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Note

Quantification of ribavirin in biological fluids and tissues by high-performance liquid chromatography

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Ribavirin (Virazole™) has recently been approved for use in the United States for the treatment of respiratory syncytial virus infections in infants and young children. Extensive reviews of pre-clinical toxicology and clinical applications, contained in recent monographs on ribavirin [1–3], describe the therapeutic activity of ribavirin in the treatment of a variety of viral infections, as well as the relative lack of detectable toxicity in clinical trials. While some mild hematological abnormalities have been reported when ribavirin was administered orally, these effects have not been seen with small-particle aerosol administration of the drug [4]. With these properties, ribavirin has the potential to see wide-spread use in treatment of many human viral diseases, including the possibility of acquired immune deficiency disease.

One factor restricting the scope to which ribavirin might be used has been a relative lack of pharmacokinetic data under various clinical settings. Although a highly sensitive radioimmunoassay has been developed [5] and used in preliminary kinetic studies [6] and a gas chromatographic–mass spectrometric technique has been described [7], neither of these techniques has been widely employed in clinical studies to measure ribavirin concentrations in patients and to compare clinical efficacy to blood and/or tissue levels.

We present here a reversed-phase high-performance liquid chromatographic (HPLC) assay for free ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) in biological fluids and tissues capable of detection at and below therapeutic concentrations. This technique, based on methods for purification and quantification of ribonucleosides described by Davis et al. [8], is simple to perform, requires only commercially available reagents, and allows processing of

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many samples with reasonable rapidity. This method should prove to be useful to investigators wishing to correlate drug levels with therapeutic efficacy and to study the pharmacokinetics of ribavirin.

EXPERIMENTAL

Chemicals and reagents

Ribavirin was provided by Viratek (Costa Mesa, CA, U.S.A). HPLC-grade water, methanol, and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade ammonium phosphate (monobasic) and phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Ammonium acetate, ammonium hydroxide, and formic acid were of the highest grade obtainable; all solutions containing these chemicals were made in HPLC-grade water. Ultrafiltration of biological samples was performed using Amicon CF25 ultrafiltration cones (Amicon, Danvers, MA, U.S.A.) soaked in HPLC-grade water for 1 h prior to use.

Apparatus and conditions

The high-performance liquid chromatograph employed for these studies was a Waters 840 system (Waters Assoc., Danvers, MA, U.S.A.) equipped with two Model 510 pumps, a Model 710B automatic sample injector, and a Model 490 multi-channel, variable-wavelength UV–VIS detector. Detector signals were recorded and analysed on a Professional 350 computer (Digital Equipment, U.S.A.) controlled by Waters Expert™ chromatography software. All separations were performed at ambient temperature on a Microsorb™ C_{18} stainless-steel HPLC column (5 μm particle size 25 cm $\times$ 4.6 mm I.D. (Rainin Instruments, Emeryville, CA, U.S.A.) protected by a Guard-Pak™ pre-column with a C_{18} insert (Waters Assoc.). Analysis of ribavirin was performed using 0.02 *M* ammonium phosphate buffer, pH 5.10 (adjusted with 10% ammonium hydroxide or 8% phosphoric acid), containing 1% methanol as the mobile phase with detection at 207 nm. Strongly adsorbed substances were eluted from the column following each analysis with a gradient of 0 to 80% acetonitrile in water. Flow-rate was maintained at 1.0 ml/min throughout. Mobile phase was filtered through a 0.22-μm filtration unit (Falcon 7105, Becton Dickinson Labware, Oxnard, CA, U.S.A.) and degassed under 600 mmHg vacuum with sonication for 5 min prior to use.

Phenyl boronate affinity (PBA) chromatography

To achieve reproducible separation of ribavirin in biological fluids and tissues we found that an initial class separation with immobilized phenyl boronate affinity gel was necessary in most samples. Matrex™ PBA-60 gel (Amicon; 88.87 μmol boron per ml) was slurry packed to a bed volume of 1.0 ± 0.1 ml into 4×0.7 cm I.D. columns (Flex-column™, Kontes, Vineland, NJ, U.S.A.) using 0.25 *M* ammonium acetate, pH 8.8, as the packing buffer. Ribavirin binding capacity was measured by frontal analysis [9, 10]. Briefly, columns were equilibrated to binding conditions by allowing 50 ml of 0.25 *M* ammonium acetate buffer, pH 8.8, to

