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## Determination of ribavirin in human serum and plasma by capillary electrophoresis

The electrophoretic separation of ribavirin and 5-methylcytidine (internal standard) by capillary electrophoresis was examined. Separation was achieved using reverse polarity in a 100 mM borate electrolyte, pH 9.1, with 5 mM spermine added to reduce the electroosmotic flow. Sample preparation based on acetonitrile protein precipitation was found to be unsuitable for ribavirin analysis in patient samples due to insufficient sensitivity and interferences. Solid-phase extraction employing phenyl boronic acid cartridges provided cleaner separations. Using this approach with 500  $\mu$ L sample and reconstitution of the dried extract into 100  $\mu$ L of 33% v/v 100 mM phosphate buffer, pH 6.4 / 67% v/v acetonitrile, the detection and quantitation limits were determined to be 0.05 and 0.10  $\mu$ g/mL, respectively, a sensitivity that is suitable for therapeutic drug monitoring of ribavirin in human plasma and serum samples. The method was validated and compared to a high-performance liquid chromatography (HPLC) method, showing excellent agreement between the two for a set of samples that stemmed from patients being treated with ribavirin and interferon- $\alpha$ -2b for a hepatitis C virus infection.

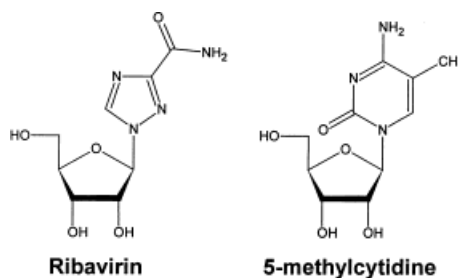
**Keywords:** Capillary electrophoresis / High-performance liquid chromatography / Ribavirin / Therapeutic drug monitoring  
DOI 10.1002/elps.200305819

### 1 Introduction

Ribavirin (1- $\beta$ -D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide, Fig. 1) is a purine nucleoside analogue with broad spectrum activity against both DNA and RNA viruses. Ribavirin is a hydrophilic molecule that does not bind to plasma proteins. Until recently its main clinical use was in the treatment of respiratory syncytial viral infections, but it has since been found to be effective when used in combination with interferon- $\alpha$ -2b for the treatment of chronic hepatitis C. In several clinical trials, combination therapy of interferon plus ribavirin significantly enhanced end-of-treatment and sustained virological and biochemical response rates in treatment-naïve and treatment-experienced patients [1]. Pharmacokinetic studies of ribavirin in healthy individuals and in patients with [2], and without [3] hepatitis C virus infection showed significant interindividual variation and it is estimated that dosage adjustment is required in ~26% of patients [1].

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**Abbreviations:** IS, internal standard; PBA, phenyl boronic acid; TDM, therapeutic drug monitoring



**Figure 1.** Chemical structures of ribavirin and the IS, 5-methylcytidine.

Several analytical methods have been developed for the analysis of ribavirin in biological fluids. These are based on either a radioimmunoassay [4, 5], or high-performance liquid chromatography (HPLC) with UV absorbance [6–11] or mass spectrometry (MS) [12, 13] detection. The latter approach offers adequate sensitivity and specificity for sample analysis with only protein precipitation for sample pretreatment, however the cost and expertise of employing HPLC-MS is a considerable disadvantage for many therapeutic drug monitoring (TDM) laboratories. While the use of UV detection is simpler and cheaper to operate, it requires the use of solid-phase extraction (SPE) to remove interferences and to reach suitable detection levels. This is achieved by using a highly specific phenyl boronic acid (PBA) extraction column that exclusively

extracts poly-ol species *via* interaction with surface-bound borate groups [2, 9, 10]. Due to the extremely polar nature of ribavirin, analytical separations are performed on a C<sub>18</sub> column in aqueous or near aqueous conditions (< 5% organic solvent), which can be detrimental to many analytical columns, while strongly retained analytes are removed by cleaning overnight with a mobile phase containing a high amount of organic solvent [2, 9].

A popular alternative to the use of HPLC is capillary electrophoresis (CE), in which analytes are separated on the basis of their charge-to-size ratio. This different separation mechanism provides a distinctly different selectivity to HPLC, often readily separating components that are difficult to separate by HPLC. When combined with the higher separation efficiency, CE analyses are often quicker than those by HPLC and in some cases can minimize [14] or even eliminate sample pretreatment [15, 16]. As such, CE has been used extensively to separate drugs and their metabolites [17] and is an alternative methodology for TDM.

The aim of this work was to investigate the possibility of using CE for TDM of ribavirin in human serum and plasma, particularly with regard to minimizing sample preparation and enhancing analytical separation. A fully validated CE method is presented, employing SPE of ribavirin and UV-absorbance detection. Patient data obtained with the CE assay are compared to those from a corresponding HPLC method.

