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BIOANALYSIS OF (*E*)-5-(2-BROMOVINYL)-2′-DEOXYURIDINE

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SUMMARY

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine is an antiviral drug that is experimentally used for modulation of the antitumour effect of fluoropyrimidines, such as ftorafur and 5-fluorouracil. The isolation of the analyte, in the presence of 5-fluorouracil, from the matrix is performed either by means of a simple protein precipitation (plasma) or by means of a liquid–liquid extraction with ethyl acetate (urine). Following pretreatment, the analyte is analysed by reversed-phase chromatography and quantified by absorbance detection at 307 nm. The minimum detectable concentration in plasma and urine samples is ca. 6 ng/ml. The recovery after deproteination of plasma samples is 75%, while after liquid–liquid extraction of urine the recovery amounts 92%. The degree of protein binding of the analyte, measured by ultrafiltration, is found to be 97%. These data allow the bioanalysis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine for pharmacokinetic studies.

INTRODUCTION

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVdUrd) (Fig. 1), a new and potent antiviral drug, is a nucleoside analogue that is of interest because of its therapeutic effect in the treatment of herpes infection [1–3]. Nowadays, however, it is also used in cancer research in combination therapies with, for instance, ftorafur or 5-fluorouracil (5-FUra) resulting in an enhanced antitumour effect of the fluoropyrimidines [4].

The emphasis during the development of a bioanalytical method for the bioanalysis of BVdUrd will be on the selective analysis of the analyte in the presence of endogenous and exogenous nucleosides and nucleotides.

For quantitation of the analyte some microbiological assays have been described, but these assays lack sensitivity or selectivity and are time-consuming [5,6]. Liquid chromatographic (LC) systems based on reversed-phase (RP) [7] or RP ion-pair (RP-IP) [8] chromatography have been described, but a rela-

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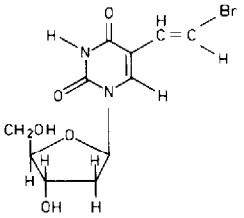

Fig. 1. Structure of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine.

tively laborious sample pretreatment procedure is needed to achieve the required sensitivity and selectivity [8] or the analysis is hindered by an interfering peak in the chromatograms [7].

The analysis of another nucleoside analogue [5′-deoxy-5-fluorouridine (5′-dFUrd)] has been described in a separate study [9]. The sample clean-up and chromatographic separation strategy of that study have been used for the development of an optimized procedure for the analysis of BVdUrd in plasma and urine samples.

Perchloric acid deproteination of the plasma proteins, followed by direct injection of the supernatant into an RP-LC system and absorbance detection at 307 nm, offers sufficient selectivity and sensitivity to carry out pharmacokinetic experiments. Furthermore, the degree of drug–protein binding is measured using ultrafiltration. For the analysis of urine samples liquid–liquid extraction is applied because of the relatively small amount of the free analyte excreted in the urine.

EXPERIMENTAL

Chemicals

BVdUrd was synthesized as previously described [10] and ethyl acetate was freshly distilled and saturated with water before use. The pH of the 50 m*M* tris(hydroxy)methylaminomethane (Tris) buffer was adjusted to pH 6.0 with 1 *M* hydrochloric acid. The phosphate buffers were mixtures of 50 m*M* disodium hydrogenphosphate, 50 m*M* sodium dihydrogenphosphate and 50 m*M* phosphoric acid. The acetate buffer consisted of 50 m*M* solutions of sodium acetate adjusted to pH 4.0 with glacial acetic acid. The perchloric acid solutions (1 *M* and 10 m*M*) were prepared by diluting concentrated perchloric acid (70%). These reagents and all other chemicals were of analytical-reagent grade and were used as such. Throughout the study deionized water (Milli-Q water purification system, Millipore, Bedford, MA, U.S.A.) was used.

