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Sensitive Radioimmunoassay for the Broad-Spectrum Antiviral Agent Ribavirin

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Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole; Viratek, Inc., Covina, Calif.), has a broad spectrum of antiviral activity. However, the study of the absorption, metabolism, and excretion of this compound has been limited by the lack of an appropriate assay for ribavirin and its metabolites. Since ribavirin has definite potential for therapeutic use, we developed a radioimmunoassay to measure ribavirin levels in clinical specimens. To prepare an effective immunogen, ribavirin was monosuccinylated and coupled to ovalbumin. The competitive binding radioimmunoassay, in which tritium-labeled ribavirin and rabbit antiribavirin serum were used, was quantitative for ribavirin at concentrations of 1 pmol/100 μl in urine or plasma samples. The rabbit antibody cross-reacted with the major metabolite of ribavirin, 1,2,4-triazole-3-carboxamide, at a low level (2 to 5%) which did not interfere with ribavirin binding until concentrations of 1,2,4-triazole-3-carboxamide 10- to 100-fold higher than ribavirin were present in mock samples, a condition not present in biological specimens. We used the ribavirin radioimmunoassay to determine the ribavirin concentration in mouse plasma after intraperitoneal administration, in the sera of adults from Sierra Leone after oral or intravenous administration for treatment of suspected Lassa fever, and in the sera of children in the United States after small-particle aerosol administration. Our experience with the radioimmunoassay indicates that it is sensitive, accurate, and reproducible. The assay will permit studies leading to a better understanding of the pharmacology and pharmacokinetics of this potentially useful antiviral drug.

The synthetic nucleoside-like compound ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazole; Viratek, Inc., Covina, Calif.) has in vitro and in vivo antiviral activity against RNA and DNA viruses (5, 9, 14). Human RNA viruses, such as influenzas A and B and respiratory syncytial virus, are very susceptible to the antiviral action of ribavirin in vitro. Influenza A and parainfluenza virus infections in mice have been shown to respond to ribavirin therapy administered by aerosol (1a, 5, 6), and hemorrhagic-fever viruses producing viremic infection in primates respond to ribavirin given parenterally (11). Ribavirin is activated by cellular adenosine kinase (13) to ribavirin 5'-monophosphate and subsequently to ribavirin 5'-diphosphate and -triphosphate. In vitro studies suggest that ribavirin triphosphate can selectively block capping of viral mRNA (3) and also may directly inhibit influenza virus RNA polymerase (2). Ribavirin also blocks GMP synthesis through competitive inhibition of IMP dehydrogenase (10, 12) and has a significant effect on purine metabolism in vitro (13). Such observations suggest that ribavirin may be a significant broad-spectrum antiviral drug, potentially useful in the treatment of epidemic and severe human viral infections.

The drug is well tolerated in small animals and in primates over a wide dosage range (4). However, with large parenteral doses of ribavirin in primates, anemia develops over a therapeutic course of 10 to 14 days, with hematocrit levels returning to normal after the end of therapy (P. G. Canonico, personal communication). Generally, oral therapy has been well tolerated in humans, although laboratory evidence suggests erythrocyte toxicity in controlled therapeutic trials (7).

The potential of ribavirin as a broad-spectrum antiviral agent in clinical practice clearly indicates the need for more extensive pharmacokinetic and toxicity studies in patients as further therapeutic trials are conducted. Such investigations have been restricted by the lack of a convenient and sensitive method to detect and quantitate ribavirin and its metabolites in clinical specimens. In this report we describe the development of a sensitive and specific radioimmunoassay (RIA) for ribavirin and its use in

quantitating ribavirin in plasma after several dosing regimens in humans.

