven solutions containing different concentrations of BVdU or BVU but fixed concentration of IVdU, were injected into the chromatograph The areas of the BVdU or BVU peaks were then plotted against the ratios of their peak areas relative to the internal standard The linear plots which resulted allowed calculation of the response correction factors (*KF*) for BVdU and BVU by the following equation *KF* = concentration of BVdU (or BVU) × area of IVdU/ concentration of IVdU × area of BVdU (or BVU) These *KF* values were entered into the data system to quantitate BVdU and its metabolites in HSV-1-

infected Vero cells Similar calibration of spiked urine and plasma samples allowed quantitation of BVdU and its metabolites in these matrices The temperature of the HPLC oven was 48°C and the detector was set at 292 nm, 0 04 a.u.f s A concave gradient in methanol rising from 25% (v/v) to 50% (v/v) at 12 min was used with aqueous methanolic eluents (pH adjusted to 5 0 with sodium hydroxide) containing 0.0125 *M* tetrabutylammonium bromide and 0 05 *M* sodium dihydrogen phosphate. Each sample (injector loop volume 13 μl) was eluted at a flow-rate of 1 ml/min on a 33-min analysis cycle. Cell extracts and serum samples gave a minimum reliable detection level (two times the signal-to-noise ratio) of 1.6 μM for all compounds in the reconstituted samples. Urine samples gave a minimum reliable quantitative concentration measurement of 3.0 μM Identification of the compounds was based on their retention times, which under typical operating conditions were BVU (4 7 min), BVdU (5 2 min) BVdUMP (6.3 min), IVdU (7 3 min), BVdUDP (11 4 min), BVdUTP (13.7 min) In addition, all compounds were identified as BVdU derivatives by their unique ratios of absorbance (absorbancy at 292 nm/absorbance at 254 nm).

RESULTS

In serum and cell extracts, BVdU and IVdU were well resolved from the void volume and other endogenous peaks (Figs 1 and 3) In urine, an endogenous compound was found to co-elute with BVdU (Fig 2) and this made quantitation below 24 μM in undiluted urine difficult. This effect diminished at higher BVdU concentrations. The BVdU metabolites, BVU, BVdUMP, BVdUDP and

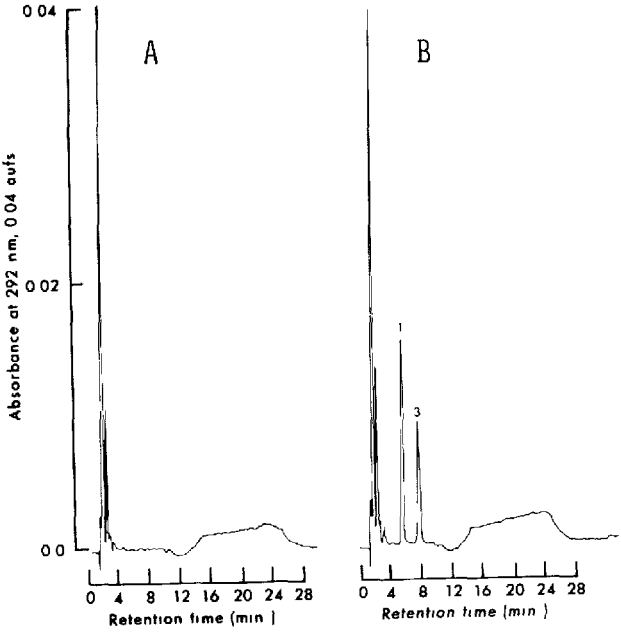

Fig 1 Typical HPLC profiles of (A) blank serum and (B) serum spiked with 46 7 μM BVdU (1) and 60 3 μM IVdU, internal standard) (3)

