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Development and validation of a bioanalytical assay for (*E*)-5-(2-bromovinyl)-2′-deoxyuridine in plasma by capillary zone electrophoresis

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Abstract

A capillary zone electrophoretic method for the quantification of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine in plasma has been developed and validated. Separation was performed with a 25 mmol/l borate buffer, pH 9.0, after an initial rinsing step with sodium hydroxide. The rinsing step was necessary for reproducible analyses of aqueous samples and plasma extracts obtained by C_{18} solid-phase extraction after deproteination with perchloric acid. No interferences with plasma compounds were observed. The calibration graph was linear over the range of 30 to 3000 ng/ml using 5-fluorouracil as external standard. The limit of quantification was 24 ng/ml. The CZE method is fast, reproducible, linear and is therefore a good alternative for the already established HPLC methods.

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

1. Introduction

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) is an antiviral drug, which is used against herpes simplex virus [1] and in anticancer therapy as a stand-alone medication [2] or sometimes in combination with other nucleoside analogues such as 5′-deoxy-5-fluorouridine (DFUR) [3], 5-fluorouracil (FU) [4], ftorafur [5] or acyclovir [6].

A simple, but not selective, BVDU bioassay in serum has been described in the literature, although this method lacks sensitivity for pharmacokinetic studies (LOD=0.2 μg/ml) [7]. Furthermore, three high-performance liquid chromatography (HPLC) methods dealing with BVDU are described [8], using either liquid–liquid extraction or a deproteination step with perchloric acid as sample pretreatment method. The detection limits of these methods range from 6 to 55 ng/ml.

Recently, many HPLC routine analytical methods have been transposed to capillary zone electrophoresis (CZE). A lot of them concern acidic as well as basic drugs, with impurity determination, but the number of routine validated bioanalysis of a drug is increasing [9].

Sample pretreatment for CZE is sometimes more extensive than for HPLC, although at sufficiently high concentrations, direct injection of the sample or direct injection after dilution can be used. To avoid

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clogging of the capillary, sodium dodecyl sulphate (SDS) can be added to the sample to solubilize or denature the plasma proteins [10].

However, when the analyte concentration is low and/or the detection characteristics are limited, the well-known off-line sample pretreatment methods like solid-phase extraction (SPE), liquid–liquid extraction, deproteination or dialysis [11], supported liquid membranes [12] or a combination of deproteination with liquid–liquid extraction [13], can be used for clean-up and lowering detection limits. Sometimes stacking procedures after sample pretreatment must be used to obtain the desired detection limits [13].

To the best of our knowledge, only one CZE separation [14] has been described which concerns the separation of fluoropyrimidines, compounds which are comparable with our analyte. However, this method has not been applied to biological samples.

In spite of a more extensive sample pretreatment in bioanalysis, we transposed the routine HPLC method [15] to CZE and validated the developed system. As a consequence of the unfavourable detection limits an off-line sample pretreatment, a C_{18} SPE column is applied for clean-up and concentration of the sample.

2. Experimental

2.1. Chemicals

BVDU, DFUR and FU were kindly supplied by the Leiden University Medical Centre. Perchloric acid (70%), methanol, boric acid and hydrochloric acid were obtained from Baker (Deventer, Netherlands). Acetonitrile was HPLC grade and purchased from Rathburn (Walkerburn, UK). Sodium hydroxide was obtained from Merck (Darmstadt, Germany); Tris was purchased from Aldrich (Bornem, Belgium) and cetrimonium bromide from BDH (Brunschwig, Amsterdam, Netherlands). All reagents were analytical grade. Heparin plasma was purchased from the Leiden University Medical Centre. For the preparation of the stock solution of the analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). One millilitre C_{18} extraction columns (Baker) were used for the sample pretreatment.

