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Determination of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine in plasma and urine by capillary electrophoresis

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Abstract

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine is an antiviral drug used for treatment of infections with Herpes simplex virus type 1 as well as Varicella zoster virus. Two fast methods for the determination of the drug and its metabolite in plasma and urine by capillary electrophoresis have been developed. The plasma method can be used for measurement of total as well as unbound drug and metabolite. Plasma and urine samples are prepared for measuring by liquid/liquid extraction resulting in a limit of quantification of 40 ng/ml for total and 10 ng/ml for free BVdU in plasma and 170 ng/ml in urine. Inter- as well as intra-day precision were found to be better than 10% and both methods have been used for drug monitoring of patients.

Keywords: (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine

1. Introduction

The thymidine analogue (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) is a highly potent inhibitor of Herpes simplex virus type 1 (HSV-1) [1] and Varicella zoster virus (VZV) [2] but shows only minor activity against Herpes simplex virus type 2 [3]. Minimal inhibitory concentrations in vitro against HSV-1 and VZV are between 1 and 10 ng/ml [4].

Owing to its unusual metabolism with rapid degradation of BVdU to its ineffective metabolite (*E*)-5-(2-bromovinyl)-uracil (BVU) followed by resynthesis of the original drug from its metabolite [5] (Fig. 1), the terminal half life of BVdU is significantly increased. Thus, a dosage regimen of one dose per day can be considered, resulting in a significant advantage in patient compliance as compared to acyclovir.

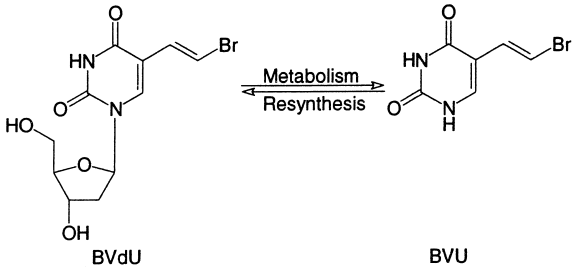

Fig. 1. Structure of BVdU and its metabolite BVU.

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BVdU has been marketed in Germany since 1990 under the proprietory name of Helpin® although only few pharmacokinetic data were reported. Plasma and urine levels have been determined using HPLC [6,7] or the biological VZV focus inhibition assay [8]. Pharmacokinetic data on BVdU after application to children are still lacking almost completely. This article describes two methods for determination of BVdU and BVU in plasma and urine using capillary electrophoresis. The sample volumes required are very small and enable the monitoring of the analytes in children from capillary blood.

2. Experimental

2.1. Chemicals

BVdU and BVU were supplied as a kind gift of Berlin Chemie, Berlin, Germany. Di-sodium-tetraborate-decahydrate, boric acid, sodium hydroxide and sodium chloride were purchased from E.Merck, Darmstadt, Germany. Tris-(hydroxymethyl)-aminomethane hydrochloride (Trizma®) was purchased from Sigma, St. Louis, USA.

2.2. Apparatus

The analysis was carried out using a P/ACE 2100 capillary electrophoresis system equipped with a P/ACE UV absorbance detector, both from Beckman, Munich, Germany. Capillaries were fused silica, effective length of 40 cm with extended light path (‘bubble cell’) from Hewlett-Packard. Cut-off filters used for deproteination of plasma were Microcon 10 100 PK, purchased from Amicon Inc., Beverly, MA, USA.

2.3. Buffer preparation

The extraction buffer used for determination of total concentration of the analytes in plasma as well as in urine consisted of 50 m*M* Trizma® hydrochloride saturated with sodium chloride and adjusted to pH 6.0 with 0.1 *M* sodium hydroxide. For the determination of the unbound analytes in plasma 200 m*M* Trizma® hydrochloride saturated with sodium chloride and adjusted to pH 5.5 with 0.1 *M* sodium hydroxide was used.

The running buffer used for determination in plasma was 50 m*M* di-sodium-tetraborate adjusted to pH 9.6 by 5 *M* sodium hydroxide. For the assay of urine a 40 m*M* di-sodium-tetraborate buffer adjusted to pH 10.5 by 5 *M* sodium hydroxide was used.