Apparatus

The LC system consisted of a Model 421A controller, two Model 110B solvent-delivery systems, a Model 340 organizer, a Model 163 variable-wavelength detector, used at 307 nm, and a Model 210A injection valve (all Beckman Instruments, Berkeley, CA, U.S.A.) with a fixed 100-μl loop. To optimize the detection system a photodiode array (PDA) detector (Model 1040A, Hewlett-Packard, Avondale,

PA, U.S.A.) equipped with a controller unit (Model 85B, Hewlett-Packard) was applied.

Chromatography

The LC system consisted of stainless-steel columns (100 mm×3.0 mm I.D.) packed with 10-μm PRP-1 (Hamilton, Reno, NV, U.S.A.) or 5-μm Nucleosil-100 C_8 (Macherey & Nagel, Düren, F.R.G.) particles. The columns were packed by the pressurized slurry technique [11,12]. The eluent for the PRP-1 system was 50 m*M* Tris (pH 8.2)–5 m*M* cetrimonium bromide, and the eluent of the C_8 system was methanol–10 m*M* perchloric acid (pH 2) (12.5:87.5, v/v). Chromatography was performed at ambient temperature, and a flow-rate of 0.5 ml/min was maintained.

Protein binding

Plasma samples of 1.0 ml, used for protein-binding studies, were spiked with 500 or 5000 ng/ml of the compounds BVdUrd and 5′-dFUrd and incubated overnight at ambient temperature. After incubation, the plasma samples were transferred to the sample reservoir of the ultrafiltration system, the micropartition system (MPS-1) containing a YMT membrane, with a cut-off value of 30 000 (Amicon, Oosterhout, The Netherlands). The whole device was centrifuged (Sorvall RC-2, DuPont Instruments, Newton, CN, U.S.A.) for 15 min at 25°C at 2000 *g* using a fixed-angle (34°) rotor. Subsequently, 50 μl of the resulting filtrate was analysed with the described Nucleosil system. The eluent for the analysis of 5′-dFUrd (model compound) consisted of 100% 10 m*M* perchloric acid (pH 2), and the mobile phase for the analysis of BVdUrd was methanol–10 m*M* perchloric acid (pH 2) (12.5:87.5, v/v).

Sample preparation and storage

Blood samples were taken during 48 h after the administration of BVdUrd and collected in heparinized polyethylene tubes. The tubes were centrifuged immediately at 4°C at 3000 *g* for 10 min and stored at −30°C until the actual analysis took place.

Urine samples were collected every 6 h during 48 h and stored in 10 ml fractions at −30°C until analysis.

Sample pretreatment

Plasma (method 1). Defrosted plasma samples (0.5 ml) were mixed with 0.5 ml of Tris buffer (pH 6.0) and 7 ml of ethyl acetate for 30 min using a rotating mixing device (60 rpm) and subsequently centrifuged for 10 min at 4000 *g*. The resulting organic phase was evaporated to dryness under a stream of dry nitrogen and the residue was dissolved in 0.25 ml of the eluent. A 0.1-ml aliquot was injected into the LC system.

Plasma (method 2). Defrosted plasma samples (0.25 ml) were vigorously mixed for 60 s with 0.075 ml of 1 *M* perchloric acid in a capped polyethylene vial. After a stabilization period of 30 min at ambient temperature the tubes were centri-

fuged for 10 min at 3000 *g*, and 0.1 ml of the clear supernatant was injected into the LC system.

Urine (method 1). Defrosted urine samples (0.25 ml) were diluted with the same volume of water, mixed with 0.5 ml of Tris buffer (pH 6.0) and the same extraction procedure was applied as discussed for plasma (method 1), but in this case only 0.05 ml of the dissolved residue was injected.

