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High-Performance Liquid Chromatography (HPLC) Assay for Ribavirin and Comparison of the HPLC Assay with Radioimmunoassay†

GEORGE G. GRANICH,[1] DONALD J. KROGSTAD,[1]* JAMES D. CONNOR,[2] KELLY L. DESROCHERS,[2] AND CHARLES SHERWOOD[2]

Divisions of Laboratory Medicine and Infectious Diseases, Department of Medicine, and Division of Laboratory Medicine, Department of Pathology, Washington University School of Medicine, and Therapeutic Drug Monitoring Laboratory, Barnes Hospital, St. Louis, Missouri 63110,[1] and Department of Pediatrics, University of California at San Diego, La Jolla, California 92093[2]

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The use of high-performance liquid chromatography (HPLC) to measure ribavirin in serum and other biological fluids has been limited by endogenous interfering substances. We report an HPLC procedure based on the extraction of ribavirin from serum, plasma, or cerebrospinal fluid with a boronate affinity gel, which uses a 3-methylcytidine internal standard. This assay is sensitive (to 0.4 μM), specific (no interference with 34 commonly prescribed drugs), reproducible (coefficients of variation from 5.4 to 22.4%), and linear ($r = 0.999$) over the range of clinically relevant concentrations in serum (from 0.5 to 50.0 μM). It also correlates well with the ribavirin radioimmunoassay ($r = 0.992$). This HPLC assay should be useful for measuring ribavirin in serum and other body fluids during clinical trials.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazole) is a purine nucleoside analog which resembles guanosine (13). It is active in vitro against a wide variety of DNA and RNA viruses (9, 18). Controlled in vivo studies have shown that ribavirin is effective against respiratory syncytial virus (8, 16), influenza A and B virus (7), and Lassa fever virus (11) infections. Ribavirin is being studied currently in clinical trials to further evaluate its activity against human immunodeficiency virus infections (5). Oral ribavirin also has been employed in the treatment of measles (2) and acute hepatitis A (17) infections.

Hematological toxicity, consisting of intravascular erythrocyte losses and reticulocytosis, has been associated with the use of oral ribavirin. Headache, insomnia, fatigue, and exertional dyspnea have been reported in patients receiving large oral or parenteral doses (10, 11, 14). These and other observations suggest that ribavirin toxicity is dose related and reversible. Sites of toxicity include circulating erythrocytes, the bone marrow, and the central nervous system.

Methods available currently for quantitating ribavirin in serum or plasma include bioassay (19), radioimmunoassay (RIA) (1), gas chromatography-mass spectroscopy (15), and high-performance liquid chromatography (HPLC) (3, 20). The use of HPLC to measure ribavirin has advantages over bioassay and gas chromatography-mass spectroscopy in terms of speed (turnaround time) and ease of performance. HPLC does not require the use of radioisotopes, as does the RIA. In this report we describe a rapid, sensitive, and specific HPLC assay for ribavirin in serum, plasma, and cerebrospinal fluid. This technique is potentially applicable to both pharmacokinetic studies and routine clinical assays.

* Corresponding author.

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MATERIALS AND METHODS

Reagents and samples. Ribavirin was obtained from Viratek, Inc., Covin, Calif. The internal standard (3-methylcytidine methosulfate), ammonium acetate, formic acid (99%), and ammonium phosphate were obtained from Sigma Chemical Co., St. Louis, Mo. Acetonitrile (HPLC grade) and phosphoric acid were purchased from Fisher Scientific Co., Fairlawn, N.J. Affi-Gel 601, a boronate affinity gel, was obtained from Bio-Rad Laboratories, Richmond, Calif., and polypropylene minicolumns (Extract-Clean columns, 1.5 ml) were obtained from Alltech Associates, Inc., Deerfield, Ill.