2.2. Buffer and sample preparation

A stock solution of BVDU (0.18 mg/ml) was prepared in an acetonitrile–water (25:75, v/v) mixture while stock solutions of DFUR and FU (1.0 mg/ml) were prepared in water. Working solutions were prepared by dilution of the stock solution. The perchloric acid (1 mol/l) was prepared by dilution of concentrated perchloric acid (70%) with water. The 10 and 25 mmol/l borate buffers were prepared by mixing 10 and 25 mmol/l boric acid and 10 and 25 mmol/l sodium hydroxide solution until the desired pH was reached. The 10 mmol/l Tris buffer was prepared by mixing 10 mmol/l Tris and 10 mmol/l hydrochloric acid. The buffer solutions were filtered through a 0.2-μm nylon Acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, USA). For the plasma calibration curves, 900 μl blank plasma was spiked with 100 μl of an aqueous analyte solution.

2.3. Equipment and conditions

The experiments were performed on a P/ACE system Model 2200 (Beckman, Fullerton, CA, USA) at ambient temperature. For data collection and sample handling System Gold software v.7.12 was used.

Fused-silica capillaries (100 μm I.D.) were obtained from SGE (Ringwood, Australia). New capillaries were rinsed for 10 min with a 0.1 mol/l sodium hydroxide solution, followed by water for another 10 min.

During initial experiments to optimise the CZE system, a capillary of 47 cm (length to the detector 40 cm)×100 μm I.D. was used and for the routine bioanalysis a capillary of 67 cm (length to the detector 60 cm)×100 μm I.D. was used; the detection wavelength was set to 254 nm. The running buffer was 25 mmol/l borate buffer, pH 9.0 and the capillary was rinsed with 5 mmol/l sodium hydroxide solution during 0.5 min and with running buffer during 3 min, before every injection (12 s, hydrodynamic). The applied potential was +20 kV.

For the sample pretreatment a Megafuge 1.0 centrifuge (Heraeus Sepatech, Osterode, Germany), a

Baker SPE-24G column processor (Baker) and a Haake-Büchler (Lenexa, KS, USA) vortex evaporator operating at 30°C were used.

2.4. Sample pretreatment

Defrosted plasma samples (1.0 ml) were mixed with 0.3 ml perchloric acid solution (1 mol/l) for 30 s. After a stabilization for 30 min at 4°C, the samples were centrifuged at 5200 *g* for 10 min and the supernatant was transferred to a pretreated C_{18} column, which was flushed with 3 ml methanol and 3 ml water. The SPE column was washed with 2 ml water and 2 ml methanol–water (10:90, v/v), respectively. After drying the column under vacuum, the analyte was eluted with 0.6 ml methanol. The methanol fraction was evaporated to dryness at 30°C and the residue was dissolved in 0.1 ml water which contained the external standard 5-fluorouracil.

2.5. Procedures

Before starting routine analysis, buffer solutions were prepared freshly every day, and a system suitability test was done with a solution containing the analytes to be determined at the concentration of 6 μg/ml BVDU and 10 μg/ml FU ($n=6$).

2.6. Quantification

For the quantification of the analyte BVDU, FU as standard was added to the residue after sample pretreatment and calibration graphs were prepared using peak area ratios. Data points for CZE were collected at an acquisition rate of 5 Hz and a rise time of 1 s was applied. Peaks were integrated using the system Gold software.

2.7. Validation

The limit of quantification (LOQ), recovery, day-to-day variation, within-day variation, accuracy and precision were calculated from the calibration curves. For the plasma calibration curves blank plasma was spiked with 30, 60, 120, 300, 600, 1200 and 3000 ng analyte per ml. On three different days three plasma calibration curves and three extra plasma samples spiked with 120 and 1200 ng/ml, were pretreated with the SPE sample pretreatment method and the results compared with two aqueous reference calibration curves. Data were fitted by weighted regression using the reciprocal of the squared peak area ratio values as weighting factor $(1/y^2)$.

For the determination of LOQ plasma was spiked in triplicate with 12, 18, 24, 30, 36 and 42 ng/ml. The LOQ was defined as the concentration at which the relative standard deviation (RSD) of repeated analyses ($n=3$) amounts to 20%.