## 2 Materials and methods

### 2.1 Drugs, chemicals, and origin of samples

Ribavirin and 5-methylcytidine (internal standard (IS), see Fig. 1 for structure) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Sodium tetraborate (borax) was obtained from Hanseler (Herisau, Switzerland) and H<sub>3</sub>PO<sub>4</sub>, HCl and NaOH were from Merck (Darmstadt, Germany). Acetonitrile (ACN) was purchased from Biosolve (Walkenswaard, The Netherlands) and methanol, ammonium acetate and concentrated ammonia were from Merck. Spermine and diethylamine were obtained from Fluka (Buchs, Switzerland). Bond Elut PBA cartridges were obtained from Varian (Middelburg, The Netherlands). Patient plasma and serum samples stemmed from individuals in combination treatment of hepatitis C with ribavirin and pegylated interferon- $\alpha$ -2b and were from the departmental drug assay laboratory where they were received for TDM of ribavirin by HPLC. Blank bovine plasma was obtained from the local slaughter house and was used for the preparation of calibration and control samples.

### 2.2 Solutions

Stock solutions of 1000  $\mu$ g/mL of ribavirin and 5-methylcytidine were prepared in H<sub>2</sub>O, and diluted as required. A stock solution of borate buffer was prepared from 100 mM borax and titrated to pH 9.1 with H<sub>3</sub>PO<sub>4</sub>. CE running buffer was prepared daily by dissolving 3 or 5 mM spermine in the borate buffer. For investigation of different pH, pH adjustment was performed after addition of spermine with either H<sub>3</sub>PO<sub>4</sub> or NaOH.

### 2.3 Capillary electrophoresis

All CE experiments were performed on a Beckman 5510 CE system (Beckman, Fullerton, CA, USA) with a Beckman P/ACE station Version 1.1 used for data acquisition and using fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50  $\mu$ m ID with a length of 47.0 cm (40.0 cm to the detector) and a voltage of -20 kV (current of about 73–78  $\mu$ A for the pH 9.1 buffer). Sample was injected for 60 s at low pressure (about 4 cm sample plug length) followed by a 10 s injection of running buffer. Detection was performed using UV absorbance at 214 nm with the capillary temperature set at 25°C. Capillaries were conditioned daily with 1 M NaOH for 20 min, followed by 1 M HCl for a further 20 min. Between separations, the capillary was rinsed with 0.1 M HCl for 1 min, and electrolyte for 2 min.

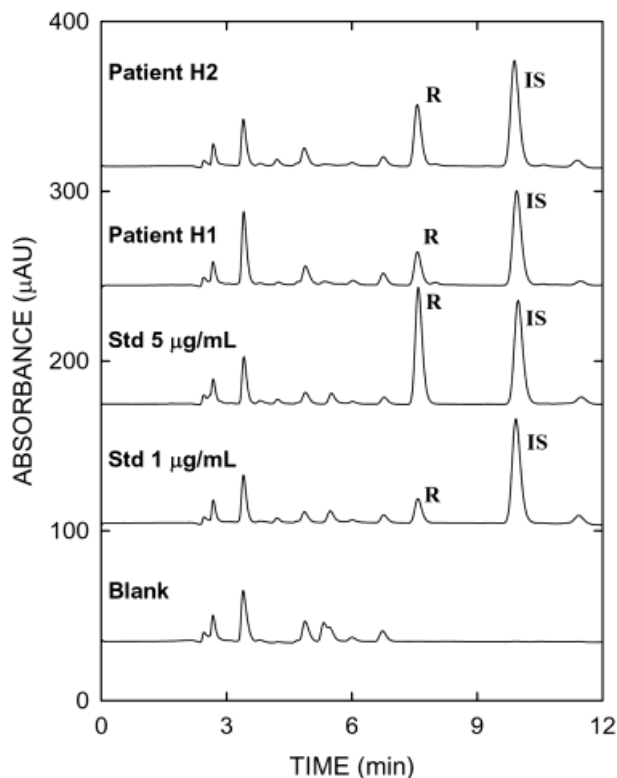
### 2.4 Sample pretreatment and quantification

For the initial work with pretreatment by protein precipitation, 100  $\mu$ L of bovine plasma was mixed with 200  $\mu$ L of ACN, vortexed for 15 s and centrifuged for 5 min at 10 000  $\times$  g. The supernatant was either injected as is or evaporated under a gentle air flow at 45°C and reconstituted in 67% v/v ACN / 33% v/v 100 mM phosphate buffer, pH 6.4 to give the desired concentration factor. For application to calibrator and patient samples, 100  $\mu$ L of sample (blank and spiked bovine plasma and patient samples) was mixed with 20  $\mu$ L of 25  $\mu$ g/mL 5-methylcytidine and 240  $\mu$ L of ACN, vortexed for 15 s and centrifuged for 5 min at 10 000  $\times$  g. The supernatant was evaporated under a gentle air flow at 45°C and reconstituted in 100  $\mu$ L of 67% v/v ACN / 33% v/v 100 mM phosphate buffer, pH 6.4. to give a 1  $\times$  concentration. For SPE, PBA cartridges were conditioned with 1 mL methanol containing 0.5% H<sub>3</sub>PO<sub>4</sub> (pH 2) and twice with 1 mL of 250 mM ammonium acetate buffer that was adjusted with concentrated ammonia to pH 8.5. An aliquot of 500  $\mu$ L of sample (blank and spiked bovine plasma and patient samples) was mixed with 20  $\mu$ L of 25  $\mu$ g/mL 5-methylcytidine and 500  $\mu$ L of 250 mM ammonium acetate, pH 8.5. After sam-