A stock solution of ribavirin, corrected for drug potency, was prepared at a concentration of 1.0 mM in distilled water and stored at −70°C until needed. Reference samples containing 0.5, 1.0, 10.0, 30.0, and 50.0 μM concentrations of ribavirin were prepared by diluting this stock solution with drug-free pooled human sera. These reference samples were used to assess intrarun and interrun precision. A single calibration standard was prepared in pooled human sera (at a concentration of 1.0 μM) independently of the reference samples and was used in all quantitations. Serum specimens spiked with ribavirin (prepared as described above), plasma specimens spiked with ribavirin by Viratek, and plasma specimens from patients receiving ribavirin were analyzed by both HPLC and RIA.

Chromatographic conditions. The chromatographic system consisted of a U6K injector, a model 510 pump, and a model 481 UV detector operating at a wavelength of 235 nm with a sensitivity of 0.01 absorbance units full scale, all of which were linked to an 810 Baseline Chromatography Workstation (Waters Associates, Milford, Mass.). Separation was achieved at ambient temperature on two μBondapak C18 reversed-phase columns (0.4 by 30 cm; Waters) connected in series. The mobile phase consisted of 10 mM ammonium phosphate buffer adjusted to pH 2.5 with 85% phosphoric acid, and the flow rate was 1.5 ml/min.

Sample preparation for HPLC. Boronate affinity gel columns were prepared by packing 0.1 g of the dry gel into a

polypropylene minicolumn (6.5 by 0.6 mm) and then adding 10 ml of ammonium acetate buffer (250 mM, pH 8.8). The columns could be stored at 4°C for up to 6 months. Immediately before use, two 1.0-ml volumes of ammonium acetate buffer were allowed to percolate through the column. Serum, plasma, or cerebrospinal fluid samples were prepared by adding 0.5 ml of the specimen to 0.5 ml of ammonium acetate buffer and 0.025 ml of a 100-mg/liter solution of 3-methylcytidine methosulfate (the internal standard) in distilled water (stock concentration, 271 μM; final concentration, 6.7 μM). This mixture was loaded onto the boronate affinity column and allowed to drain through. The column was then washed five times with 1.0-ml volumes of ammonium acetate buffer. The ribavirin and internal standard were eluted from the column with two 1.0-ml volumes of 100 mM formic acid, dried under a stream of nitrogen, and reconstituted with 0.1 ml of the mobile phase. Alternatively, ribavirin and the internal standard were eluted from the column with three 0.3-ml volumes of 100 mM formic acid by discarding the first two eluates and retaining the third. Because batch-to-batch variation among lots of boronate affinity gel and variations in column preparation may affect column performance, the fraction containing the ribavirin and internal standard was verified with each new group of minicolumns prepared. The minicolumns were regenerated after each use by being washed with two 1.0-ml volumes of 100 mM formic acid and then with 10 ml of ammonium acetate buffer. With this procedure, the minicolumns were reused up to 10 times without any decrease in recovery. With either elution method, a 100-μl sample was injected into the HPLC system.

RIA. The RIA was performed as described previously (1). Briefly, 100-μl portions of the samples to be tested were mixed with 200 μl of buffer (phosphate-buffered saline with 1% bovine serum albumin), 100 μl of ribavirin antiserum obtained from rabbits immunized with monosuccinylated ribavirin coupled to ovalbumin, and 100 μl of [^{3}H]ribavirin (0.4 pmol; specific activity, 15 Ci/mmol) (ICN Corp., Irvine, Calif.). After these components were mixed, the tubes were incubated overnight at 4°C. Bovine gamma globulin (50 μl of a 10-mg/ml solution; Miles Laboratories, Inc., Elkhart, Ind.) and cold saturated ammonium sulfate (550 μl, pH 7.2) were added, and the contents of the tubes were mixed again. After incubation for 2 h at 4°C, the tubes were centrifuged for 20 min at 1,400 × *g*. The supernatant was decanted, and the pellet was washed with 1 ml of 50%-saturated ammonium sulfate (pH 7.2) at 4°C. After an additional 10 min at 4°C and a second centrifugation at 1,400 × *g*, the pellets were collected and dissolved in 100 μl of distilled water. The contents of the tubes were mixed and counted in a liquid scintillation counter after the addition of 100 μl of 4 N HCl and 2 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.).