percolate through by gravity. After equilibration was achieved, 15 ml of a feed solution containing ribavirin (1.0 mg/ml, in 0.25 *M* ammonium acetate) were applied and 1-ml fractions were collected for HPLC analysis. Following collection of the binding fractions, the columns were cleaned and equilibrated to non-binding conditions by allowing 50 ml of 0.1 *M* formic acid to percolate through. Non-binding fractions (1 ml) were collected after application of 10 ml of ribavirin (1.0 mg/ml, in 0.1 *M* formic acid). The area under the peak corresponding to ribavirin was then determined for each fraction and each feed solution by HPLC and was used to calculate the percentage of feed for each fraction. Binding capacity for ribavirin was calculated for each column using the following formula: binding capacity $= (V_f/V_t) \cdot [T]$; where V_f = the difference in 50% saturation points for binding and non-binding conditions, V_t = the column bed volume, and $[T]$ = the concentration of the feed solution.

Recovery of ribavirin from the PBA chromatography step was assessed by chromatographing 1.0 ml of ribavirin (10.0 μg/ml, in 0.25 *M* ammonium acetate) on each of several PBA columns (using conditions described in *Sample preparation*) and comparing areas under the ribavirin peak on HPLC analysis to that of the standard solution. Lyophilization recovery experiments, in which 1.0 ml of ribavirin (10.0 μg/ml, in 0.25 *M* ammonium acetate) was diluted with 5.0 ml of 0.1 *M* formic acid prior to freezing and lyophilization, were conducted simultaneously with PBA recovery experiments.

Standard curve preparation and calibration

A stock solution of ribavirin was prepared by dissolving 100.00 mg of pure drug in 100 ml (volumetric) of HPLC-grade water. Standard solutions for determination of linearity of detector response and recovery experiments were then prepared from this stock by making serial dilutions using HPLC mobile phase (0.02 *M* ammonium phosphate–1% methanol, pH 5.10), PBA binding buffer (0.25 *M* ammonium acetate, pH 8.8), and human plasma or serum as dilutants. Quantification of ribavirin peak area following HPLC analysis was performed using the external standard method after calibration of the computer with a 10.00 μg/ml (volumetric) standard solution.

Sample preparation

The same general scheme of sample preparation was used for each type of biological sample analysed with the only differences being in collection procedures as noted below. In general, 1.0 ml of sample was first deproteinized by ultrafiltration through CF25 cones for 30 min at 500 relative centrifugal force (RCF). The volume of the resulting clear ultrafiltrate was measured and an appropriate volume of 2.5 *M* ammonium acetate buffer, pH 8.8, added to adjust the sample pH and ionic strength prior to PBA chromatography. The adjusted ultrafiltrate of sample was then applied to a PBA column pre-equilibrated to binding conditions by allowing 50 ml of 0.25 *M* ammonium acetate, pH 8.8, buffer to percolate through. Unretained substances were washed from the column with 7.0 ml of the ammonium acetate buffer, ribavirin and other bound substances were then eluted with 6.0 ml of 0.1 *M* formic acid. The eluate was collected, frozen, and lyophilized to

dryness. Following lyophilization, the sample was resuspended in 0.02 *M* ammonium phosphate–1% methanol, pH 5.10 (HPLC mobile phase), using the volume measured after ultrafiltration and before addition of 2.5 *M* ammonium acetate to the sample. Following elution of ribavirin, the PBA column was washed with 50 ml of 0.1 *M* formic acid to ensure removal of bound substances and either re-equilibrated to binding conditions and used to process another sample or stored at 4 °C in 0.02 *M* phosphate buffer, pH 6.8, containing 0.1% sodium azide. Under these conditions we found that between five and ten samples could be processed on a single column before a loss in separation efficiency was observed and repacking of the column became necessary.

Serum. Peripheral blood from healthy individuals was collected and allowed to clot for 20 min at room temperature, serum was separated by centrifugation at 500 RCF for 15 min prior to ultrafiltration and PBA chromatography. If processing could not proceed immediately, serum was stored at −20 °C.

Plasma. Peripheral blood from healthy individuals was collected into heparinized syringes and plasma was separated by centrifugation at 500 RCF for 15 min prior to ultrafiltration and PBA chromatography. Plasma was stored at −20 °C as for serum.