ple loading, the column was washed with 1000  $\mu\text{L}$  of ammonium acetate buffer followed by 1000  $\mu\text{L}$  of methanol before the column was air-dried. Elution was performed with 1000  $\mu\text{L}$  of 3% v/v formic acid in methanol, evaporated under a gentle air flow and reconstituted in 100  $\mu\text{L}$  of 67% v/v ACN / 33% v/v 100 mM phosphate buffer, pH 6.4, to give a 5  $\times$  concentration. For quantitation of ribavirin in serum and plasma extracts, calibrators containing 0, 0.1, 0.5, 1.0, 2.5, and 5.0  $\mu\text{g}/\text{mL}$  were prepared by dilution of the standard drug solution in blank bovine plasma, and stored at  $-20^\circ\text{C}$  until required. Control samples containing 0.6 and 3.5  $\mu\text{g}/\text{mL}$  ribavirin were prepared in the same way. Quantitation of ribavirin was based upon multilevel internal calibration using relative peak areas, *i.e.*, peak areas divided by detection time.

## 2.5 HPLC assay

An HPLC method similar to that described by Larrat *et al.* [2] was employed. The system consisted of a Waters LC-Module I plus autosampler, absorbance detector and pump (Waters, Milford, MA, USA). Chromatography was performed at ambient temperature using a  $4.6 \times 250$  mm Atlantis dC18 5  $\mu\text{m}$  column (Waters) and an acidic, aqueous mobile phase comprising 1 mL of diethylamine per liter whose pH was adjusted with  $\text{H}_3\text{PO}_4$  (49%) to pH 2.5. The flow rate was 1.2 mL/min, the total run time was 15 min and detection occurred at 215 nm. Chromatograms were registered and evaluated with an HP ChemStation Rev. A.06.01 (Agilent Technologies, Basel, Switzerland). The assay is based upon SPE of ribavirin using PBA cartridges (Varian). Sample cleanup was achieved as described above with the following modifications: 250  $\mu\text{L}$  of sample and 25  $\mu\text{L}$  of a 50  $\mu\text{g}/\text{mL}$  5-methylcytidine (IS) solution were used and analyte elution was achieved using 1 mL methanol with 0.5%  $\text{H}_3\text{PO}_4$ . The extract was evaporated at  $40^\circ\text{C}$  under a gentle stream of air, reconstituted in 200  $\mu\text{L}$  water and 50  $\mu\text{L}$  was injected. The recovery of ribavirin was assessed with fortified bovine plasma samples ( $n = 3$ ) and determined to be 79.1% and 88.0% for ribavirin concentrations of 0.5  $\mu\text{g}/\text{mL}$  and 5.0  $\mu\text{g}/\text{mL}$ , respectively. Correspondingly, the recovery of 5  $\mu\text{g}/\text{mL}$  of 5-methylcytidine was found to be 88.2%. Typical chromatograms obtained with calibrators and patient samples are presented in Fig. 2. Calibration and control samples were prepared with fortified bovine plasma. Quantitation of ribavirin was based upon five-level internal calibration using peak areas (calibration range: 0.1–5  $\mu\text{g}/\text{mL}$ ). Linear calibration graphs (peak area ratio vs. concentration) were obtained with mean (SD) values ( $n = 5$ ) of the slopes,  $y$ -intercepts and regression coefficients being 0.18880 (0.01318) (unit:  $(\mu\text{g}/\text{mL})^{-1}$ ), 0.00947 (0.00705) and 0.99878 (0.00075), respectively.



**Figure 2.** Representative HPLC separations obtained (from bottom to top) with blank bovine plasma, plasma fortified with 1 and 5  $\mu\text{g}/\text{mL}$  ribavirin, and two patient sera containing 1.61 and 2.68  $\mu\text{g}/\text{mL}$  ribavirin. Except for the bottom graph which was obtained without IS, the concentration of the IS is 5  $\mu\text{g}/\text{mL}$  in all cases. Other conditions as described in Section 2.5. R refers to ribavirin.

Intraday precision RSD values ( $n = 5$ ) assessed for drug levels of 0.6 and 4.0  $\mu\text{g}/\text{mL}$  were determined to be 3.38% and 4.53%, respectively. Corresponding interday RSD values ( $n = 5$ ) were 5.55% and 4.61%, respectively. After each set of data, the column is rinsed with an equi-volume mixture of water and ACN at a flow rate of 0.7 mL/min for 2 h.