RESULTS

Representative chromatograms of a drug-free serum sample and a serum sample spiked with ribavirin and the internal standard are shown in Fig. 1. The retention times for ribavirin and the internal standard were 8.0 ± 0.5 and 11.0 ± 1.0 min, respectively. Ultrafiltration of the sample improved neither the quality of the chromatogram (Fig. 2) nor the sensitivity of the assay (0.4 μM). As measured by HPLC, ribavirin was stable at 4, −20, or −70°C for up to 30 days. Heat treatment (56 to 60°C for 30 min) did not affect the HPLC assay.

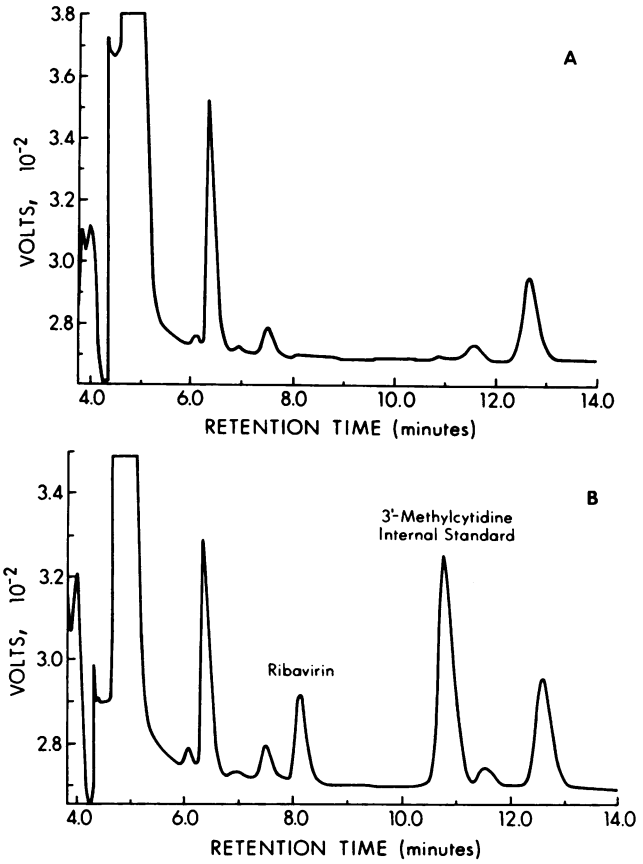

FIG. 1. Representative chromatograms for human serum without either ribavirin or the 3-methylcytidine internal standard (A) and for serum spiked with ribavirin and the internal standard (B).

Sensitivity. The limit of detectability for this assay with the first method of elution (two 1.0-ml volumes of 100 mM formic acid) was 0.1 μM in serum and plasma. With the second method of elution (three 0.3-ml volumes of 100 mM formic acid), the sensitivity was 0.4 μM in serum, plasma, and cerebrospinal fluid. Because the sensitivity of the HPLC assay is below the therapeutic levels in serum and plasma reported previously for ribavirin (1, 10, 14) and because interassay precision was reduced at ribavirin concentrations of less than 0.5 μM, the precision and recovery studies were performed at ribavirin concentrations of between 0.5 and 50 μM.

Specificity. Thirty-four commonly prescribed drugs were tested and shown not to interfere with this assay. These included four antiviral compounds, i.e., acyclovir, 2',3'-dideoxycytidine, azidothymidine, and 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine. Other drugs which were tested and did not interfere with the assay included five aminoglycosides (amikacin, gentamicin, netilmicin, kanamycin, and tobramycin), chloramphenicol, vancomycin, digoxin, theophylline, seven anticonvulsants (diazepam, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, and carbamazepine), four antiarrhythmics (procainamide and its *N*-acetyl metabolite, disopyramide, quinidine, and lidocaine), four antidepressants (amitriptyline, desipramine, imipramine, and nortriptyline), two analgesics (acetaminophen and salicylate), caffeine, cyclosporin A, methotrexate, and lithium. No interferences were observed with serum specimens from patients with hepatic disease, hemol-