3. Results and discussion

3.1. Method development

3.1.1. CZE separation

Initial separations of BVDU, FU and DFUR (Fig. 1), were investigated over the whole pH range, concerning acidic media with acetate buffers the compounds migrated as very broad peaks, while all three compounds have the same migration time. Probably, adsorption to the silica wall influences the peak shape. In alkaline media separation of the negative compounds was initially performed with Tris as well as borate buffers (Fig. 2). The zwitterionic Tris buffer shows a broad BVDU peak and no separation between FU and DFUR at pH 8 and a good peak shape for BVDU at pH 9.0, but the other peaks are broad. The borate buffer gave much better peak efficiencies than the Tris buffer, possibly due to complexation with the ribose hydroxyl group. At pH 8.0 the compounds migrate in the order of BVDU–DFUR–FU, and the peaks are not well shaped. At pH 9.0 the BVDU peak shows the best peak efficiency and the other two peaks change in migration order, while DFUR gives a very broad peak. At pH 10.0 the electropherogram shows very inefficient separation with Tris as well as with borate buffer.

Addition of cetrimonium bromide (10 mmol/l) to the two buffer solutions offered in most cases, at all three measured pH values, a better separation (Fig. 3) between the three compounds.

Addition of cetrimonium bromide to the running buffer caused a reversal of electroosmotic flow; for the migration and separation of the compounds, a negative high voltage must be applied.

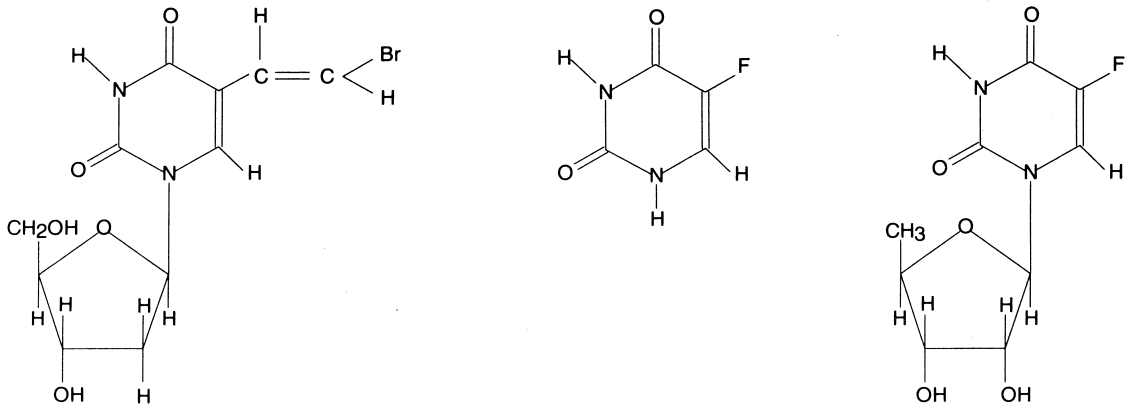

Fig. 1. Structures of BVDU [1], FU [2] and DFUR [3].