RESULTS AND DISCUSSION

Chromatography and detection

BVdUrd was first chromatographed with the cetrimonium bromide containing RP-IP system as described for 5′-dFUrd [9]. This resulted in a broad peak with a capacity ratio (k') of ca. 180. Although it was possible to decrease the retention

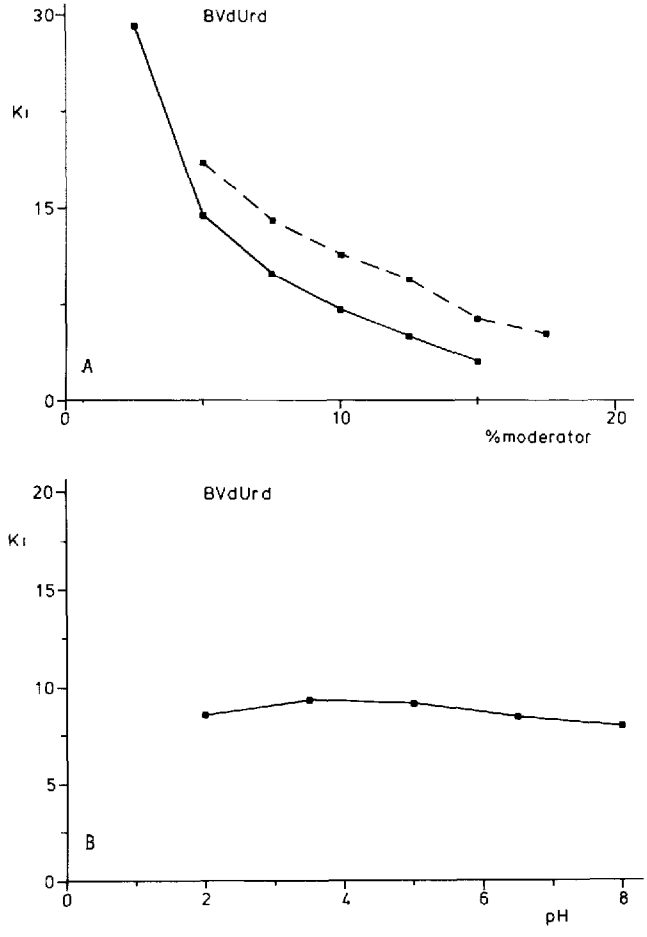

Fig. 2. (A) Capacity ratio of BVdUrd versus percentage of modifier: (——) acetonitrile, (---) methanol. (B) Capacity ratio of BVdUrd versus pH: the eluent was acetonitrile–50 m*M* phosphate buffer (pH 3.5) (10:90, v/v).

of the analyte, for instance, by the addition of an organic modifier (methanol, acetonitrile, tetrahydrofuran) to the eluent, some inconveniences were inherent in the use of such a phase system. The most pronounced ones were the long system equilibration time, the temperature dependence of the system and consequently the limited system stability.

Therefore, the usefulness of simple RP systems was investigated, using acetonitrile or methanol as modifier (Fig. 2A). As can be seen from this figure, acetonitrile influences the k' value of the analyte more strongly than methanol and, furthermore, the influence of the pH on the k' ratio is negligible (Fig. 2B). The k' value of BVdUrd in the applied RP systems is about five times larger than the k' of 5′-dFUrd; this increase is mainly due to the presence of the bromovinyl group in BVdUrd.

Because of the chosen sample pretreatment procedure, denaturation with perchloric acid, a diluted (10 mM) perchloric acid solution was used as the aqueous component of the mobile phase. The k' value in the mobile phase containing perchloric acid was 10.2 and in a mobile phase containing 50 mM phosphate buffer (pH 2.0) it was 8.56. In both cases acetonitrile (10%, v/v) was used as a modifier. The difference in k' values is probably caused by the difference in ionic strength.