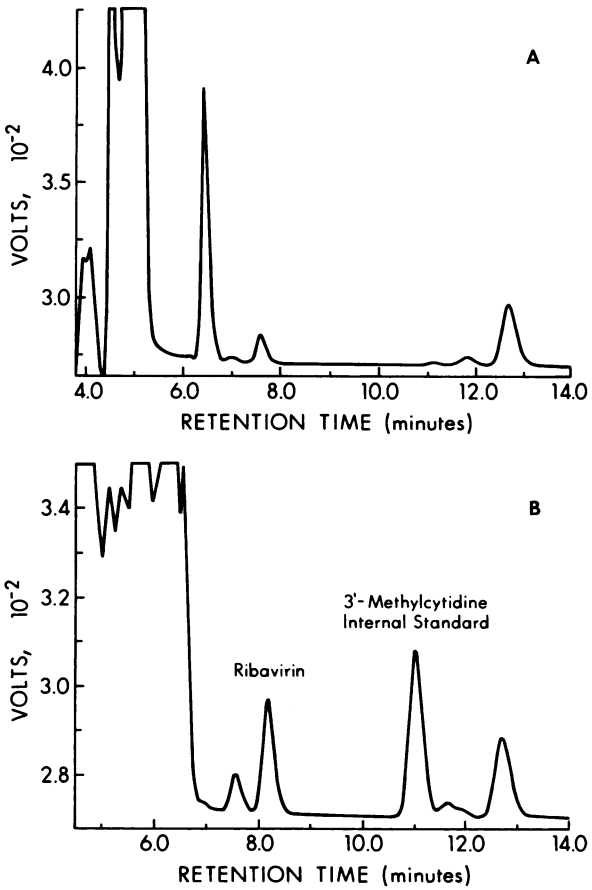

FIG. 2. Effects of ultrafiltration on the ribavirin HPLC. Ultrafiltration did not improve the base-line variations in absorbance observed with normal human serum (A) or the quality of the chromatograms observed with ribavirin and the internal standard (B).

ysis, or hyperlipidemia. Some interference was noted with serum specimens from patients with renal dysfunction (serum creatinine level of >3.0 mg/dl). This interference increased with increasing serum creatinine concentrations, although creatinine itself did not interfere, and is probably related to endogenous ribonucleosides in serum. Nucleic acid bases, deoxyribonucleosides, and deoxyribonucleotides do not bind to the boronate affinity gel (6) and did not interfere with the assay. Ribonucleosides bind to the boronate affinity gel. Eight ribonucleosides, which have retention times close to that of ribavirin or the internal standard, were tested and shown not to interfere with the assay. These ribonucleosides included cytidine, uridine, 1-methyladenosine, 5-methylcytidine, 2-thiocytidine, 7-methylinosine, 7-methylguanosine, and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside. No significant levels of endogenous 3-methylcytidine were detected in serum.

Recovery. The absolute recovery with the method was determined by adding sufficient ribavirin to serum or cerebrospinal fluid samples to achieve concentrations of 0.5, 1.0, 10.0, and 50.0 μM and comparing the ribavirin concentrations measured in those specimens by HPLC with those measured in aqueous samples spiked with similar concentrations of ribavirin. Mean (n = 3) recoveries ranged from 78.2 to 86.8% from serum and from 68.6 to 80.6% from cerebrospinal fluid (Table 1). Mean recoveries (n = 3) of the internal standard were 72.4% from serum and 71.2% from cerebrospinal fluid.

TABLE 1. Ribavirin recovery in the HPLC assay

Ribavirin concn (μM)	Recovery (%) from:	
	Serum	Cerebrospinal fluid
0.5	79.0	68.6
1.0	78.2	80.6
10.0	78.9	69.3
50.0	86.8	73.4

Precision. To define intrarun and interrun precision, we analyzed each of the reference samples 10 times in a single run and 10 times in separate runs (Table 2). Coefficients of variation for intrarun and interrun data ranged from 3.07 to 11.11% and from 5.36 to 22.41%, respectively.