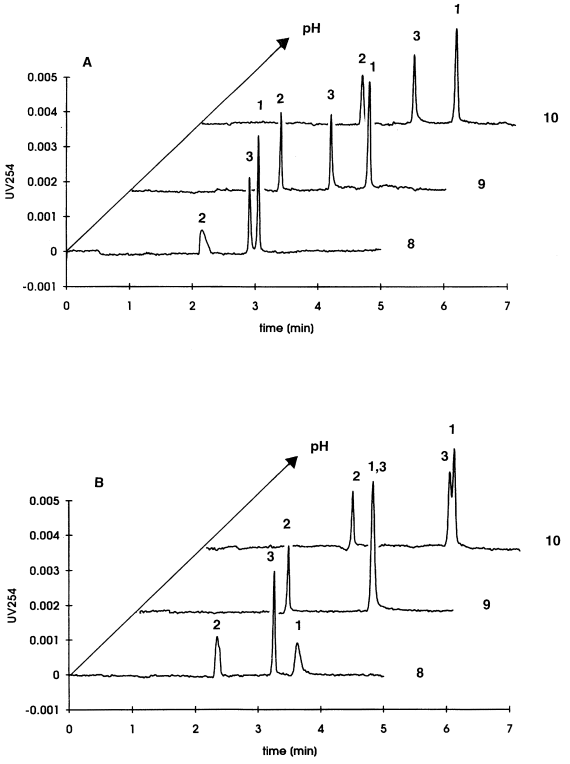

Fig. 2. Electropherograms of a mixture of BVDU [1], FU [2] and DFUR [3] in water (respectively, 6, 10 and 10 μg/ml) with different buffers and different pH: 10 mmol/l Tris buffer (A) and 10 mmol/l borate buffer (B). Conditions: capillary 47 cm×100 μm, potential +20 kV, hydrodynamic injection 5 s and detection at 254 nm.

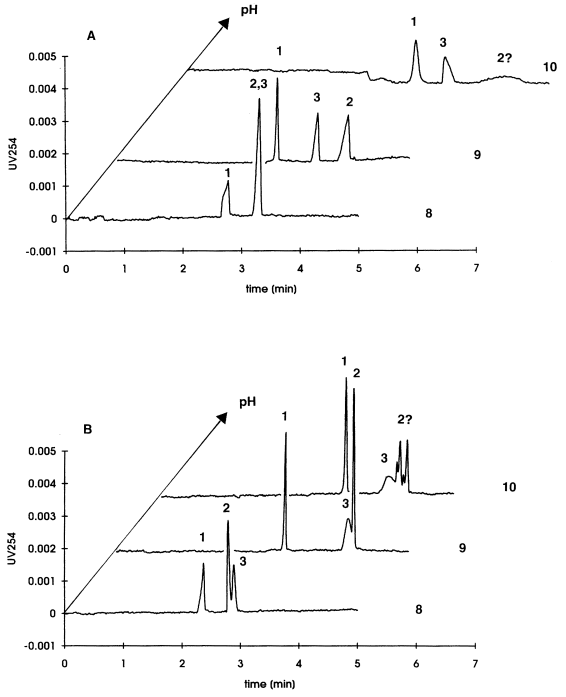

Fig. 3. Electropherograms of a mixture of BVDU [1], FU [2] and DFUR [3] in water (respectively, 6, 10 and 10 μg/ml) with different buffers and different pH: 10 mmol/l Tris buffer with 10 mmol/l cetrimonium bromide (A) and 10 mmol/l borate buffer with 10 mmol/l cetrimonium bromide (B). Conditions: capillary 47 cm×100 μm, potential −20 kV, hydrodynamic injection 5 s and detection at 254 nm.

Furthermore, the DFUR peak migrates with a much better efficiency than as shown in Fig. 2. Possible compound–wall interactions play an im-

portant role, especially with compounds of this type. Comparison of Figs. 2 and 3 shows that the borate buffer at pH 9.0 and the Tris buffer, pH 9.0 with cetrimonium bromide results in the best signal-to-noise ratio.

To test the long-term reproducibility with the borate buffer, pH 9.0 system, a standard solution which contained 6 μg/ml BVDU, 10 μg/ml DFUR and 10 μg/ml FU was injected every 7 min. After measuring during 8 h the migration times were increased by about 50%, the peaks were broad and the signal-to-noise ratio was reduced to about 40% with respect to the initial value.

Testing the Tris, pH 9.0 buffer with cetrimonium bromide showed nearly the same phenomenon: after 4.5 h measuring the peaks of BVDU and DFUR were broad, while the peak of FU was small. However, in this system the migration times were not changed.

Both Tris and borate buffers are widely applicable in CZE in the pH range of 8 to 10. They are good and stable buffering agents. We believe that the changes observed were due to alterations in the capillary surface.