The analyte shows, in aqueous solutions, two absorption maxima in the UV region: at 251 nm with a molar absorptivity (ϵ) of 14 400 and 10 600 at 293 nm. For routine bioanalysis the wavelength of 293 nm is preferred because of the multitude of endogenous interfering compounds present in the matrix. At 251 nm a number of interferences were observed in the chromatograms; detection at 293 nm resulted in one small interfering peak in the plasma samples. In order to optimize the detection conditions, three-dimensional plots of blank plasma samples were recorded using the PDA detection system. It could be concluded that the best compromise between sensitivity and selectivity was obtained at 307 nm. Although detection at 307 nm resulted in a 25% loss in sensitivity, in comparison with detection at 293 nm, a better signal-to-noise ratio was obtained owing to a lower background signal.

Sample pretreatment

For the clean-up of the plasma samples two different methods have been tried: a liquid–liquid extraction similar to the procedure used for the determination of 5′-dFUrd [8] and a protein precipitation method.

Addition of a buffer solution was not necessary for the liquid–liquid extraction procedure, but after addition of the Tris buffer (pH 6.0) or the acetate buffer (pH 4.0) cleaner blank chromatograms were obtained. The best results, however, were obtained after the addition of a Tris buffer. The use of methanol as a modifier in the mobile phase is preferable to acetonitrile or tetrahydrofuran (Fig. 3A–C). However, an interfering component still elutes with BVdUrd, resulting in an unfavourable determination limit (ca. 50 ng/ml). The presence of this interfering peak was previously reported in the literature [7]. The difference between the application of a phosphate buffer (pH 3.5) or 10 mM perchloric acid as the aqueous component of the mobile phase is shown in Fig. 3B and D. The difference is

probably due to a somewhat lower pH and lower ionic strength in the perchloric acid system.

The precipitation procedure, with perchloric acid, resulted in cleaner blank chromatograms (Fig. 3D and E), but in order to achieve a clear supernatant, the mixture was allowed to stand for 30 min at room temperature before centrifugation.