Linearity. The linearity of the HPLC assay in serum was assessed by comparing the peak-height ratios (y) obtained for the ribavirin reference samples at concentrations of 0.5, 1.0, 10, 30, and 50 μM in 10 independent runs with their theoretical values (x), using linear regression (4). The equation of the regression line for the HPLC assay was $y = 0.040x - 0.005$, and the correlation coefficient was 0.999 (Fig. 3). The assay was also linear with cerebrospinal fluid ($y = 0.049x - 0.009$; $r = 0.999$ [data not shown]).

Comparative accuracy. In a direct comparative study, 41 specimens were assayed by both HPLC and RIA. These specimens included 20 spiked serum samples, 10 spiked plasma samples, and 11 plasma samples from patients receiving ribavirin. The concentrations determined by the two methods were compared with each other by using linear regression and showed good agreement. The correlation coefficient was 0.992, and the equation of the regression line was $y = 0.908x + 0.906$. When those samples which fell within the more representative clinical range of 0.5 to 50.0 μM were used, the correlation coefficient became 0.988, and the equation of the regression line became $y = 0.950x + 0.488$ (Fig. 4).

DISCUSSION

HPLC has been the method of choice for the detection and quantitation of nucleosides and can be adapted readily for the measurement of different nucleoside antiviral agents. However, the use of HPLC to measure ribavirin in human serum has been limited by the presence of endogenous interfering compounds. Use of a minicolumn filled with a boronate affinity gel (based on the previous studies of Davis et al. [6]) provides a simple, rapid sample clean-up procedure which removes most endogenous interfering compounds and

TABLE 2. Precision of ribavirin HPLC assay[a]

Ribavirin concn (μM) in reference standard	Intrarun precision		Interrun precision	
	Mean (μM)	CV (%)	Mean (μM)	CV (%)
0.5	0.54	11.11	0.58	22.41
1.0	1.09	9.17	1.05	11.43
10.0	9.78	3.07	10.07	5.36
50.0	51.08	3.45	52.05	6.09

[a] Each reference standard was tested 10 times in a single run and 10 times in separate runs for the intrarun and interrun data, respectively. CV, Coefficient of variation.

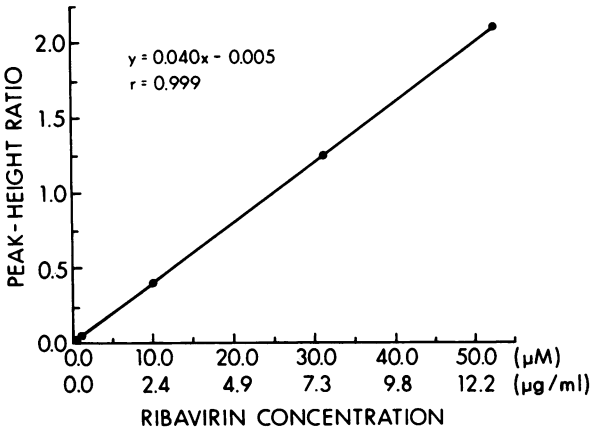

FIG. 3. Linearity of the ribavirin HPLC. The peak-height ratios for ribavirin divided by the internal standard (y) are plotted against the concentration of ribavirin (x). The data form a straight line and indicate a linear relationship between the peak-height ratio and the ribavirin concentration (4).

is relatively specific for ribonucleosides (6, 12). Bio-Rad Affi-Gel 601, which has been used in this and other (12) studies, has a polyacrylamide gel base with aminoethyl succinyl aminophenyl boronate functional groups covalently bonded to the matrix. These groups form a stable complex at alkaline pH (e.g., in the presence of ammonium acetate buffer, pH 8.8) with coplanar *cis*-diol groups, such as ribonucleosides; the complex dissociates at an acid pH (in the presence of 100 mM formic acid). The low pH of the mobile phase (pH 2.8) had no detectable adverse effects on column function. More than 500 ribavirin assays were performed on a single C18 column over 12 months with no evidence of deterioration. Recently, Smith and Gilbert used a similar boronate affinity gel (Matrex PBA-60; Amicon, Danvers, Mass.) to prepare specimens for the measurement of ribavirin (20). Differences between their studies and those reported here include our comparison of HPLC results with the ribavirin RIA, development of an internal standard, testing for interference with commonly prescribed drugs, use of a minicolumn for the boronate extraction procedure, and observation of no effect with ultrafiltration.