Tracheal aspirates. Respiratory secretions, collected as part of the routine care of intubated patients receiving ribavirin therapy by small-particle aerosol, were collected in suction traps using standard techniques. The secretions were diluted 10–100 fold (depending on sample consistency) in 0.25 *M* ammonium acetate buffer prior to ultrafiltration. Again, if processing could not proceed immediately, specimens were stored at −20 °C. We found that the PBA step was not required for analysis of tracheal aspirates.

Lung tissue. Lungs from six-week-old white Swiss mice were used for determination of ribavirin concentration following administration of drug by small-particle aerosol [11]. After receiving ribavirin or placebo, mice were sacrificed by cervical dislocation and lungs were removed. Lung tissue was washed free of surface blood, homogenized in 1.0 ml of water using a glass/PTFE homogenizer, and centrifuged at 13 000 *g* to remove large debris prior to ultrafiltration and PBA chromatography. Tissue homogenates were stored at −70 °C if immediate processing was not possible.

RESULTS

Binding capacity of phenyl boronate affinity colums

Fig. 1 shows a representative frontal analysis plot for measurement of ribavirin binding capacity and was generated as described in Experimental. In the example shown the column bed volume was 1.0 ml and the calculated binding capacity for ribavirin was 6.04 mg/ml of gel. In this experiment, a small fraction of ribavirin, an additional 10% of the feed, eluted from the column at the end of the formic acid step. The average (±S.D.) binding capacity for five columns tested from the same lot of phenyl boronate gel was 6.50 ± 0.85 mg of ribavirin per ml of gel.

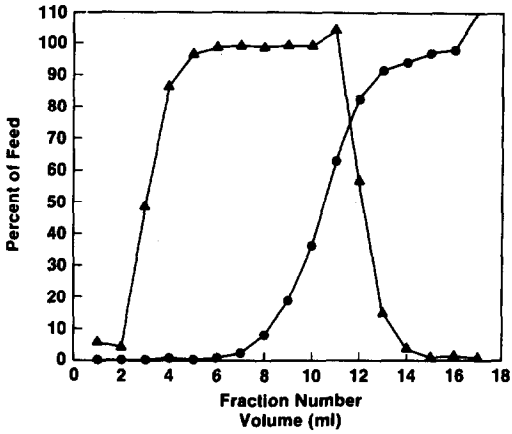

Fig. 1. Frontal analysis plot for determination of ribavirin binding capacity on PBA columns. The column was slurry-packed to a bed volume of 1.0 ml and equilibrated to binding conditions by allowing 50 ml of 0.25 *M* ammonium acetate, pH 8.8, to percolate through. A 15-ml volume of binding (●) feed solution (ribavirin, 1.0 mg/ml in 0.25 *M* ammonium acetate) was then applied and 1-ml fractions were collected. The column was then cleaned and equilibrated to non-binding conditions with 50 ml of 0.1 *M* formic acid. Non-binding (▲) fractions (1 ml) were collected after application of 10 ml of non-binding feed (ribavirin, 1.0 mg/ml in 0.1 *M* formic acid). Each fraction was analysed by HPLC for ribavirin content and plotted as percentage of original feed. Ribavirin binding capacity for the plot shown was calculated to be 6.04 mg/ml of PBA gel

Recovery and reproducibility

As summarized in Table I, recovery of ribavirin standards from the PBA chromatography and lyophilization steps is quantitative. Overall recovery of ribavirin from spiked human plasma and serum samples is also shown in Table I. Intra- and inter-day reproducibility of the HPLC analysis is summarized in Table II

TABLE I

RECOVERY OF RIBAVIRIN

Percentage recovery calculated as: (peak area sample/peak area standard) · 100. C.V. = Coefficient of variation.

Purification step	Number of samples	Recovery (%)	C.V. (%)
PBA chromatography★	8	100.0	2.33
Lyophilization★	9	96.7	2.95
Overall recovery★★			
+0.1 μg/ml (0.41 μ*M*)	4	70.7	19.5
+1.0 μg/ml (4.1 μ*M*)	6	68.2	10.2
+5.0 μg/ml (20.5 μ*M*)	6	84.8	1.4
+10.0 μg/ml (41.0 μ*M*)	4	72.5	0.8

★Starting material: 1.0 ml of 10.0 μg/ml ribavirin.

★★Overall recovery from human plasma or serum spiked with shown concentration of ribavirin prior to ultrafiltration.

TABLE II

INTRA-DAY AND INTER-DAY REPRODUCIBILITY

Amount per injection		*n*	Coefficient of variation (%)	
ng	nmol		Intra-day	Inter-day
5	0.02	6	5.8	7.1
50	0.20	12	0.3	4.4
500	2.05	22	0.5	5.6

and most probably accounts for the slight loss of ribavirin measured in the lyophilization step.