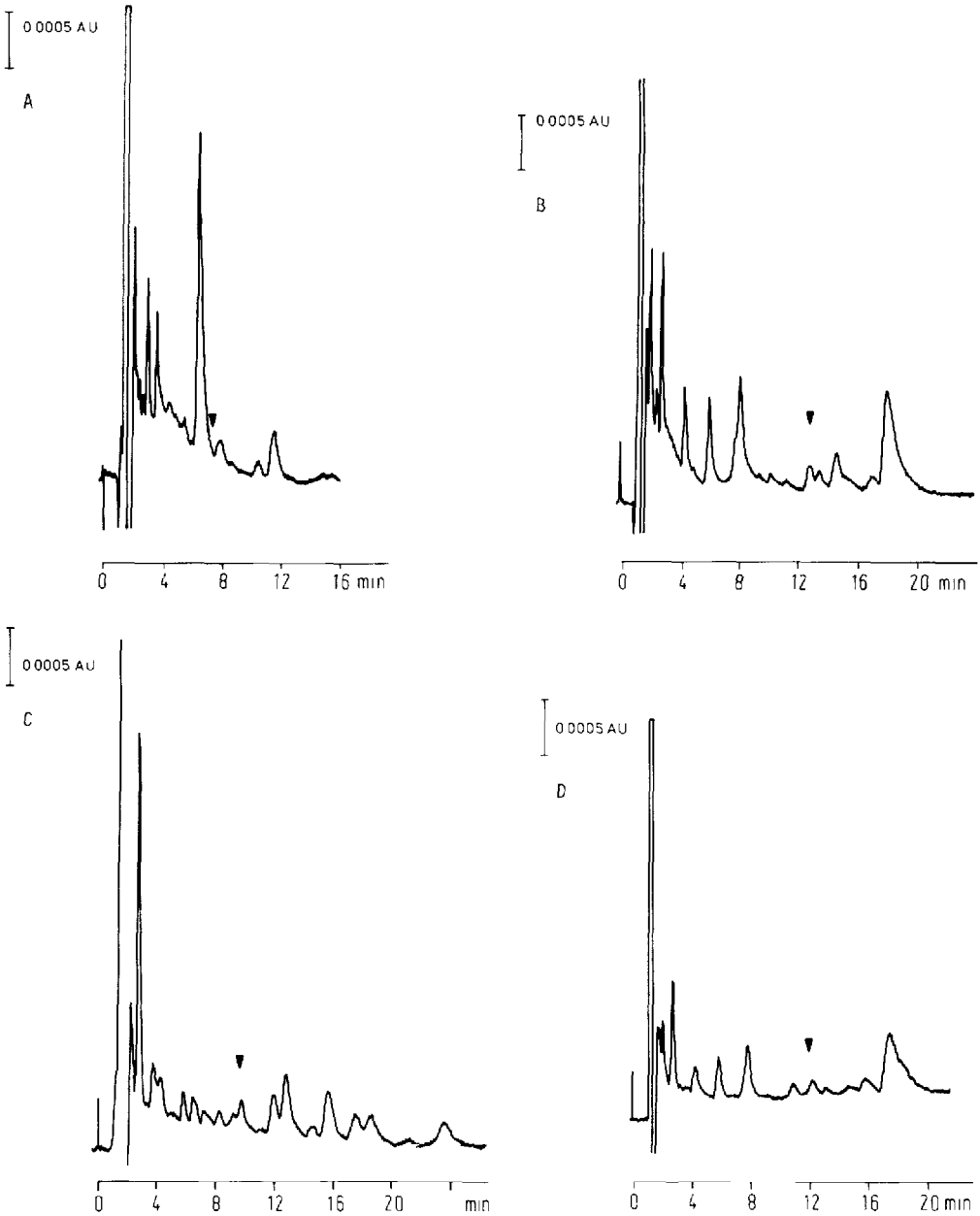

Fig. 3.