3. Sample preparation

3.1. Total plasma content

For quantification and validation 50 μl of blank plasma was spiked with 10 μl of an aqueous solution containing BVdU and BVU in the desired concentrations as well as 12.5 μg/ml of 5-fluorouracil (5-FU) as the internal standard. After vortexing for 10 s the samples were stored for 1 h at room temperature in order to allow the protein binding of the analytes and the internal standard to be established. After addition of 55 μl of the extraction buffer extraction was carried out using 1000 μl of ethyl acetate and vortexing for 30 s. Subsequently, the samples were centrifuged at 1500 *g* for 5 min and the supernatant was evaporated under a gentle stream of nitrogen at 55°C. The residue was redissolved in 10 μl of doubly-distilled water and used for the assay.

3.2. Unbound plasma content

In order to determine the amount of BVdU and BVU not bound to proteins plasma samples were filtered through 10 kD cellulose cut-off filters. For quantification purposes 300 μl of blank plasma were filtered by centrifugation at 8000 *g* for 10 min. The filter was discarded and 100 μl of the filtrate were spiked with 10 μl of a solution containing the analytes as well as 40 μg/ml of 5-FU. This mixture was extracted using 25 μl of the extraction buffer and 1000 μl of ethyl acetate following the procedure given above.

3.3. Urine

Urine samples were handled in the same way as plasma samples except that the 1 h period for

establishment of the protein binding was omitted and only 25 μl of extraction buffer were used.

4. Results

4.1. Recovery

Recovery was measured in 6 samples each spiked with 1 μg/ml of the respective substance. Using the extraction procedures given above the rate of recovery from plasma was 86.2%±7.5% for BVdU, 85.1%±6.4% for BVU and 46.4%±5.8% for 5-FU. From filtered plasma results were 84.1%±6.0% for BVdU, 82.3%±6.2% for BVU and 47.2%±5.2% for 5-FU. The extraction of urine yielded 90.2%±7.4% for BVdU, 87.3%±7.2% for BVU and 67.5%±5.9% for 5-FU.

The recovery for 5-FU was relatively low, but sufficiently constant in order to serve as internal standard. A severe interaction between BVdU and 5-FU which dramatically increases the toxicity of 5-FU [9] precludes comedication. Thus, 5-FU is well suited for use as internal standard in the two methods described.

4.2. Sample stability and storage

In order to determine conditions required for storing samples of BVdU and BVU dissolved in both doubly-distilled water and plasma samples each were measured directly as well as after one day, one week, one month and three months of storage at −20°C, 4°C, room temperature (all under exclusion of light) and at room temperature with the influence of daylight (6 samples each).

Fig. 2 shows the stability in plasma of BVdU and BVU, respectively. It can be noted that both substances showed sufficient stability at all temperatures over the three months period with no significant deterioration being detectable. Under the influence of daylight both substances degraded quickly. After one day of storage 6% of BVdU and 26% of BVU were lost.

Stock solutions of BVdU and BVU were stored at −20°C after preparation. Patient samples were transferred from the hospital to the laboratory at room temperature under the exclusion of light and stored at −20°C.

5. Analytical separation

5.1. Untreated plasma samples

The capillary was prerinsed for 2 min with 0.1 *M* sodium hydroxide and for 3 min with running buffer. The sample was injected using 0.5 p.s.i. (1 p.s.i.= 6894,8 Pa) of pressure for 30 s. Application of 25 kV (532 V/cm) enabled the separation to be achieved in less than 10 min resulting in a current of 98 μA. The UV-detector was set to 254 nm. The running buffer was replaced after twelve runs with no changes in migration times detected.

The initial extraction buffer was taken from [7] where 50 m*M* Tris buffer pH 6.0 was used for extraction of plasma for the determination with HPLC. Using this buffer an interference occurred with BVdU and blank plasma. The coeluting substance could be removed from the organic phase by saturation of the buffer with sodium chloride, although this slightly diminished the recovery of the two analytes.

As can be seen in Fig. 3, BVdU, BVU and the internal standard are well separated from the matrix. The separation of BVU from hypoxanthine might be difficult in patients with extremely high hypoxanthine plasma levels. For test purposes plasma samples received from twelve different patients were analyzed and no problems were encountered with the determination of BVdU. In two cases determination of BVU was prevented by hypoxanthine.