## 3 Results and discussion

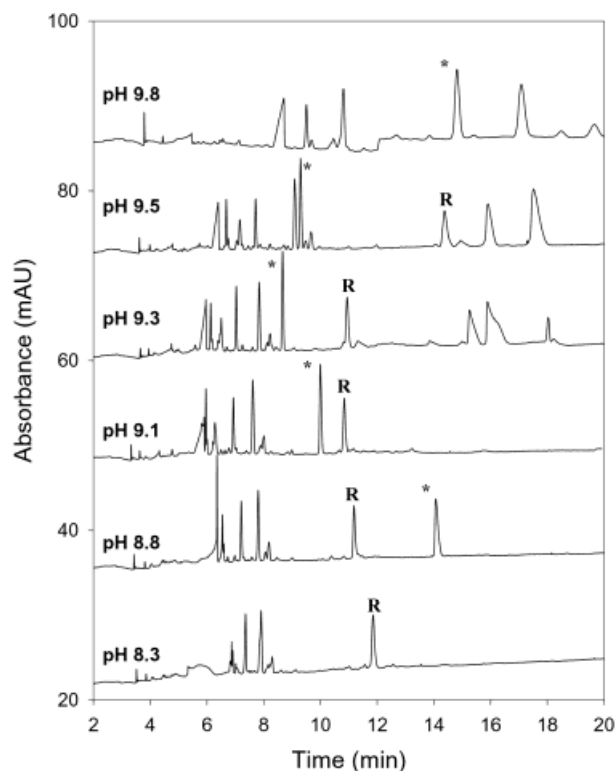
### 3.1 Method development and optimization

Ribavirin is neutral over the majority of the pH range, thus to separate it by CE, it is necessary to impart some charge to the compound. It is well known from the separation of carbohydrates by CE, that borate is able to form complexes with compounds containing two or more hydroxy groups within close proximity to form negatively charged complexes [18]. Preliminary experiments using a 100 mM borate buffer, pH 9.1, showed that ribavirin could be read-

ily separated from the IS and the electroosmotic flow (EOF). However, poor efficiency ( $\sim 30\,000$  plates) and resolution were observed due to the analytes migrating after the EOF. In order to enhance efficiency and resolution, the EOF was suppressed by adding 5 mM diaminobutane and the polarity reversed, however, migration times were exceptionally long (30 min) due to incomplete suppression of the EOF. Replacement of diaminobutane with 3 or 5 mM spermine proved more successful and provided adequate separation of ribavirin from IS within 12 min, with efficiencies of over 100 000 plates. The quality of the separation was strongly dependent on the preconditioning procedure, with a between-separation rinse for 1 min with 0.1 M HCl required to maintain the separation time and efficiency. With the satisfactory performance of this electrolyte system, focus shifted to whether simple sample pretreatment methods could be exploited.

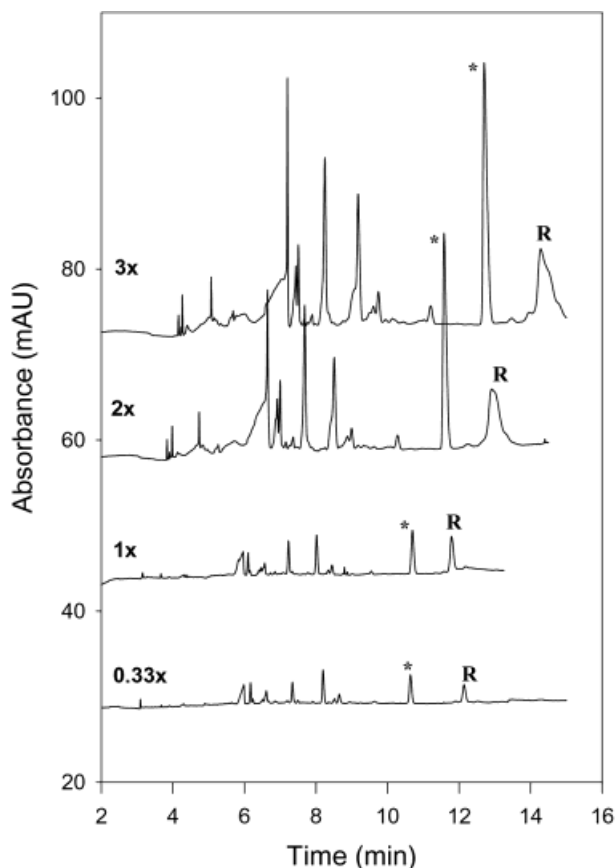
### 3.1.1 Acetonitrile protein precipitation

As discussed previously, the enhanced resolving power of CE when compared to HPLC may enable quicker and cheaper sample preparation procedures. Typically, laborious and expensive SPE steps are replaced with simpler liquid-liquid extraction or protein precipitation procedures. In the case of ribavirin, its highly hydrophilic nature ensures that liquid-liquid extraction is not a viable option, thus sample pretreatment with protein precipitation is the only alternative to SPE. The most common protein precipitation approach used in CE is by using ACN, which can also be used to enhance the sensitivity by improving stacking efficiency [14]. However, the lack of specificity of ACN protein precipitation ensures a large number of endogenous compounds will be present and may potentially interfere with the analysis. To examine this, bovine plasma was spiked with 10  $\mu\text{g}/\text{mL}$  ribavirin, treated by ACN protein precipitation (1:2 plasma:ACN) and separated in a phosphate-borate electrolyte with pH between 8.3 and 9.8. It can be seen from Fig. 3 that variation of the pH has two main influences on the separation. Firstly, the separation selectivity changes significantly, with several peaks changing position relative to ribavirin, the most notable indicated with an asterisk. And secondly, as the pH is increased to 9.8, the magnitude of the EOF increases due to an increase in the number of dissociated silanol groups and the inability of spermine to adequately neutralise these, thus elongating the separation time resulting in no ribavirin peak migrating before 20 min. Concentrations of spermine were increased up to 20 mM, but failed to provide any significant improvement. The optimum pH was judged to be 9.1 due to the separation time and best resolution between ribavirin and other endogenous compounds.