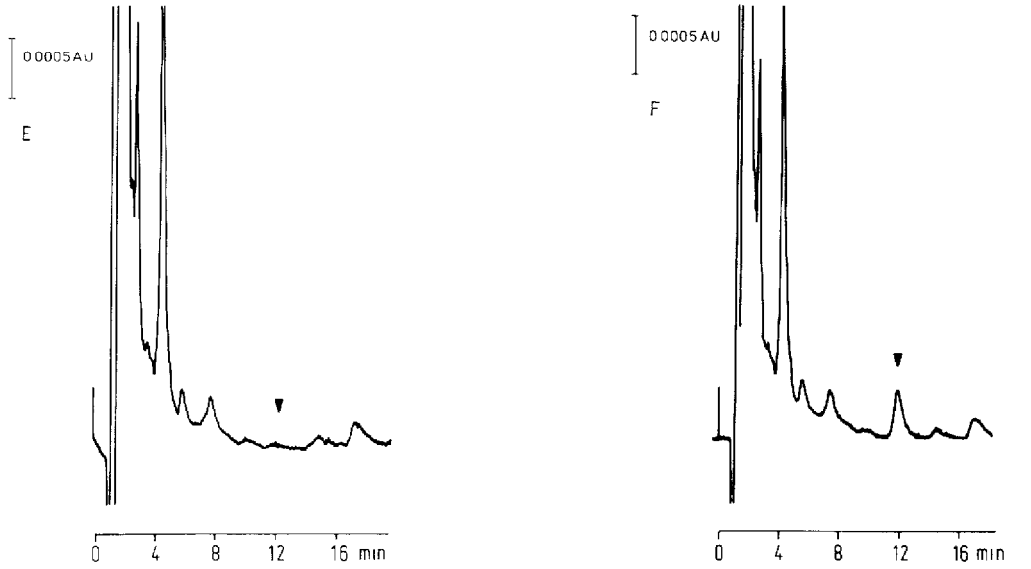

Fig. 3. Chromatograms of blank plasma samples (A–E) obtained with different eluents and sample pretreatment procedures. (A) Method 1 and acetonitrile–50 m*M* phosphate buffer (pH 3.5) (10:90, v/v); (B) method 1 and methanol–50 m*M* phosphate buffer (pH 3.5) (12.5:87.5, v/v); (C) method 1 and tetrahydrofuran–50 m*M* phosphate buffer (pH 3.5) (10:90, v/v); (D) method 1 and methanol–10 m*M* perchloric acid (pH 2.0) (12.5:87.5, v/v); (E) method 2 and methanol–10 m*M* perchloric acid (pH 2.0) (12.5:87/5, v/v). Chromatogram F was a plasma sample spiked with 104 ng/ml BVdUrd, obtained with method 2 and methanol–10 m*M* perchloric acid (pH 2.0) (12.5:87.5, v/v).

The extent of degradation of the stock solutions (0.1 mg/ml), stored at 4 °C was less than 10% after three months. In perchloric acid (pH 0.6 and 2.0) the analyte was stable for at least 24 h.

Dilution of the urine samples with water or the mobile phase resulted in unacceptable blank chromatograms and low concentrations of BVdUrd. For this reason, the liquid–liquid extraction procedure was used for urine samples.

Analytical variables

In order to investigate the linearity of the procedures, blank plasma and urine samples were spiked with different amounts of BVdUrd to obtain concentrations of 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml. For all the experiments described in this section the plasma samples were pretreated by the precipitation procedure and the urine samples by the liquid–liquid extraction procedure. Calibration curves showed good linearity, as can be seen from the following equations: plasma: $y=0.114\ (\pm 0.001)x-4\ (\pm 9)$ $(r=0.9997)$; urine: $y=0.139\ (\pm 0.002)x-20\ (\pm 13)$ $(r=0.9995)$. Both calibration lines were obtained after analysing seven samples; x and y are the concentration of the solute in ng/ml and the peak height, respectively, and r is the correlation coefficient.

The recovery of the analyte from plasma was $75 \pm 3\%$ after the analysis of fourteen samples in the concentration range 50–5000 ng/ml. The within-assay precision for the analysis of BVdUrd in plasma samples was determined for six

samples at two different concentrations (102 and 510 ng/ml) and the resulting coefficients of variation (C.V.) were 4 and 3%, respectively.

The recovery from urine samples was $92 \pm 4\%$, and the within-assay precision was determined with four samples at two different BVdUrd concentrations (204 and 2040 ng/ml) in urine. The within-day C.V. were 8 and 6%, respectively. The minimum detectable amount, with a signal-to-noise ratio of 3, is ca. 6–7 ng/ml in plasma and in urine.

Drug–protein binding

In a separate study a fully automated continuous-flow system for the cytostatic agent mitomycin C is described [12]. In this assay for BVdUrd a relatively high degree of protein binding of analyte was observed. In order to calculate this degree of protein binding, the bound and the free fractions were separated by ultrafiltration. This micropartition technique is based on filtering of the free analyte through an anisotropic, hydrophylic ultrafiltration membrane [13]. The ultrafiltrate, which contains the free analyte, was collected after centrifugation in a polyethylene filtrate cup and separated from the protein-bonded fraction and other macromolecular compounds. The possibility of irreversible adsorption of the analytes onto the membrane was checked and was found to be negligible. The results with BVdUrd were compared with those with 5′-dFUrd, because for the latter nucleoside a relatively high degree of protein binding has been described [14].

The degree of protein binding in plasma samples spiked with 500 ng/ml was ca. 56% ($n=2$) for 5′-dFUrd and 97% ($n=2$) for BVdUrd. Plasma samples spiked with 5000 ng/ml were diluted after incubation and before filtration with nine volumes of water. The degree of protein binding was found to be 27% ($n=2$) for 5′-dFUrd and 92% ($n=2$) for BVdUrd.