Standard curve and minimum level of detection

Fig. 2 depicts the lower end of the standard curve for ribavirin at 207 nm (from 1 to 100 ng per injection) and shows a representative chromatogram of ribavirin at the 1 ng (4.1 pmol) per injection level. The detector response is linear over a range of ribavirin concentrations from 1 ng to 10 μg per injection with a linear correlation coefficient of 0.9998. Injection volumes of 10 to 100 μl had no effect on ribavirin peak area but reduced peak heights were noted when large injection volumes (100 μl) were used. The minimum detectable amount of standard ribavirin is 1 ng (4.1 pmol) injected. Under the standard conditions described here for analysis of biological samples (50 μl injected without concentration of the sample following lyophilization) we find that the minimum detectable level of ribavirin in serum, lung tissue or tracheal aspirates is 0.1 μg/ml (0.41 μM).

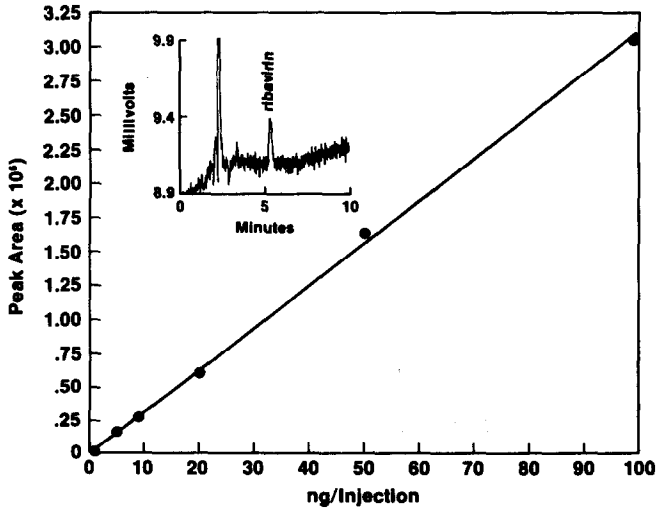

Fig. 2. Standard curve for ribavirin at 207 nm. Each point shown is the mean of at least three determinations. Inset: chromatogram of ribavirin showing minimum detection at 1 ng injected. Conditions: mobile phase, 0.02 *M* ammonium phosphate–1% methanol, pH 5.10; flow-rate, 1.0 ml/min; column, Microsorb C_{18} (25 cm × 4.6 mm) protected by Guard-Pak with C_{18} insert; detection, 207 nm.

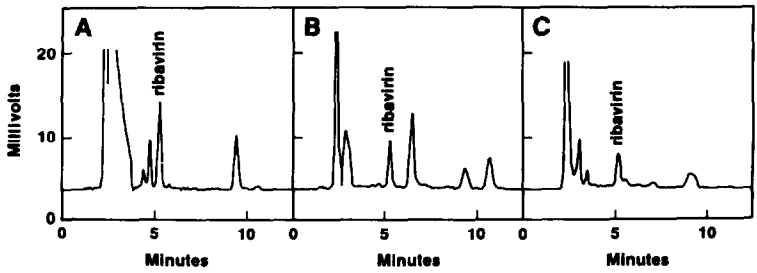

Fig. 3. Detection of ribavirin in various biological samples. Conditions: mobile phase, 0.02 *M* ammonium phosphate–1% methanol, pH 5.10; flow-rate, 1.0 ml/min; column, Microsorb C_{18} (25 cm×4.6 mm) protected by Guard-Pak with C_{18} insert; injection volume, 50 μl; detection, 207 nm. (A) Human plasma spiked with 1.0 μg/ml ribavirin prior to ultrafiltration, PBA chromatography, and lyophilization; 0.89 μg/ml ribavirin detected. (B) Mouse lung tissue after 11 h of continuous ribavirin administration by small-particle aerosol (20 mg/ml in aerosol generator reservoir). Lungs were removed immediately following administration, rinsed, and homogenized in 1 ml water. Homogenate was diluted 1:10 in 0.25 *M* ammonium acetate, pH 8.8, centrifuged at 13 000 *g* for 10 min, ultrafiltered, PBA-chromatographed, lyophilized, and resuspended. Concentration of ribavirin: 5.27 μg per lung. (C) Tracheal aspirate from a patient receiving ribavirin therapy by small-particle aerosol. Secretions were aspirated into a suction trap 2 h post-treatment, diluted 1:100 in 0.25 *M* ammonium acetate, ultrafiltered, and analysed by HPLC without PBA chromatography and lyophilization. Concentration of ribavirin: 31.8 μg/ml of secretion.