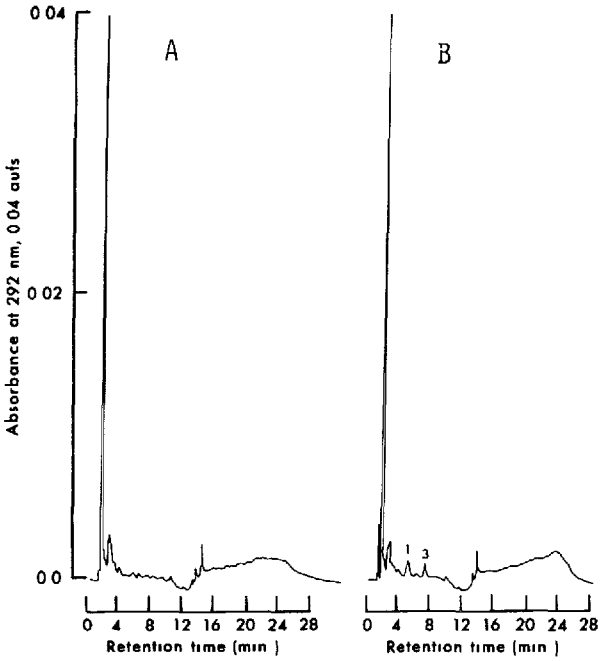

Fig 2 Typical HPLC profiles of (A) blank urine and (B) urine spiked with 46 7 μM BVdU (1) and 60 3 μM IVdU, internal standard (3)

BVdUTP, were resolved from each other and other endogenous peaks in serum, urine and cell extracts

Serum validation

The HPLC method showed a linear response to BVdU (and to BVU) in reconstituted serum over the concentration range 3.5—467 0 μM A plot of peak area against BVdU concentration gave a linear calibration curve (r = 0 996, n = 7). The mean recovery of BVdU form serum (n = 7) was 55 3 ± 10% (S D) The BVdU concentration in serum samples was measured using IVdU as an internal standard In four replicate samples of BVdU in serum, the mean BVdU concentration was 45 1 ± 3 14 μM (S D) (46.7 μM calculated value) and the coefficient of variation was 7 05%

Urine validation

The HPLC method showed a linear response to BVdU (and to BVU) in reconstituted and diluted urine over the concentration range 0.175—23 3 μM However, the endogenous substance which co-eluted with BVdU raised the lower reliable BVdU detection limit to 3 0 μM in the diluted samples A plot of peak area against BVdU concentrations higher than 3 0 μM gave a straight line which went through the origin (r = 0 988, n = 7). The recovery of BVdU was variable (120—286%) at concentrations below 3 0 μM, but at higher concentrations the recovery was 50.23 ± 4 2% (mean ± S D) BVdU concentrations in urine extracts were calculated as above, using the appropriate response correction factor In four replicate analyses of BVdU in urine the

maesured BVdU concentration was 47 5 ± 1 6 μM (46.7 μM nominal concentration) and the coefficient of variation was 3.38%.

Quantitative analysis of BVdU and its metabolites in cell extracts

The metabolic fate of BVdU in HSV-1-infected Vero cells is illustrated in Figs. 3 and 4 BVdU and its metabolites were not detected in mock-infected Vero cells. In HSV-1-infected Vero cells, however, BVdU, BVdUMP, BVdUDP and BVdUTP were detected. Time-dependent increases in the intracellular quantities of BVdUMP, BVdUDP and BVdUTP were noted

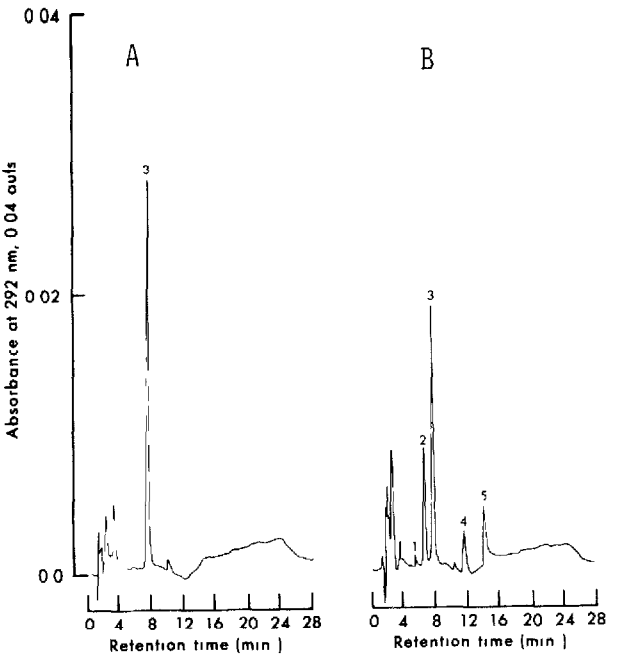

Fig 3 Typical HPLC profiles of (A) mock-infected cell extracts showing IVdU, internal standard (3) and (B) HSV-1-infected cell extracts showing BVdU (1), BVdUMP (2), IVdU, internal standard (3), BVdUDP (4) and BVdUTP (5)