5.2. Filtered plasma samples

The running buffer used for determination of filtered plasma was identical to that used for plasma. Thus, the electropherogram essentially looks similar except for a more stable baseline (Fig. 4). Determination without prior extraction is possible using the same system, but is insufficient with regards to the limit of quantification.

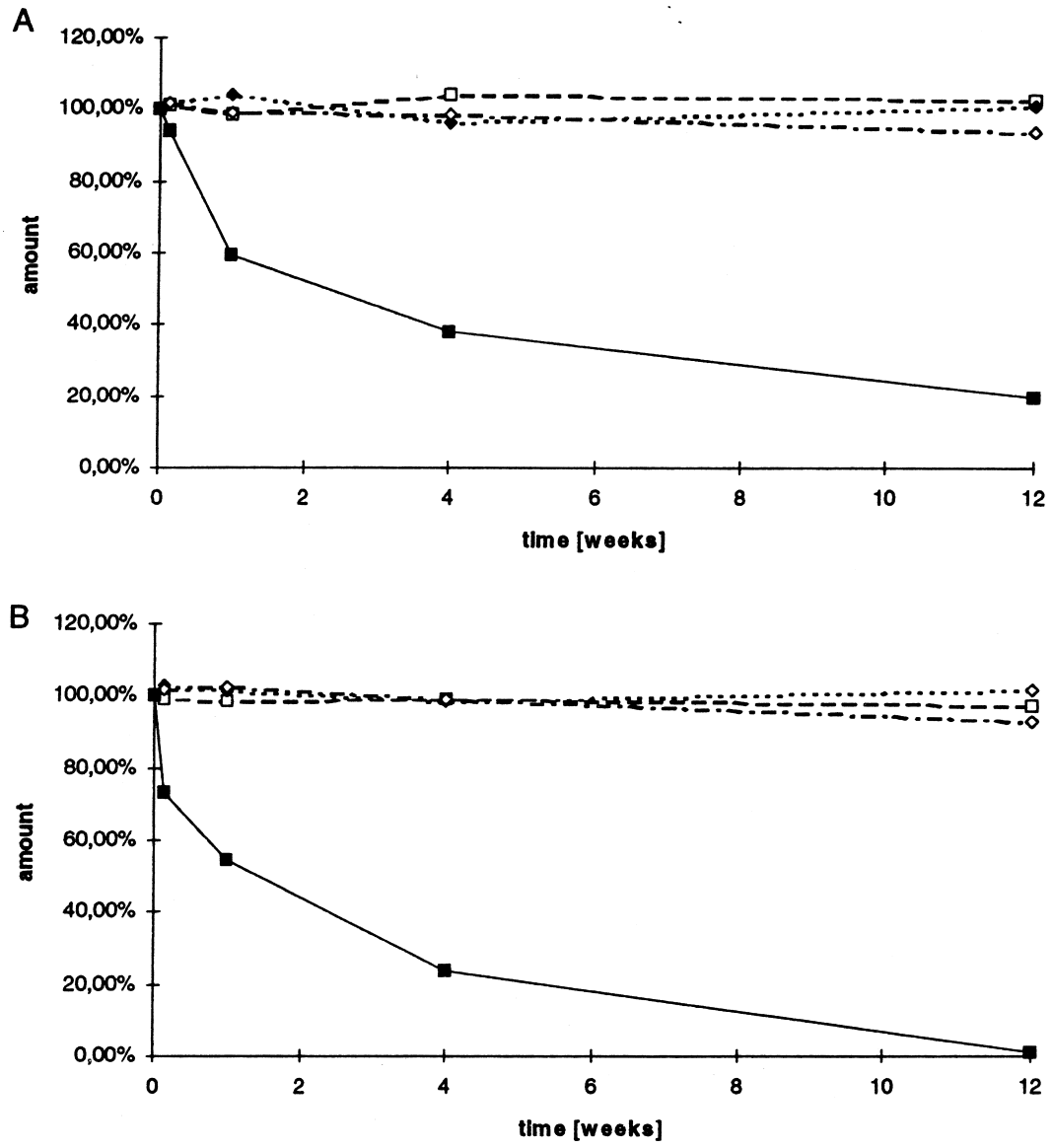

Fig. 2. Stability of BVdU (A) and BVU (B) in plasma; content of samples after storage under different conditions: −20°C (— —), 4°C (– – –), room temperature (– — –) and room temperature with daylight (——).