The problem of an unstable system could be solved by cleaning the capillary with a small plug of sodium hydroxide. Unfortunately, this method cannot be used in the system with cetrimonium bromide.

Reproducible measurements were obtained with a 25 mmol/l borate buffer at pH 9.0 with a rinsing step of 5 mmol/l sodium hydroxide solution during a 0.5 min and 3 min rinsing with the running buffer. During 7 h the migration times varied by 2.0% and the peak area ratios had a RSD of 2.5% for BVDU/DFUR and 3.6% for BVDU/FU, respectively.

A rinsing step with sodium hydroxide, even when injecting only reference solutions, appeared to be a necessity, which is also mentioned by other researchers [16,17], although they do not explain. It is known that these compounds easily adsorb onto glass surfaces; when the capillary wall is activated with sodium hydroxide, the wall will become more negative. Therefore repulsion of the negatively charged analyte ions will diminish the adsorption of the glass wall. Regular refreshment of the glass surface by rinsing with sodium hydroxide appeared to improve both the efficiency and reproducibility needed for validation of routine bioanalysis.

For optimization of the detection, different filters of the UV absorbance detector were tested for best signal-to-noise ratio and the optimum parameters of data rate and rise time were investigated. The best signal-to-noise ratio for BVDU was obtained at 254 nm, a data acquisition rate of 5 Hz and a rise time of 1 s.

Although, the highest signal-to-noise ratio was obtained for a 47 and 67 cm capillary at 5 kV, still 20 kV was chosen since nearly the same signal-to-noise ratio and the shortest analysis time was obtained.

In this study two different lengths of the capillary were used, i.e., 47 cm for the initial experiments with reference solutions and 67 cm for the extracts of the plasma samples. Use of the long capillary results in a better separation between analyte and residual plasma compounds.

To provide enough sensitivity and a robust experimental set-up in all experiments 100 μm capillaries were applied. Capillaries with smaller internal diameters were easily blocked by residual plasma constituents.

The optimal hydrodynamic injection time for a 67 cm capillary appeared to be 12 s corresponding to a plug of about 185 nl. All the residual samples were dissolved in water, which induced stacking of the analytes in the capillary. Commonly only 1% of the total volume of the capillary can be injected, which was now 3.5-times higher, implying an increase in sensitivity and a decrease in sample concentration detection limit. Longer injection times influence the selectivity dramatically by peak broadening.

In Fig. 4 the results obtained with the validated procedure are depicted. Fig. 4A shows the electropherogram of a blank plasma extract, while Fig. 4B and 4C show the electropherogram of a plasma extract spiked with 60 and 600 ng/ml BVDU (peak 1), respectively.

3.1.2. Sample pretreatment

A sample pretreatment for bioanalysis in CZE is needed because of the presence of biological matrix ions in the residue can disturb the current and therefore the reproducibility in CZE. Furthermore, a concentration step must be used because of the relatively low plasma levels. The simple deproteination step used in earlier work [15] cannot be used in this CZE procedure, since the perchloric acid dis-

