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Development and validation of a plasma assay for acyclovir using high-performance capillary electrophoresis with sample stacking

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Abstract

A sensitive plasma assay for acyclovir has been developed and validated. Acyclovir was separated from plasma components using Oasis HLB columns. Separation was obtained with no plasma interference using micellar electrokinetic chromatography (175 m*M* SDS) and hydroxypropyl-β-cyclodextrin (100 m*M*) in 90 m*M* borate buffer (pH 8.8) containing 0.2% NaCl. High sensitivity was achieved by large volume sample introduction and stacking. The linear range was from 20 to 10 000 ng/ml with a limit of quantitation of 20 ng/ml. This method is a viable alternative to HPLC because of its high separation and sensitivity, reproducibility, and adaptability to other nucleoside analogs.

Keywords: Sample stacking; Therapeutic drug monitoring; Acyclovir; Herpes simplex virus

1. Introduction

Acyclovir [9-(2-hydroxyethoxylmethyl) guanine, ACV] is a guanine derivative nucleoside analog with strong antiviral activity against herpes simplex and varicella zoster viruses [1]. The utility of acyclovir, and its prodrug valacyclovir [2], as a prophylaxis in pregnancy [3] and as treatment in neonatal herpes [4] is currently being investigated as a standard form of therapy in these clinical conditions.

Analysis of ACV in biological matrices has been made possible by a number of HPLC methods employing different modes of detection such as fluorescence [5–7] direct UV [8–11], and HPLC–MS [12]. Sample preparation for these HPLC methods includes mainly a deproteinization step (using perchloric acid or organic solvents such as acetonitrile and methanol) and/or a solid-phase extraction step (using a hydrophobic column) as well as other techniques such as ultrafiltration [13]. There have been only two reported assays for acyclovir analysis in biological fluids using high-performance capillary electrophoresis [14,15] that employed UV and amperometric modes of detection, with limits of detection in the high hundreds of the μg/ml range.

The use of high-performance capillary electrophoresis (HPCE) for the determination of drug levels in pharmacokinetic studies and drug level monitoring has provided investigators a viable alternative to HPLC in recent years. With its high separation

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efficiency, ease of operation, speed, and small sample volume requirements, HPCE is gaining popularity as a suitable tool in the bioanalysis of pharmaceuticals [16–18]. Here we report the use of on-line sample concentration by large volume sample stacking in a MECC–cyclodextrin buffered system to improve both sensitivity and separation in HPCE.

2. Experimental

2.1. Chemicals

Acyclovir and Borax were purchased from Sigma (St. Louis, MO, USA). The internal standard 5-(2-hydroxyethyl)-2′-deoxyuridine (HedU, I.S.) was from Berry & Associates (Dexter, MI, USA). Acetonitrile and methanol were HPLC grade from Fisher Scientific (NJ, USA). Hydroxypropyl-β-cyclodextrin (HPβCD) was obtained from Aldrich (Milwaukee, MI, USA). The Oasis HLB solid-phase extraction column and the vacuum manifold were from Waters (Milford, MA, USA). Uncoated capillaries were from Polymicro Technologies (Phoenix, AZ, USA). All other reagents were of analytical grade. Water for the preparation of buffers and solutions was filtered and deionized using a ModuLab 2020 system from Continental Water Systems (San Antonio, TX, USA). Pooled plasma was from 20 healthy volunteers.

2.2. Preparation of plasma standards

Stock solutions of ACV (1.0 mg/ml) and HEdU (0.5 mg/ml) were prepared and stored at −80 °C. Serial dilutions of stock ACV and HedU were done in water. Drug-free pooled plasma (600 μl) was spiked with 20 μl containing ACV (at final concentrations of 20, 100, 1000, 5000, and 10 000 ng/ml) and HEdU (final concentration 1.0 μg/ml) to prepare the plasma standards.

2.3. Solid-phase extraction

The Oasis solid-phase extraction HLB column was conditioned with 2 ml of methanol followed by 2 ml of deionized water using a vacuum manifold. The pooled plasma sample (600 μl) was passed through the column under vacuum. The column was washed with 0.5 *M* phosphate buffer pH 7.4 containing 2 m*M* EDTA under high vacuum. Elution was achieved at a flow-rate of 0.2 ml/min using 200 μl of a solution containing 15% acetonitrile and 0.9% NaCl. The sample was stored at 4 °C before analysis.