Detection of ribavirin in biological fluids and tissue

Fig. 3 shows chromatograms of spiked human plasma, mouse lung tissue following administration of ribavirin by small-particle aerosol, and human tracheal aspirate after treatment by small-particle aerosol. The ribavirin peak is well separated from other naturally occurring substances and has a retention time of 5.3 min. Ribavirin standards were processed along with the samples and recovery was 95.7 ± 3.6%. Metabolites of ribavirin which do not contain the ribose moiety are not retained by the PBA chromatography step and therefore are not detected. Other ribose-containing metabolites of ribavirin which are retained in the PBA chromatography step are completely resolved from ribavirin (data not shown) and were not detected in the samples analysed. Assay of phosphorylated metabolites of ribavirin, not retained by the C_{18} column, may be possible with ion-pairing or ion-exchange HPLC techniques.

DISCUSSION

Immobilized PBA chromatography has long been used for purification and analysis of naturally occurring ribonucleosides and ribonucleotides [12]. The combination of reversed-phase HPLC coupled with an initial class separation by PBA chromatography, as described by Davis et al. [8], has proven to be a powerful technique for analysis of naturally occurring ribonucleosides in human urine and other biological fluids [13, 14]. We have extended this technique to include analysis of ribavirin in a number of biological fluids and tissues (Fig. 3) with sufficient sensitivity and reproducibility to be of use in the analysis of clinical samples from patients receiving ribavirin therapy for viral disease.

Although ribavirin does not exhibit the characteristic UV absorption spectra

of other antiviral nucleoside analogues and exhibits only a modest maximum UV absorption at 207 nm (extinction coefficient $= 1.17 \cdot 10^{-3}$ l mol^{-1} cm^{-1} [15], the sample preparation scheme described here clearly allows for detection and accurate quantification of ribavirin in the range of concentrations reported in the serum (0.4–24 μM) of patients receiving ribavirin by current dosing regimens [6]. Furthermore, the combination of PBA chromatography and lyophilization during sample preparation allows for a sample concentration step (10- to 20-fold) thus markedly increasing the sensitivity of the assay without effecting selectivity.

The variability of this assay at low ribavirin concentrations (0.1 μg/ml) is 19.5%. We feel this variability is due to the low absorbance of ribavirin and can be improved by sample concentration techniques. Although the processing of a single sample (including lyophilization) can require about a day, multiple samples can be processed with reasonable ease and rapidity. We routinely process 20–30 samples in a day. If necessary, the flow-rate through PBA columns can be increased to 3 ml/min or more without loss in efficiency if the length to diameter aspect of the column is increased to 6.5×0.5 cm. Furthermore, utilization of an automatic sample injector has been useful for analysing multiple samples as the current method, employing a 0 to 80% acetonitrile gradient following each analysis, requires at least 40 min between injection of samples. We have found that without utilization of such a gradient, approximately 60 min are required for elution of all material contained in biological samples.

As with the other two methods that have been described previously for the quantification of ribavirin in biological tissues, i.e., radioimmunoassay [5] and chemical ionization mass fragmentography [7], the HPLC method described here can determine ribavirin concentrations at and below therapeutic dose levels. However, the HPLC method is simpler than the combined gas chromatographic–mass spectrometric technique which requires in addition to the specialized equipment, molecular filtration, lipid extraction, and acidic and basic ion-exchange chromatography; the level of detection of ribavirin in biological fluids by these two methods is similar, 10 ng/ml of serum.

The radioimmunoassay developed by Austin et al. [5] which exhibits a higher degree of sensitivity for ribavirin than the HPLC method (four-fold) is also rather cumbersome for single sample processing, requiring an overnight incubation. Furthermore, production of highly specific antisera to ribavirin which is crucial for this assay has proven difficult, thereby limiting the usefulness and overall availability of this assay.

The sample preparation scheme and HPLC assay we described here is relatively simple and requires only commercially available materials and reagents. While other methods have been available, for whatever reason, they have not been widely used. We feel that the current HPLC method should prove to be a more useful alternative for future therapeutic studies of ribavirin.

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