From these experiments it may be concluded that the degree of protein binding of BVdUrd in plasma is too high to apply a continuous-flow dialysis technique, without destroying the protein binding before the dialysis.

Interference studies

The possible interferences of other cytostatic agents, 5′-dFUrd, 5-FUra, 5′-fluorodeoxyuridine and mitomycine C, and other similar compounds or metabolites of BVdUrd, such as dimethyluracil, flucytosine, chlorouracil, uracil and 2-thiouracil, were studied with the described procedures for the determination of BVdUrd. The k' values of all of these compounds were less than 6, except for mitomycine C which broke down (in the acidic eluent) into four different components, the last of which interfered with the analysis of BVdUrd.

The interferences of some co-administered drugs, such as the anti-inflammatory agent ibuprofen, the antiemetic primperan and the tranquilizers diazepam and lorazepam, were also investigated, but none hindered the analysis.

Drug monitoring

In order to evaluate the described procedure some plasma and urine samples of a patient treated three times orally (0.8 and 16 h) with 250 mg of BVdUrd and intravenously with 180 mg of 5-FUra (90–105 min) were analysed. The protein

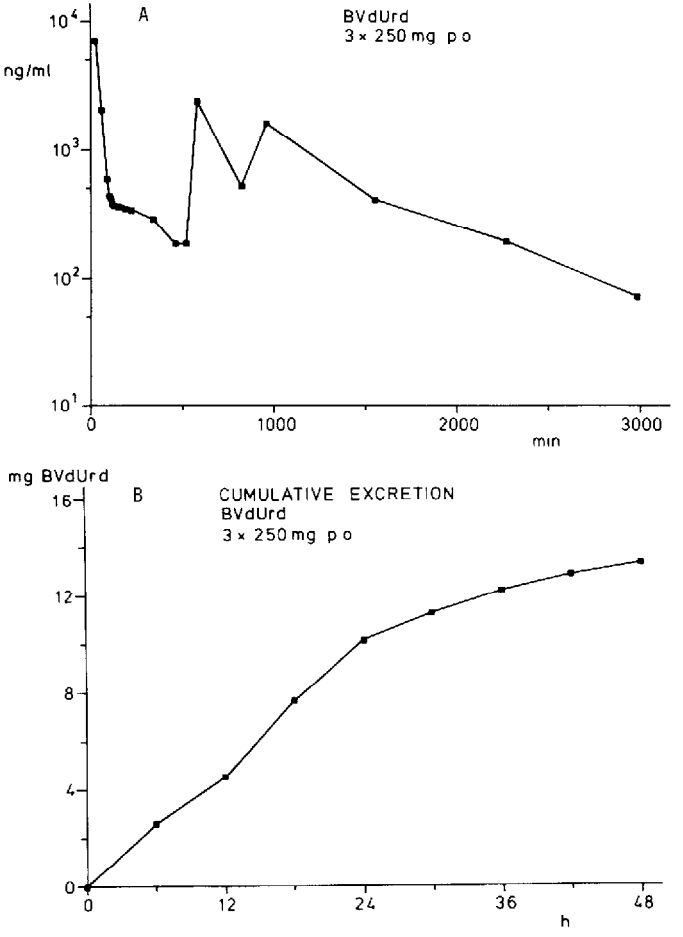

Fig. 4. Concentration–time curve for BVdUrd in plasma (A) and excretion curve of BVdUrd in urine (B) after oral administration of three times 250 mg of BVdUrd (0.8 and 16 h).

precipitation procedure, using the perchloric acid-containing mobile phase was used for the plasma samples (Fig. 3F), and for the urine samples the liquid–liquid extraction was used. The plasma concentration–time curve of BVdUrd and the urinary excretion curve are shown in Fig. 4A and B. The plateau in the concentration–time curve is due to the co-administration of 5-FUra and to the subsequent second and third oral administration of BVdUrd, and can be explained by drug interactions at the enzymatic breakdown level.

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