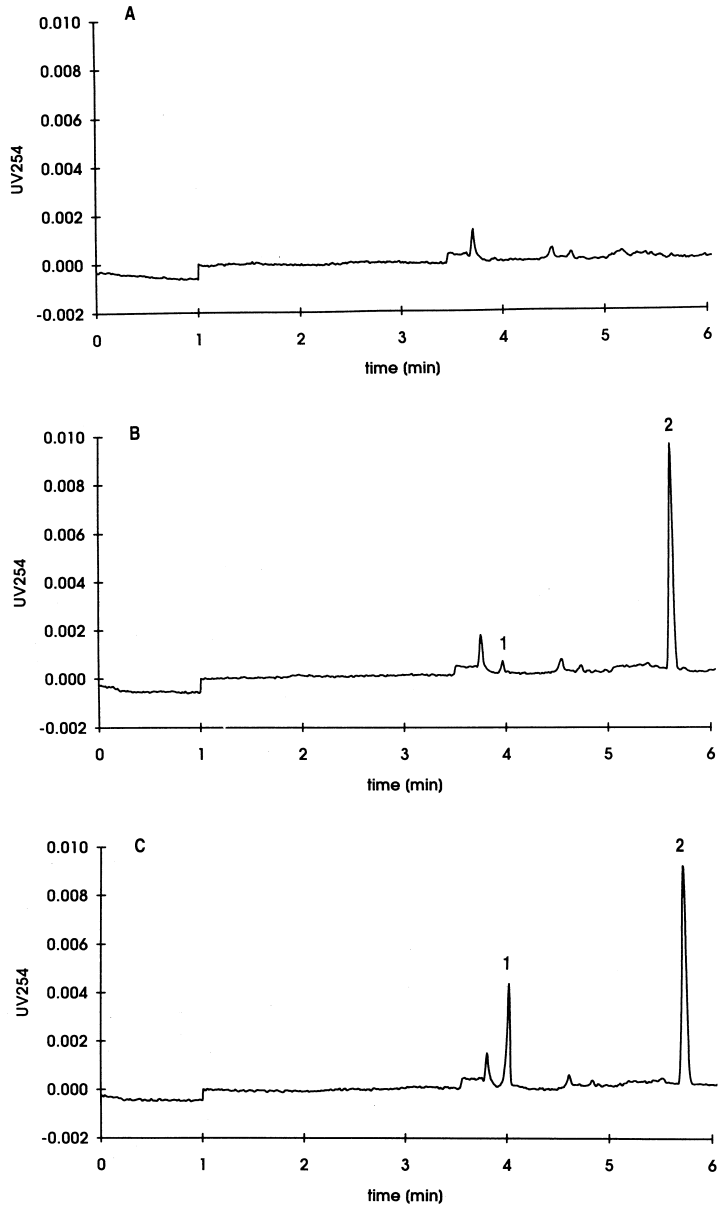

Fig. 4. Electropherogram of a plasma extract spiked with BVDU [1] and FU [2] as the external standard; blank extract (A), extract of plasma spiked with 60 ng/ml (B) and extract of plasma spiked with 600 ng/ml (C). Conditions: capillary 67 cm×100 μm, the running buffer was 25 mmol/l borate buffer, pH 9 with a pre-rinse step of 5 mmol/l sodium hydroxide during 0.5 min and borate buffer during 3 min, potential +20 kV, hydrodynamic injection 12 s and detection at 254 nm.

turbs the separation of the compounds. Also, the liquid–liquid extraction used for urine extraction cannot be used directly for this determination; because of the high solubility of water in ethyl acetate a relatively dirty residual sample is obtained. A pre-extraction with chloroform could overcome this problem, but the sample pretreatment is very time consuming. Furthermore, there were interferences

migrating at the time of the external standard. For that reason, a SPE sample pretreatment method was developed.

The described method requires a deproteination step, because of the high protein binding (92%) of the analyte. Perchloric acid, used for deproteination, did not influence the recovery of the analyte from SPE. When the sample is transferred to the SPE column, the column can be washed with maximally 4 ml methanol–water (10:90, v/v), before the analyte breaks through the column and recovery is decreased. Two millilitres appeared to be enough to wash the column from all interfering and disturbing compounds.

A very selective sample pretreatment was applied, because many compounds, such as the here used external standard FU, elute in the washing step with the methanol–water mixture.

3.2. Method validation

Before start of the sample analysis, a system suitability test was carried out with an aqueous sample which contained 6 μg/ml BVDU and 10 μg/ml FU, respectively, for testing the performance of the CZE system with respect to migration times, peak performance and sensitivity. For the quantitative determination of the analyte no related compound could be found for use as an internal standard before sample pretreatment, since most related compounds did not adsorb to the SPE column or eluted in the wash step. Therefore, we chose to add a standard before CZE determination. The developed method was validated for the quantitative determination of BVDU in plasma, except for the robustness.