2.4. Instrument and separation procedures

Separations were performed on a Beckman MDQ automated capillary electrophoresis system (Beckman Instruments, Missisauga, ON, Canada). Uncoated capillaries were used with internal diameters of 75 μm, external diameters of 360 mm and lengths of 60.2 cm (50 cm to detector). New capillaries were first rinsed with 1.0 *M* NaOH (10 min, 20 p.s.i.), followed by rinsing with the separation buffer (20 min, 20 p.s.i.). The new capillary was then left to equilibrate overnight in the separation buffer prior to operation. Each separation was preceded by a 1 min rinse with 1.0 *M* NaOH, a 1 min rinse with deionized water, followed by a 3 min rinse with the separation buffer. The sample was introduced using low-pressure (0.5 p.s.i.) injection for 100 s. All separations were carried out at 22 °C using a voltage of 20 kV throughout the experiment. Detection of acyclovir was monitored at 254 nm using UV. Stock borate buffer (600 m*M*) was adjusted to pH 8.8 using HCl(aq) and was used to dissolve SDS and HPβCD powders to make 90 m*M* borate pH 8.8 containing 175 m*M* SDS and 100 m*M* HPβCD with 0.2% NaCl, which is the separation buffer. The buffer was passed through a 0.22 μm filter (Sartorius, Gottingen, Germany) before analysis.

2.5. Method validation

Blank pooled plasma was spiked with 0, 20, 100, 1000, 5000 and 10 000 ng/ml of ACV and 1000 ng/ml of HedU. Validation samples were prepared with the SPE column and 50 μl was analyzed by HPCE. Calibration was obtained by plotting the corrected peak-area ratios (ACV/HEdU) versus ACV concentrations. Peak area was corrected by using the migration time of the analyte (area/migration time). Linear least-squares analysis was performed using StatView and the calibration plot was used to determine the concentrations of ACV in unknown samples. Intra-day accuracy, precision, recovery,

linearity, and range were determined. The validation was repeated on different days to determine the inter-day precision.

3. Results and discussion

3.1. Sample preparation with SPE

Plasma samples were loaded directly onto a Waters Oasis HLB column that had been pre-conditioned with methanol and deionized water. Phosphate buffer (0.5 *M*) was used for washing the column instead of water to improve sample clean-up. A pH range of 7.4 to 9.5 was tested to optimize the recovery of ACV. A buffered solution of 0.5 *M* phosphate at pH 7.4 yielded the best washing conditions with the best recovery of the analyte. The elution buffer of 15% acetonitrile containing 0.9% NaCl was chosen because it provided the best ratio of analyte recovery to plasma interference while providing optimal conditions for sample stacking during HPCE separation (see below).

3.2. HPCE conditions

3.2.1. Method development

Initial separations of ACV in the plasma matrix were investigated over a range of pH from 8 to 10. Acyclovir, with a pK_a in the purine ring of ~9.2, would have the greatest charge switching in this pH range and hence its mobility would be affected the most. Results indicated that ACV mobility was affected little over this range; however, the presence of plasma interference peaks produced a large effect. At certain pH values, the interference overlapped with the ACV signal and the best resolution and peak shape and width was in the range pH 8.6–9.2. Borate, with a natural pK_a around 9.2, was thus the buffer of choice for this condition.

The addition of HPβCD as a separation buffer additive can improve the stacking of nucleosides to allow for greater sample concentration during electrophoresis. By including HPβCD (100 m*M*) in the separation buffer for the analysis of ACV, excellent stacking was observed following a 100 s injection (Fig. 1). While the inclusion of HPβCD in the buffer produced high theoretical plates, separation of ACV from endogenous plasma interference was poor. By including SDS in the presence of HPβCD, ACV could be separated from plasma interference and high resolution could be achieved. However, the presence of SDS reduced the stacking efficiency of HPβCD slightly, as demonstrated in Fig. 2. Increasing the SDS concentration led to a reduced theoretical plate for ACV, suggesting competition between the interaction between ACV with HPβCD and SDS micelle complexes. As a result, the best compromise between SDS and HPβCD leading to a good separation efficiency (plates) and also good resolution from plasma interference was determined to be 175 m*M* SDS and 100 m*M* HPβCD.