**Figure 3.** Influence of pH on the separation of blank bovine plasma spiked with ribavirin and treated by ACN protein precipitation. Electrolyte: 100 mM borate buffer, 3 mM spermine, pH adjusted to 8.3 with  $\text{H}_3\text{PO}_4$  and then stepwise to 9.8 with NaOH. Conditions: 47.0 cm  $\times$  50  $\mu\text{m}$  ID (40.0 cm to the detector),  $-20$  kV,  $25^\circ\text{C}$ , 214 nm. Sample: 100  $\mu\text{L}$  of blank bovine plasma spiked with 10  $\mu\text{g}/\text{mL}$  ribavirin, treated by protein precipitation with 200  $\mu\text{L}$  of ACN. Injection: 60 s low pressure + 10 s injection of buffer. R refers to ribavirin and the asterisk indicates an unknown component of the blank bovine plasma.

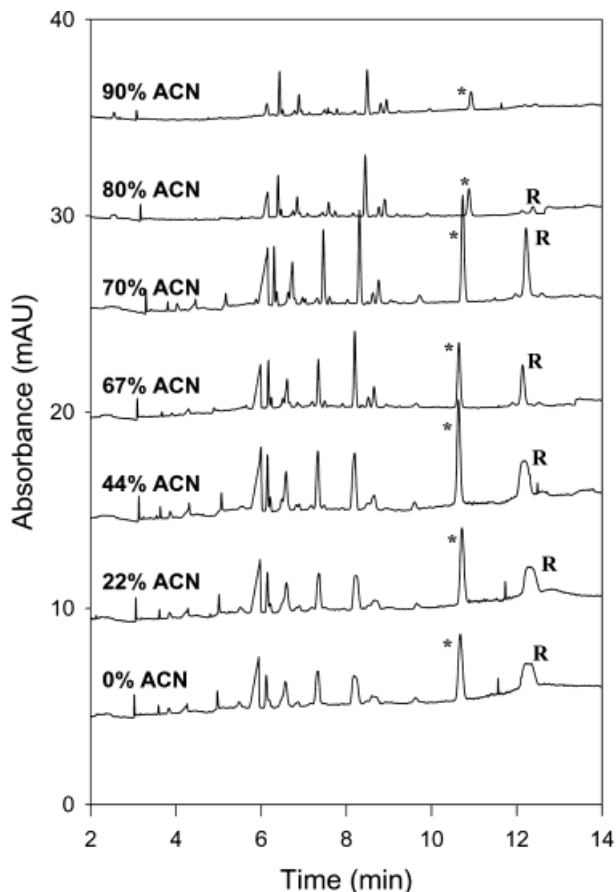
While the separation selectivity was adequate in the above electrolyte, the sensitivity was unsatisfactory. Ribavirin is routinely analyzed between 0.1 and 5.0  $\mu\text{g}/\text{mL}$  [2], and thus the LOD of 0.66  $\mu\text{g}/\text{mL}$  of the above procedure was inadequate for TDM. Alternatively, the use of a bubble-cell capillary would possibly provide sufficient sensitivity. To enhance the sensitivity with the plain capillary, supernatant from the protein precipitation was evaporated and reconstituted in 67% ACN/33% 100 mM phosphate buffer, pH 6.4 to obtain a 1 $\times$ , 2 $\times$  and 3 $\times$  concentration effect, with 1 $\times$  corresponding to reconstitution in the same volume as the original sample, while 0.33 $\times$  is the equivalent concentration of the supernatant without evaporation. As can be seen from the separations in Fig. 4, a 1 $\times$  concentration can be used without any significant loss in resolution or efficiency. However, higher concentration factors produce



**Figure 4.** Preconcentration factor obtained during reconstitution of spiked blank bovine plasma after protein precipitation. The solution used for reconstitution contained 67% v/v ACN / 33% v/v 100 mM phosphate buffer, pH 6.4. The pH of the running buffer was 9.1, with all other conditions as in Fig. 3.

poor peak shapes for ribavirin, which we speculate is due to the high concentration of salt disrupting the stacking mechanism.