5.3. Urine

The capillary was pre-rinsed with 1 *M* sodium hydroxide for 1 min, doubly-distilled water for 1 min, 0.1 *M* sodium hydroxide for 1 min and finally running buffer for 2 min. The sample was injected hydrodynamically with 0.5 p.s.i. for 10 s. Separation was achieved in less than 15 min by applying 25 kV with a typical current of 108 μA. The detector was set to 254 nm.

Owing to the many interfering peaks found in blank urine (Fig. 5) separation was difficult and could only be achieved by the use of borate buffer at pH 10.5. Due to this increase in pH as well as the

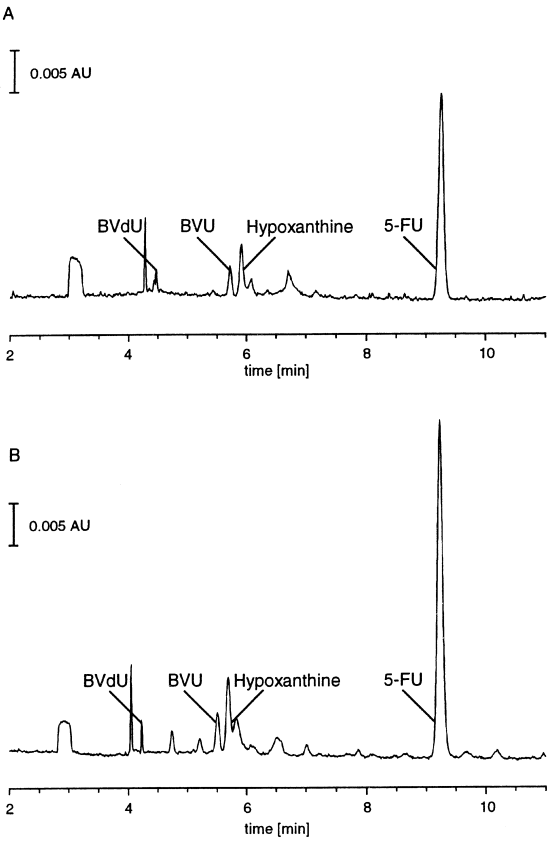

Fig. 3. Electropherograms of blank plasma spiked with 78 ng/ml BVdU, 150 ng/ml BVU and 2.5 μg/ml 5-FU without addition of the buffer prior to extraction (A) as well as with addition of the 50 m*M* Trizma®/sodium chloride buffer (B).

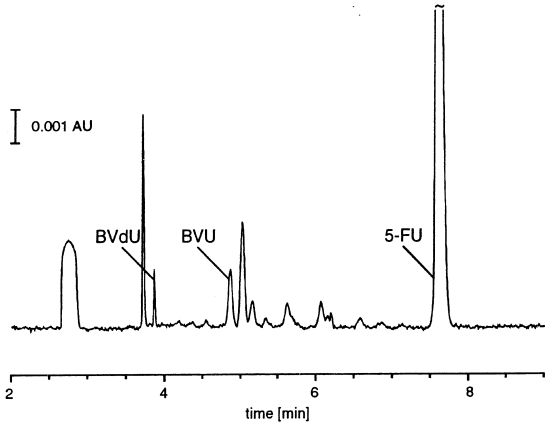

Fig. 4. Electropherogram of filtered blank plasma spiked with 40 ng/ml BVdU, 80 ng/ml BVU and 4 μg/ml 5-FU.

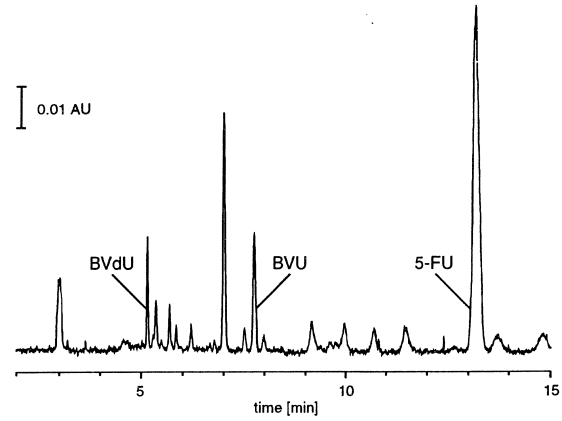

Fig. 5. Electropherogram of blank urine spiked with 680 ng/ml of BVdU, 680 ng/ml of BVU and 10 μg/ml of 5-FU.

decrease in molarity the buffering capacity of the running buffer is somewhat lowered. The resulting problem with slightly unstable migration times could be overcome using the rinsing procedure given above and replacement of the running buffer after six runs. A trial using a larger number of runs resulted in a drift of migration times towards longer values. For test purposes urine collected from three subjects was analyzed and no problems were encountered concerning the separation.