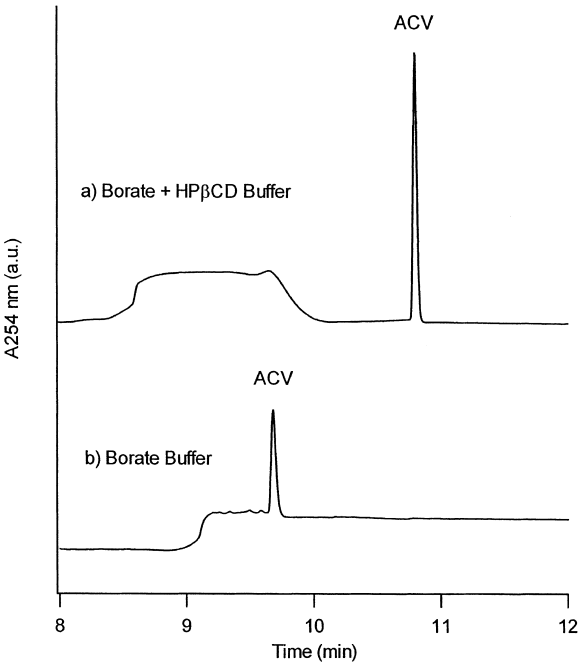

Fig. 1. Effect of HPβCD in the separation buffer. A standard solution of ACV (3.0 μg/ml) in 15% ACN containing 0.9% NaCl was analysed using the capillary conditions described in the Experimental section and separated using (a) 90 m*M* borate pH 8.6 containing 100 m*M* HPβCD and (b) 90 m*M* borate pH 8.6.

3.2.2. Effect of sample matrix on sample stacking

Traditionally, the sensitivity of the CE method using UV–Vis detection suffers because of the inherently narrow path length of the capillary bore. Extensive research has been carried out in this field to try to overcome this technical issue [19]. In

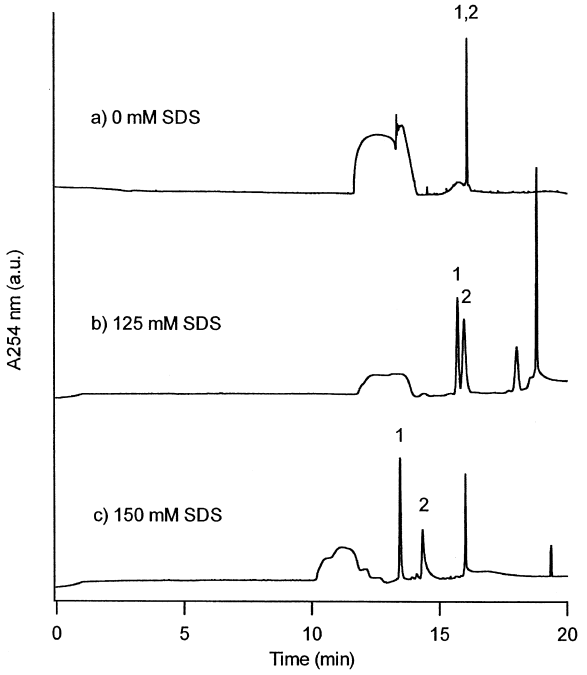

Fig. 2. Effect of SDS in the separation buffer. Plasma samples spiked with ACV (1.0 μg/ml) were prepared and injected as described in the Experimental section. Samples were separated using a 60 m*M* borate buffer pH 8.6 containing 100 m*M* HPβCD and (a) 0 m*M* SDS, (b) 125 m*M* SDS, or (c) 150 m*M* SDS. Peaks: 1=ACV, 2=unidentified plasma signal.

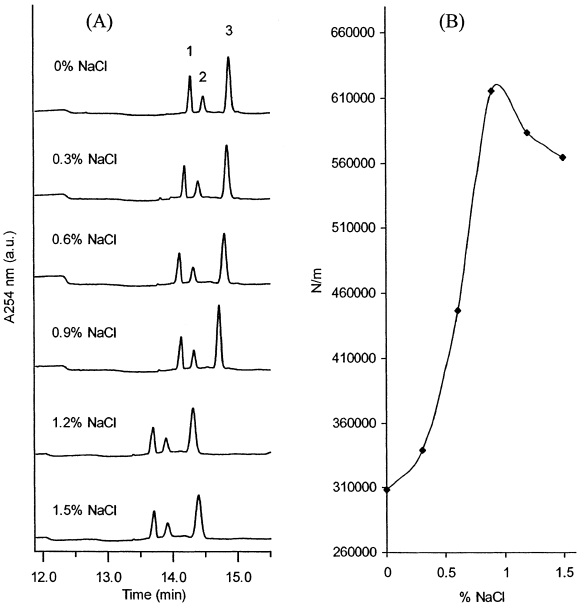

Fig. 3. Effect of salt in the sample on stacking. Plasma samples spiked with ACV and I.S. (1.0 μg/ml of each) were prepared by SPE as indicated in the Experimental section and eluted with 15% acetonitrile containing various concentrations of NaCl. The samples were analysed as described in the Experimental section. Peaks: 1=internal standard, 2=unidentified plasma signal, 3=ACV. (A) Electropherograms of the salt titration, and (B) the corresponding theoretical plates of ACV with respect to the salt titration.