3.2.1. Quantitative aspects

The LOQ was defined as the lowest concentration at which the peak area ratio shows a RSD of 20% ($n=3$). For the determination of LOQ blank plasma was spiked with 6, 12, 18, 24 and 30 ng/ml, respectively.

The concentration of 24 ng/ml had a RSD of 7.4% and the concentration 12 and 18 ng/ml higher than 20%, which means that LOQ is at least 24 ng/ml with an accuracy of 13.2% and LOD, which is defined as 1/3·LOQ, about 8 ng/ml.

For the investigation of the linearity, plasma samples were spiked with 30 to 3000 ng/ml BVDU, treated according to the sample pretreatment method, and the residues dissolved in 100 μl FU solution (10 μg/ml) and compared with reference solutions which contained 30 to 3000 ng BVDU plus 10 μg FU in 100 μl.

The linearity was determined by weighted regression ($1/y^2$). For all 15 calibration curves measured (nine plasma and six reference curves) no point deviated more by than 5%, thus there is a linear behaviour for the analyte with the here developed system at least from 30 to 3000 ng/ml.

The mean plasma calibration equation of the nine plasma calibration curves with the RSD was: $y=-3\cdot10^{-3}$ ($\pm$82.4%)$+5.76\cdot10^{-4}$ ($\pm$2.70%)$\cdot x$ with a mean regression coefficient of 0.9973 ($\pm$0.20%). The mean equation for the six reference calibration curves was: $y=-8\cdot10^{-3}$ ($\pm$58.0%)$+1.21\cdot10^{-3}$ ($\pm$2.71%)$\cdot x$ with a mean regression coefficient of 0.9971 ($\pm$0.22%).

For the calculation of the overall recovery, the slope of the mean plasma curve was related to the slope of the mean reference calibration curve. The results obtained on three different days were 47.6%, 46.7% and 46.4% respectively, with a RSD of 1.4%. The RSDs of the three different plasma curves per day were 3.6%, 5.1% and 1.4%, respectively. The recovery of the low concentration samples did not differ significantly from that of the high concentration (Table 1).

The results of the day-to-day and within-day precision are presented in Table 1: the RSDs were better than 7.1 and 5.9%, respectively. For the nominal concentrations of 120 and 1200 ng/ml, six QC samples were measured on three different days. The precision and accuracy for the low level are better than 10.0 and 8.9%, respectively and for the high level better than 6.9 and 5.3%, respectively.

4. Conclusions

The here developed method can be used as a routine bioassay. The obtained precision and accuracy are comparable with the requirements needed for registration purposes. Compared to HPLC, CZE is favourable with respect to time, cost of columns and

Table 1
Results obtained from method validation: recovery of BVDU derived from the results obtained from the plasma spiked samples ($n=3$) and the reference samples; and the means, standard deviations (SDs) and relative standard deviation (RSD) derived from within-day and day-to-day precision studies of the determined BVDU in plasma

Nominal concentration (ng/ml)	Recovery		Within-day ($n=3$)		Day-to-day ($n=3$)			
	Mean (%) ($n=3$)	RSD (%)	Mean SD (ng/ml)	RSD (%)	Mean calculated concentration	SD (ng/ml)	RSD (%)	Relative error (%)
30.0	47.6	12.3	0.60	2.0	30.7	0.62	2.0	2.4
60	46.9	7.9	3.2	5.4	58	2.0	3.5	2.9
120	49.4	8.0	4.9	4.1	117	8.2	7.0	2.4
300	47.1	4.2	17.4	5.8	302	6.2	2.1	0.6
600	45.3	2.7	19.0	3.5	600	22.8	3.8	0.1
1200	47.0	1.6	32.4	2.9	1258	48.2	3.8	4.8
3000	45.8	2.6	137	4.7	3084	64.6	2.1	2.8

chemicals, and the environmental aspects such as disposal of organic solvents. Because of the high selectivity and high sample throughput, CZE can also compete with HPLC in bioanalysis.

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