6. Quantification and validation

6.1. Plasma

Samples were spiked with eight different amounts from 40 ng/ml to 5000 ng/ml for BVdU and from 80 ng/ml to 10000 ng/ml for BVU. The ratios of the corrected (time normalised) peak areas of the analytes and the internal standard were correlated against sample concentration. The result was satisfactory with the correlation coefficient being higher than 0.99 in both cases. The limit of quantification (LOQ) could be lowered to 40 ng/ml and 80 ng/ml for BVdU and BVU, respectively by using long injection times. As the samples were redissolved in doubly-distilled water, the ionic strength in the sample zone is low and thus a very efficient sample stacking is achieved. LOQ was defined as the lowest concentration which could be determined with preci-

sion and accuracy staying within the borders specified below.

For the determination of inter- as well as intra-day precision and accuracy six samples each were measured at four different amounts on three different days. While precision was satisfactory with relative standard deviation smaller than 10%, accuracy showed a slight systematic error for BVU at low levels. This however could be corrected by dividing the calibration range into two different ranges at 1200 ng/ml. Using the two resulting calibration curves from 80 to 1200 ng/ml and from 1200 to 10 000 ng/ml accuracy stayed within ±10% for means and ±20% for single samples, as can be seen in Table 1. The systematic error can be attributed to the great width of the calibration range, as this leads to a stronger influence of the larger values.

6.2. Filtered plasma

The method used for quantification and validation was similar to that given for plasma. The calibration range reached from 10 ng/ml up to 2500 ng/ml for BVdU and 20 ng/ml to 5000 ng/ml for BVU. Both substances gave excellent linear correlation ($r>$ 0.99), but the calibration ranges had to be split at 312 ng/ml for BVdU and 625 ng/ml for BVU to meet the requirements for precision and accuracy.

6.3. Urine

The procedure used for quantification and validation of BVdU and BVU in urine, again, was the same as the one described for plasma. The calibration was done from 170 ng/ml to 11 000 ng/ml for both substances using seven different levels of content. Correlation coefficients were higher than 0.99. Precision and accuracy fulfilled the above mentioned requirements when separate calibration curves were used from 170 ng/ml to 1375 ng/ml and from 1375 ng/ml to 10 000 ng/ml.

6.4. Determination of protein binding

In order to determine the degree of protein binding six plasma samples were spiked to 2 μg/ml of BVdU and BVU. These samples were measured using the plasma method as well as after filtering using the procedure for filtered plasma. The degree of protein binding was calculated to be 99.4% and 88.5% for BVdU and BVU, respectively. These results were higher than the ones given in [7] (BVdU: 97%) and effectively prevented measurement of free BVdU in patient samples while determination of unbound BVU did not cause any problems.

Table 1
Accuracy and precision

	c Spiked[a]	Day 1			Day 2			Day 3		
		c Found[b]	Accuracy[c]	Precision[d]	c Found	Accuracy	Precision	c Found	Accuracy	Precision
BVdU	4942	4946	0.09%	1.37%	4894	−0.97%	2.82%	5266	6.56%	8.72%
	618	614	−0.65%	2.37%	602	−2.50%	1.31%	616	−0.22%	2.68%
	77	77	0.32%	5.63%	80	3.17%	7.50%	77	−0.32%	6.01%
	39	37	−4.23%	8.44%	41	6.27%	6.55%	41	6.83%	7.82%
BVU	9547	9473	−0.77%	1.21%	9379	−1.76%	4.10%	10103	5.82%	9.60%
	1193	1110	−6.97%	5.20%	1153	−3.39%	2.44%	1308	9.60%	7.59%
	149	145	−3.14%	7.69%	145	−2.57%	6.21%	142	−4.65%	5.49%
	75	80	6.91%	6.74%	74	−0.17%	5.02%	67	−9.60%	8.00%

[a] Concentration of the spiked samples and the concentrations found are given in ng/ml.
[b] Concentration found is the mean of six samples at every level.
[c] Accuracy is the deviation of means from the spiked content.
[d] Precision is given as relative standard deviation.