general, these techniques are designed to compress, ''stack'', a large volume of the sample solution on-line, effectively increasing the sample concentration band as it passes through the detection window. One of the better understood techniques is where the sample matrix is lower conducting than the BGE (a discontinuous buffer system); thus, a mobility difference is artificially induced as the analyte crosses the sample–BGE zones [20]. The greater the mobility difference, the greater the stacking effect. The use of acetonitrile in the sample matrix in MECC has previously been reported [21–23]. Sample stacking was improved with the addition of both NaCl and ACN to a sample prepared as described for the SPE process. For the ACV assay, the presence of 15% ACN in the sample and increasing concentrations of NaCl improved stacking up to an optimal concentration of 0.9% NaCl (Fig. 3). Using this specific matrix, a large volume of 100 s of plasma sample could be introduced for on-line sample stacking, achieving a high theoretical plate of 615 916 N/m (Fig. 4).

3.3. Method validation

3.3.1. Selectivity

The analytical peaks of ACV and HEdU were well resolved from one another and in plasma pooled from 20 people. There was a clear separation between ACV and the nearest plasma peak (resolution >2).

3.3.2. Sensitivity

The limit of quantitation (LOQ) was defined as the lowest drug concentration which can be determined with an absolute relative error (RE) and relative standard deviation (RSD) of <20%. The lowest concentration that was tested that met this criterion was 20 ng/ml with a RE of 15.6% and a

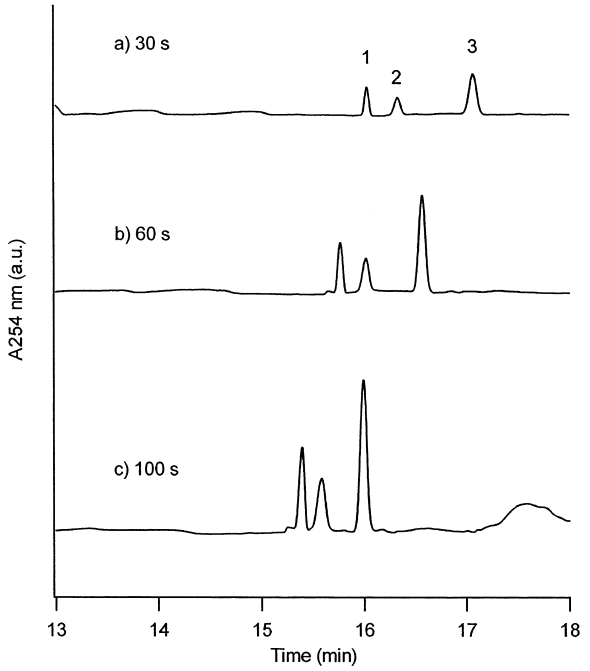

Fig. 4. Effect of injection time. Plasma samples spiked with ACV and I.S. (1.0 μg/ml of each) were prepared by SPE and analysed as indicated in the Experimental section using an injection time of (a) 30 s, (b) 60 s, or (c) 100 s. Peaks: 1=internal standard, 2=unidentified plasma peak, 3=ACV.

RSD of 10.4% (n=6). The limit of detection (LOD) was defined as the concentration giving rise to a signal three times the standard deviation of the blank, and was calculated to be 5.5 ng/ml.

3.3.3. Recovery

Recovery was determined by comparing the analysis of extracted samples with standard samples prepared in the SPE elution buffer (15% ACN containing 0.9% NaCl) at concentrations of 20, 1000, and 10 000 ng/ml (n=6). The average ACV recovery over the validation range was 102.9±14.2%.

3.3.4. Linearity and range

Linearity was determined by plotting a standard curve from the ratio between the corrected ACV peak area to the corrected HEdU peak area versus the corresponding ACV concentration in plasma. Three different standard curves were obtained on three different days and all were found to be linear over the range 20–10 000 ng/ml by linear regression analysis (R^2>0.999). Furthermore, the back-calculated mean values of the validation samples within this range were found to differ by less than 16% from the nominal values and the RSDs of the validation samples were all less than 20%.