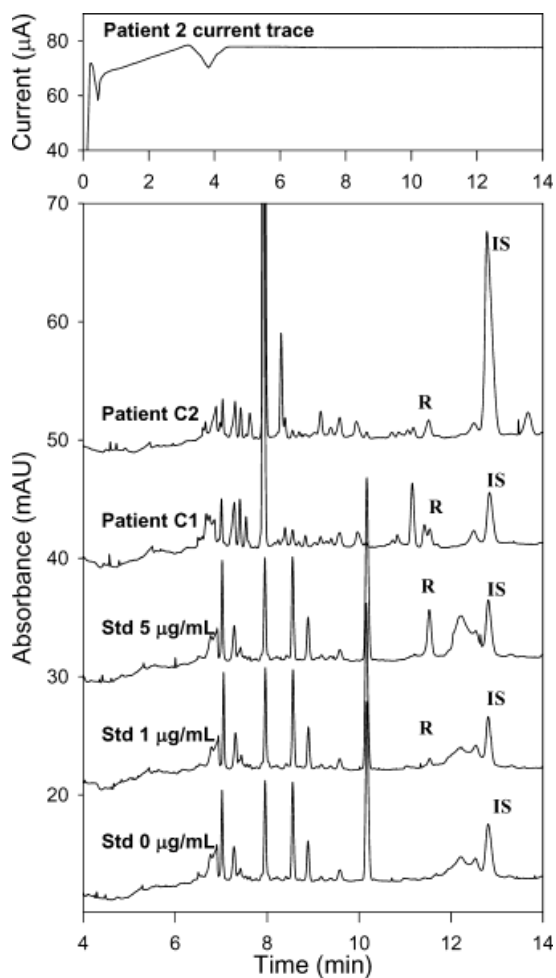
Further attempts to enhance the sensitivity by varying the amount of ACN used to reconstitute the evaporated protein precipitation supernatant are shown in Fig. 5. Below 67% ACN, the peak shape for ribavirin was poor, while above 70% the sensitivity was reduced due to limited solubility of ribavirin in organic solvents. The best sensitivity was found with 70% ACN, however, 67% was preferred due to intermittent current errors with the higher ACN concentration arising from overheating the sample zone. The slow adjustment of the current in the initial phase of the experiment is depicted with the current data presented in the top panel of Fig. 6. Together when combined with a 1× concentration after protein precipitation, the LOD ( $S/N = 3$ ) was calculated to be  $0.21 \pm 0.04$  ( $n = 3$ ), and covers most of the range required for TDM of riba-



**Figure 5.** Influence on the amount of ACN used to reconstitute after protein precipitation of spiked blank bovine plasma with a preconcentration factor of 1×. For the cases with 0–67% v/v ACN, the amount of phosphate in the sample was kept equal to that employed in Fig. 3 (33% v/v 10 mM phosphate buffer of pH 6.4 and appropriate amount of water), whereas for the cases with 70, 80, and 90% v/v ACN, the amounts of phosphate buffer were 30, 20 and 10%, respectively. The pH of the running buffer was 9.1, with all other conditions as in Fig. 4.

virin. Furthermore, an increase of the spermine concentration to 5 mM resulted in a better reproducibility of detection times.

The above method was applied to two samples from patients currently prescribed ribavirin. The separations, as well those of three standards prepared with bovine plasma, are shown in Fig. 6, where it can be seen quite clearly that sample pretreatment by ACN protein precipitation is unsuitable for TDM of ribavirin due to interferences with either ribavirin (patient C1) or the IS (patient C2). Given the high number of endogenous compounds, the insufficient sensitivity and that ribavirin patients are commonly prescribed additional medication, re-optimization of the electrolyte conditions to enhance



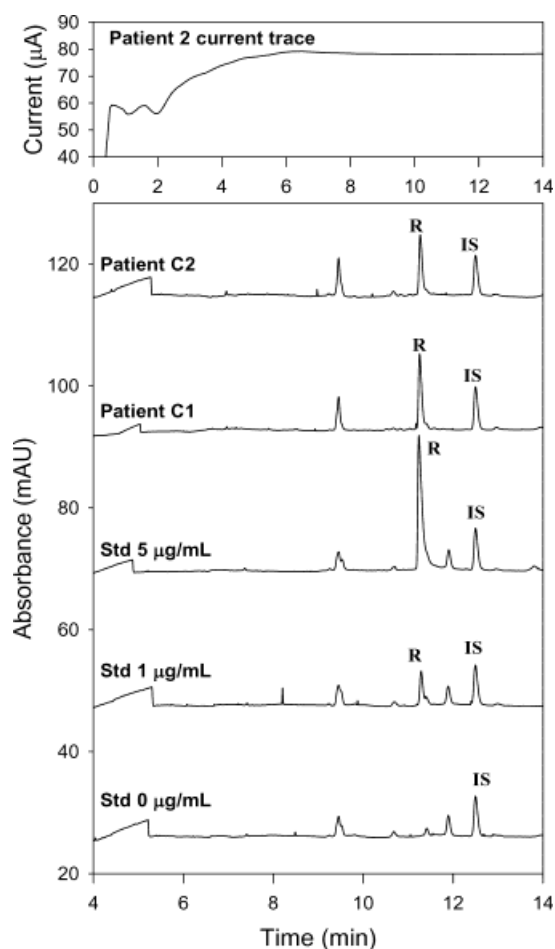
**Figure 6.** Separation of blank bovine plasma, spiked blank bovine plasma, and patient samples obtained after sample treatment with protein precipitation. The top panel shows the full current trace from the analysis of the sample from patient C2. The pH of the running buffer was 9.1 and the spermine concentration was 5 mM. Sample pre-treatment with inclusion of the IS (preconcentration factor of  $1 \times$ ) is as described in Section 2.4 and all other conditions are as in Fig. 3. Patient C1 contains 1.88 µg/mL and patient C2 contains 2.12 µg/mL ribavirin as determined by HPLC.

the selectivity from these interferences was deemed to have low probability of success and further work with ACN protein precipitation was thus not executed.