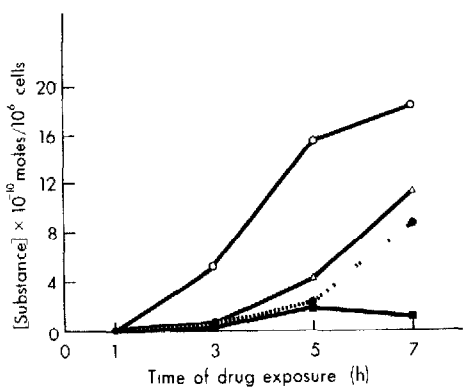

Fig 4 Effect of time of drug exposure on the amount of substance detected in HSV-1-infected Vero cells (■) BVdU, (○) BVdUMP, (●) BVdUDP, (△) BVdUTP

DISCUSSION

Pairing-ion concentrations, salt concentrations, methanol concentrations, temperature and pH were varied in order to establish HPLC conditions Column stability was attained at a pH of 5 The detector was set at 292 nm because of good absorbance at this wavelength in the chromatographic solvent, allowing clean separation from naturally occurring nucleotides The eluent- column system was chosen on the basis of good separation of BVdU and its metabolites from other endogenous peaks in cells and plasma Urine assays, on the other hand, were hampered by a small peak which co-eluted with BVdU.

In the quantitation of BVdU and its metabolites in herpes-simplex-infected cells, the same response correction factor was entered into the HPLC data system for all the compounds of interest since they share the same ultraviolet-absorbing chromophore Possible loss of BVdU and its metabolites through freeze-drying and transfer of the samples was accounted for by the addition of an internal standard (IVdU) to the samples In the case of cell extracts, the internal standard was added after perchlorate extraction Earlier additions might have led to confusion, since extracellular standard would have been compared with intracellular and partially protein-bound metabolites Whereas the freeze-drying and reconstitution parts of the sample processing were suitable for serum and cell extract samples, they were not suitable for urine samples because of the interfering endogenous peak. Accordingly, urine was first diluted twenty times The presence of a small peak co-eluting with BVdU in urine raised the reliable detection limit of this compound in diluted samples to 3 μM Thus, the reliable detection limit of BVdU in undiluted urine was 24 μM Preparation of urine samples for HPLC analysis could therefore exclude the concentration step of freeze-drying The extracted urine samples, after neutralization, were diluted and injected directly into the HPLC system The reliable detection limit of BVdU in concentrated serum and cell extracts was 1 6 μM which corresponded to 0 64 μM in the original serum samples and 20 pmol per 10^6 cells in the cell extracts, respectively

Other investigators have indicated that the isolated HSV-1-specified thymidine—thymidylate kinase converts BVdU to BVdUMP and BVdUDP in vitro [6, 7]. Our results confirm this finding in situ. Furthermore, BVdU uptake by Vero cells was enhanced by virus infection. Similar results have been obtained for acyclovir [8]. Only one strain of HSV-1 was used in these studies BVdU is a more potent inhibitor of HSV-1 replication than of HSV-2 replication [9] Further studies should therefore involve several strains of HSV-1 and HSV-2 to probe the possible mechanisms involved in the differential action of BVdU against these two virus types

The HPLC assay presented in this paper is a rapid, accurate and reproducible method for quantitative analysis of BVdU and its metabolites in serum and urine The procedures are sufficiently selective for BVdU and its metabolites to allow quantitation of BVdU and its nucleotides in plasma. In addition, they allow for assays of in situ metabolites of this drug in treated cells

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