3.3.5. Accuracy

Accuracy of the assay was defined as a percentage of the systemic error, which was calculated as the agreement between the measured value of the validation samples and the true value. The average accuracy over the concentration range studied has a relative error (RE) of 7.4%; with the highest RE in the lowest concentration (LOQ) of 15.6%. Accuracy values were determined over two days and were always within acceptable limits (<20%) at all validation concentrations (see Table 1).

3.3.6. Precision

Precision of the method was tested for both the intra-day and inter-day reproducibility of the assay. Intra-day variability of the assay was determined by repeated analysis of five validation samples on the same day (Table 2). Similarly, the inter-day variability was determined by repeated analysis of five validation samples on different days. The average intra-day precision had a relative standard deviation of 6.9% over the concentration range studied; the inter-day precision RSD value was 5.9%. The results summarized in Table 2 indicate that the assay was reproducible within the same day as well as between different days.

3.3.7. Robustness

The assay was run with the separation buffer adjusted to pH 8.8±0.4 with no significant effect on the performance parameters.

Table 1
Accuracy of the method, expressed as relative error in percent, for determining acyclovir concentrations

Accuracy (n=6)		
Conc. added (ng/ml)	Conc. found (ng/ml) (mean±SD)	Relative error (%)
20	23.1±2.4	15.6
100	107.3±11.6	7.3
1000	1089.3±28.4	8.9
5000	5176.8±114.1	3.5
10 000	10 160.6±352.5	1.6

Table 2
Intra-day and inter-day variability of the method for determining acyclovir concentrations

Conc. added (ng/ml)	Conc. found (ng/ml) (mean±SD)	RSD (%)
Intra-day (n=3)		
20	23.4±2.7	11.6
100	101.3±15.0	14.8
1000	1070.8±26.2	2.4
5000	5102.9±111.9	2.2
10 000	10 148.8±346.4	3.4
Inter-day (n=6)		
20	23.1±2.4	10.4
100	107.3±11.6	10.8
1000	1089.3±28.4	2.6
5000	5176.8±114.1	2.2
10 000	10 160.6±352.5	3.5

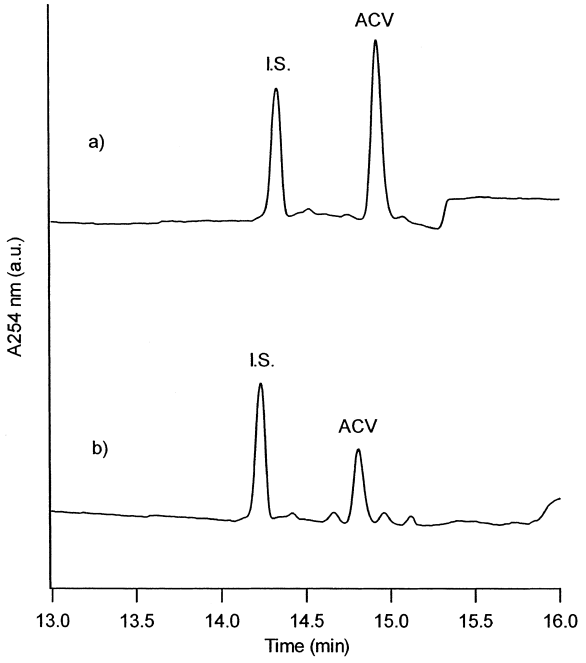

Fig. 5. Analysis of ACV in clinical samples. Plasma samples from (a) maternal plasma and (b) venous cord plasma of a newborn were collected following birth and prepared and analysed according to the method described in the Experimental section. The ACV levels in the maternal blood and in the child's venous cord blood were measured to be 0.72 and 0.29 μg/ml, respectively.

3.3.8. Clinical application

The validated assay was used to measure the ACV level in venous cord blood from a newborn child, as well as in maternal blood. The results are shown in Fig. 5.

4. Conclusion

The described method has been validated and shown to be a robust and sensitive bioassay. According to the literature, the highest LOQ for ACV measurement using CE is ~0.5 mg/ml [14,15]. By optimizing the SPE and the separation buffer using the principle of stacking, a LOQ of 20 ng/ml is achieved and thus this validated assay is more sensitive than previously reported methods. The combination of MECC and cyclodextrin buffer system is robust, sensitive, and readily adaptable to the bioanalysis of other nucleoside analogs. Acyclovir is one of the main drugs for prophylactic use and the treatment of herpes simplex virus and, in certain cases, human cytomegalovirus (CMV). Therapeutic drug monitoring of ACV levels in the clinic is an essential diagnostic tool for the effective management of these clinical conditions.

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