### 3.1.2 Phenyl boronic acid SPE

The problems of low selectivity and sensitivity of the ACN protein precipitation approach can be addressed using SPE. As previously mentioned, ribavirin can be extracted using a PBA phase, which should almost exclusively

extract ribavirin and the IS, and enable high preconcentration factors to be obtained. To examine this approach, the same patient samples and standards used to examine the protein precipitation approach were treated with SPE. The separations are shown in Fig. 7, where it can be seen that there are far fewer components after treatment by SPE than with ACN protein precipitation. The lower amount of ionic species and thus conductivity of the sample zone is also reflected in the temporal behavior of the current. The time interval required to reach a steady current level is far longer compared to the case with ACN protein precipitation (compare top panels of Figs. 6 and 7). The region surrounding ribavirin and the IS provides few interferences, in particular, the interferences present in the patient samples treated by ACN protein



**Figure 7.** Electropherograms of blank bovine plasma, spiked blank bovine plasma and patient samples after SPE using PBA cartridges as described in Section 2.4. The top panel shows the full current trace from the analysis of the sample from patient C2. Samples are reconstituted in 33% v/v H<sub>2</sub>O / 67% v/v ACN to give a  $5 \times$  preconcentration, with all other conditions as in Fig. 6. Patient C1 contains 1.88 µg/mL and patient C2 contains 2.12 µg/mL ribavirin as determined by HPLC.

precipitation are removed. Furthermore, it provides better sensitivity than with protein precipitation, with a LOD (S/N = 3) of  $0.05 \pm 0.02 \mu\text{g/mL}$  ( $n = 3$ ) and a LOQ (S/N = 10) of  $0.10 \pm 0.02 \mu\text{g/mL}$  ( $n = 3$ ) thus providing suitable sensitivity for routine TDM. It should be noted, however, that a small interference migrating slightly after ribavirin is observed in both the bovine plasma standards and in the patient samples. Separation was poor at ribavirin concentrations above  $1 \mu\text{g/mL}$ , but was almost baseline-resolved at the lowest standard concentration (data not shown). Attempts to enhance resolution by adjusting the pH, borate concentration, electric field strength and capillary length, provided no improvement. The significance of this interfering peak on the quantification of ribavirin in human serum and plasma is discussed below.

It is important to note that the composition of both, the elution and the reconstitution media, were critical to avoid current errors. Using phosphoric acid (as in HPLC) or HCl in methanol for elution provided extracts that could not be analyzed after reconstitution with 67% ACN / 33% water or pH 6.4 phosphate buffer. Furthermore, the extracts prepared with formic acid had to be reconstituted in 67% v/v ACN / 33% v/v 100 mM phosphate buffer, pH 6.4, in order to avoid any problems during analysis.

### 3.2 Determination of ribavirin in serum and plasma

#### 3.2.1 Calibration data, detection limits, repeatability, and accuracy

Analysis of blank bovine plasma fortified with 0.1–5  $\mu\text{g/mL}$  ribavirin (see Section 2.4) provided linear calibrations graphs with  $r^2$  values between 0.9964 and 0.9999 (mean: 0.9987,  $n = 6$ ). Mean values (RSD) for slopes and  $y$ -intercepts were determined to be 0.736 (6.86%,  $n = 6$ ) and  $-0.015$  (340%,  $n = 6$ ), respectively. Analysis of blank plasma spiked with 0.1  $\mu\text{g/mL}$  of ribavirin established the detection limit (S/N = 3) as  $0.05 \mu\text{g/mL}$ . In this work, we used 500  $\mu\text{L}$  of plasma for each extraction and reconstituted in a final volume of 100  $\mu\text{L}$  of 67% v/v ACN / 33% v/v 100 mM phosphate buffer, pH 6.4. The running buffer pH was 9.1 and the spermine concentration 5 mM.

The repeatability of the CE method was evaluated by analyzing a series of bovine plasma samples spiked with ribavirin prepared independently to that of the calibration graph. Intraday ( $n = 6$ ) and interday ( $n = 6$ ) variation of migration times was less than 3% RSD for both ribavirin and IS, while migration times normalized to those of the IS varied less than 1%. Relative peak heights and areas, *i.e.*, peak heights and areas divided by the detection time, were less than 3% RSD for samples spiked with

**Table 1.** Precision and accuracy data for determination of ribavirin in spiked blank bovine plasma

Duration	Added ( $\mu\text{g/mL}$ )	Found ( $\mu\text{g/mL}$ )	RSD (%)	Accuracy (%)
Intraday ( $n = 6$ )	3.50	$3.55 \pm 0.11$	3.35	101.4
	0.60	$0.61 \pm 0.02$	2.94	101.8
Interday ( $n = 6$ )	3.50	$3.53 \pm 0.08$	2.39	100.8
	0.60	$0.62 \pm 0.02$	2.60	104.9