In our use of this technique, we have found that the removal of serum proteins by ultrafiltration does not improve the recovery (which is comparable to that obtained with ultrafiltration, 68.2 to 84.8%) over a concentration range of 0.4 to 41 μM (16) or the specificity of the extraction (see Results and Fig. 2). Dispensing with the serum pretreatment by ultrafiltration reduces the expense of the procedure and permits the use of a smaller sample size (0.5 versus 1.0 ml of serum). Determining the fraction in which ribavirin and the internal standard elute and collecting only that fraction provides a more rapid extraction procedure, which can be completed within 20 min. In addition, measuring ribavirin at 235 nm (3), rather than at 207 nm, reduces the run time to approximately 20 min by eliminating peaks from late-eluting compounds which may interfere with the assay and thus obviates the need for gradient elution between samples to clear the column. These modifications to the procedure decrease sensitivity (sensitivity of 0.4 μM). However, the sensitivity is adequate for most clinical purposes because serum ribavirin levels are generally ≥1.0 μM (1, 10, 14). Although the interrun variability of the HPLC assay is high at low ribavirin concentrations (22% coefficient of variation at 0.5 μM [Table 2]), the absolute magnitude of this variation is small. We have also employed an internal standard, because we believe it is essential for any HPLC assay performed in the routine clinical laboratory, particularly for assays that require extractions.

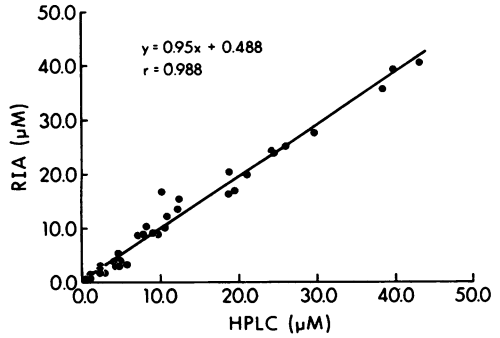

FIG. 4. Correlation between the ribavirin HPLC and RIA. Ribavirin concentrations for 34 serum and plasma specimens measured by the HPLC assay (x) are plotted against those obtained with the RIA (y). The 34 specimens plotted are those within the clinically relevant range of 0.5 to 50 μm. The data form a straight line, indicating an excellent linear relationship between results obtained with the two assays.

To be useful for both pharmacokinetic studies and routine clinical purposes, a drug assay must be simple and rapid as well as sensitive, precise, and accurate. Although bioassays have been used to measure a wide variety of antimicrobial agents, they are frequently not as reproducible or as sensitive as other methods, and they typically have longer turnaround times. In addition, bioassays for antiviral agents require expertise in tissue culture techniques, which is not available in many clinical laboratories.

The gas chromatography-mass spectroscopy method for measuring ribavirin is comparable to HPLC in precision, accuracy, and sensitivity (15, 20). However, gas chromatography-mass spectroscopy requires a specialized extraction procedure, as well as more expensive equipment and a high level of technical skill.

The RIA for ribavirin is precise, accurate, specific, and more sensitive than HPLC (sensitivity of 0.01 μM) (1). Although the RIA requires an overnight incubation, it lends itself well to the processing of large batches of samples. However, the RIA requires a specific antiserum to ribavirin, which is not available commercially. It also involves the use of radioisotopes, which many laboratories, particularly clinical microbiology laboratories, prefer to avoid.

For these reasons, we believe that HPLC is an important alternative to the RIA for the measurement of ribavirin in clinical specimens of serum, plasma, or cerebrospinal fluid. The results of these studies suggest that HPLC is equivalent to RIA within the clinically relevant range of ribavirin concentrations achieved in serum or plasma.

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