6.5. Pharmacokinetics

Kinetics of BVdU and BVU from plasma and urine were measured after oral administration of 125 mg as well as 250 mg of BVdU on empty stomach to a 83 kg male patient. Plasma samples were taken at 30 min intervals during the first 2 h, at 60 min intervals up to 6 h and after 24 and 30 h. Urine was collected over the first 24 h following administration and measured as one sample.

Fig. 6 shows the kinetics of BVdU and BVU on logarithmic scale. BVdU is quickly absorbed after oral administration and metabolised almost immediately to BVU with a half-life of about 1.5 h. From 4 to 30 h after administration plasma levels of BVdU run parallel to the curves of BVU, giving BVdU a terminal half-life of up to 20 h. This unusual fact confirms the results found by other investigators [5] stating that BVdU is resynthesised from its metabolite in vivo.

It is interesting to note that the peak plasma level of BVdU 1 h after administration is more than three times higher with a dose of 250 mg than with 125 mg. One explanation might be that the metabolic capacity of the liver on first pass can be saturated resulting in very high peak plasma levels at high dosages.

The excretion of BVdU into urine after 24 h was determined after both applications. It was found to be quite low at about 1% of the applied dose in both cases. These results are in accordance with the values reported by [7].

7. Conclusion

Two methods for the determination of total and unbound BVdU and its metabolite BVU from plasma and urine have been established. Capillary electrophoresis proved to be well suited for this task as it combines small sample volumes with short run times and good reproducibility. The common problem with low sensitivity can be overcome by bubble-cell capillaries and the use of sample stacking with long injection times from solutions with low conductivity.

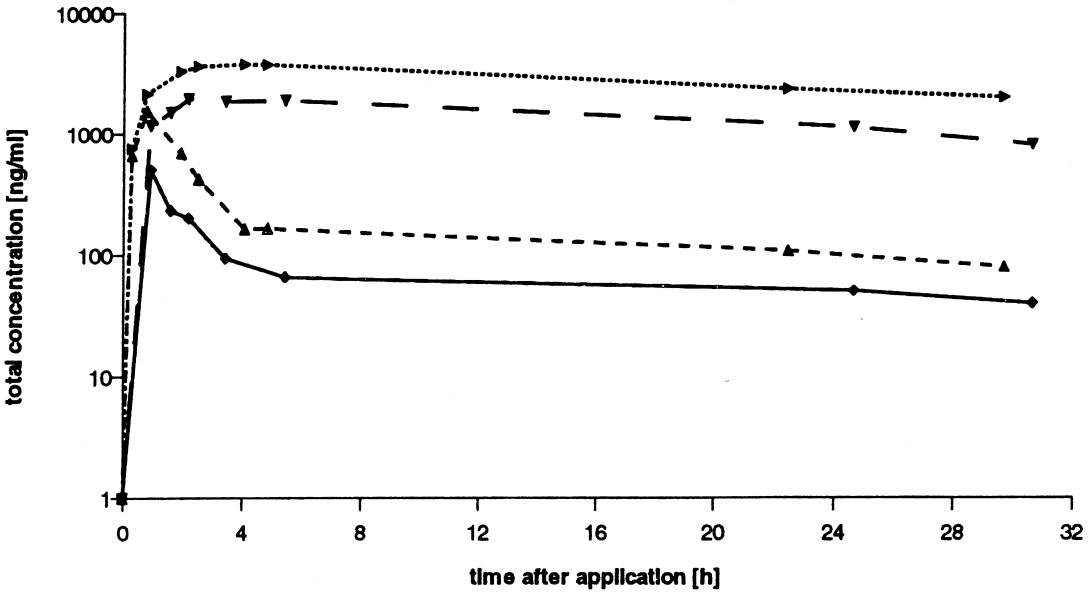

Fig. 6. Total plasma concentrations of BVdU and BVU after oral application of 125 mg and 250 mg of BVdU; BVdU 125 mg (——), BVU 125 mg (— —), BVdU 250 mg (– – –) BVU 250 mg (- - -).

These techniques allow quantification of samples with content as low as 10 ng/ml.

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