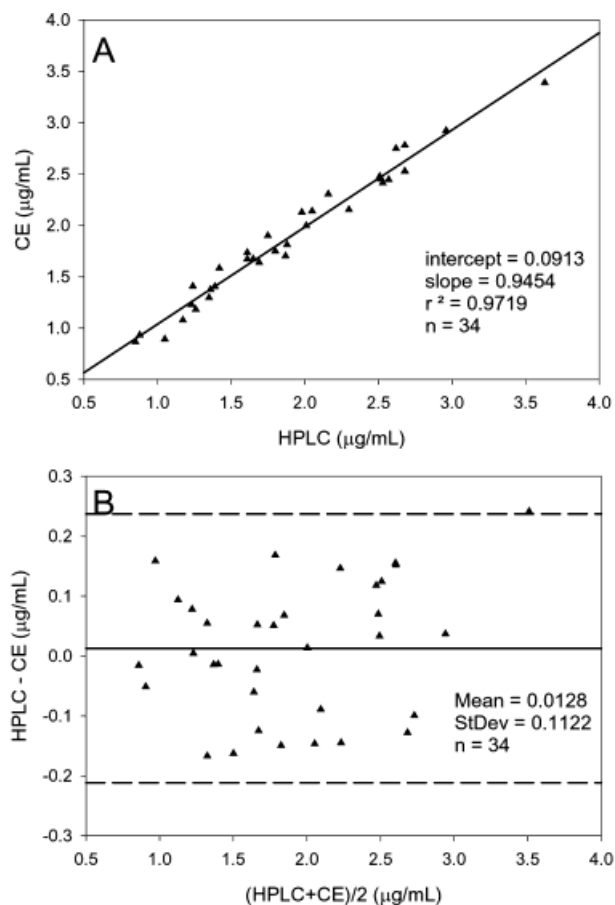
3.50  $\mu\text{g/mL}$  ribavirin, and less than 8% for samples spiked with 0.60  $\mu\text{g/mL}$  ribavirin. Accuracy and precision data for these spiked samples are shown in Table 1, where all precision data is within about 3% RSD, while accuracy values are less than 5%.

#### 3.2.2 Application to patient samples and comparison with HPLC

During a period of three months, a total of 34 patient samples were analyzed by CE and HPLC for ribavirin, with representative separations shown in Figs. 7 and 2, respectively. The mean  $\pm$  SD (median, range) of the 34 ribavirin levels determined by CE and HPLC were determined to be  $1.90 \pm 0.634$  (1.78, 0.87–3.39)  $\mu\text{g/mL}$  and  $1.91 \pm 0.661$  (1.84, 0.85–3.63)  $\mu\text{g/mL}$ , respectively. Comparison of the two sets of data with the  $t$ -test revealed not a statistically significant difference between the two input groups ( $P = 0.9352$ ). The mean ribavirin level determined in our samples was found to be somewhat lower than those reported for blood specimens taken immediately before [19] and 2–4 h after the morning dose [2].

Comparison of the levels of ribavirin found in patient serum samples using the two methods shows excellent agreement between the two methods (Fig. 8A). Plotting the difference of the two values vs. the mean, shown in Fig. 8B, again shows excellent consistency between the two methods. The mean of the differences was 0.012  $\mu\text{g/mL}$ , indicating that on average, the CE method gives slightly lower amounts than the HPLC method, however this underestimation is insignificant. The source of this underestimation is most likely due to the partial separation of the small peak migrating slightly after ribavirin, however given the excellent agreement between the two methods, this has no major influence on ribavirin determination in serum for this set of patient samples.

The excellent agreement between the data obtained by HPLC and CE indicate that the CE method may be suitable for routine monitoring of ribavirin. In both methods



**Figure 8.** (A) Correlation and (B) difference vs. mean of each HPLC and CE data pair of ribavirin concentration in 34 patient sera. The solid line represents the mean of the differences and the broken lines are the mean  $\pm$  2 SD.

SPE is required. CE capillaries are less expensive than HPLC columns and CE requires smaller amounts of chemicals and organic solvents. However, when other features of the two methods are compared, the CE method is found to be somewhat inferior. For CE 500  $\mu\text{L}$  of serum is required whereas the HPLC method is based upon 200  $\mu\text{L}$  of serum. Furthermore, the presence of an interfering peak in the CE separation provides a potential source for overestimation of ribavirin in patient samples in which this compound is elevated. Finally, sample throughputs for CE and HPLC are about three and four per hour, respectively.

In conclusion, a method based on borate complexation for the separation of ribavirin by CE has been developed and applied to the determination of ribavirin in human serum and plasma samples. Sample pretreatment using

ACN protein precipitation was found to be unsuitable for routine monitoring due to insufficient sensitivity and interferences in patient samples. SPE using PBA cartridges overcame these limitations and allowed the method to be validated. Comparison with an HPLC method showed excellent agreement indicating the suitability of the CE method for TDM of ribavirin.

*The authors acknowledge stimulating discussions with Prof. J. Reichen. This work was partly supported by the Swiss National Science Foundation.*

Received December 2, 2003

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