MATERIALS AND METHODS

Succinylation of ribavirin. Ribavirin (50 mg, 0.23 mM; Viratek, Inc., Covina, Calif.) was solubilized in 1 ml of dimethylformamide (Mallinkrodt, Inc., St. Louis, Mo.) containing 0.5 ml of pyridine (Mallinkrodt, Inc.), after which 25 mg of succinic anhydride (0.25 mM; Matheson, Coleman and Bell Co., Norwood, Ohio) was added (1, 8a). Before dimethylformamide was used, it was dried with a type 4A molecular sieve (Linde Division, Union Carbide Corp., South Plainfield, N.J.), and pyridine was dried with calcium hydride. The reaction mixture was incubated in a stoppered flask at 60°C in a water bath overnight. As a guide to optimization of the succinylation procedure, samples were tested on cellulose thin-layer chromatography plates (no. 13254 Cellulose no. 6065; Eastman Kodak Co., Rochester, N.Y.) with butanol–5 N acetic acid (2:1) as the solvent. A new spot was found which migrated faster than ribavirin and which was dependent on the ratio of ribavirin to succinic anhydride, as well as upon the time and temperature of incubation. As a tracer, 30 μl of [^{3}H]ribavirin (specific activity, 15 Ci/mmol; ICN Corp., Irvine, Calif.) was added to the mixture. Pyridine and dimethylformamide were removed with a rotary evaporator under vacuum, after which the remaining syrup-like residue was dissolved in 100% methanol and the evaporation procedure was repeated twice more.

To separate succinylated from non-succinylated ribavirin derivatives, the final residue was dissolved in 3 ml of water, adjusted to pH 5.0 with 1 M ammonium hydroxide, and placed on a 7.0-ml quarternary aminoethyl (QAE) Sephadex A-25 anion-exchange column (HCO_3^- form) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The column was eluted with water, and then a linear gradient from 0 to 0.5 M ammonium bicarbonate was established and monitored by conductivity.

NMR. Nuclear magnetic resonance (NMR) spectra were obtained with an EM-360 60-MHz NMR spectrometer (Varian Instruments, Palo Alto, Calif.). Succinic acid shows a sharp singlet signal at δ2.6, whereas ribavirin shows no signal at δ2.6.

HPLC. An Altex high-pressure liquid chromatograph (Beckman Instruments, Inc., Fullerton, Calif.) with a C-18 micro Bondapak reverse-phase column (Waters Associates, Milford, Mass.) was used for high-pressure liquid chromatography (HPLC) analysis. Buffer A was 5 mM KH_2PO_4 (pH 3.34), and buffer B was 60% acetonitrile in water. Samples were separated in 15 min, using a linear gradient of 0 to 15% buffer B, a combined flow rate of 1.5 ml/min, and an operating pressure of 810 lb/in^2.

Protein coupling of ribavirin. Succinylated ribavirin was coupled to ovalbumin (OVA; Miles Laboratories Inc., Elkhart, Ind.), using the water-soluble carbodiimide coupling method. Briefly, 50 mg of lyophilized monosuccinylated ribavirin was dissolved in 0.5 ml of water and added to 50 mg of OVA in 5 ml of water. The reaction mixture was adjusted to pH 6.0 with 0.1 M NaOH, after which 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (ECDI; Sigma Chemical Co., St. Louis, Mo.) was added. After 1 and 2 h, an additional 40 and 20 mg, respectively, of ECDI were added. During the procedure, a constant pH was maintained with 0.1 M NaOH. After overnight incubation at room temperature on a rotator, the reaction mixture was centrifuged to remove particles and filtered through a 0.8-μm Millex filter (Millipore Corp., Bedford, Mass.). The reaction was terminated by passage over Sephadex G-25 (Pharmacia Fine Chemicals) equilibrated with water. The eluted protein was dialyzed against phosphate-buffered saline (pH 7.2) and stored at 4°C.

Immunizations. Adult New Zealand white rabbits were immunized subcutaneously at multiple sites on the back, using 125 to 500 mg of ribavirin-succinyl-OVA in complete Freund adjuvant; over the next 15 months, the rabbits were given booster injections monthly with 250 μg of the ribavirin-succinyl-OVA conjugate in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). At 1 to 2 weeks after each booster injection, the rabbits were bled, the serum was collected, and the antiribavirin titer was determined.

RIA. The RIA was performed in a single polypropylene tube (12 by 75 mm; W. Sarstedt, Inc., Princetown, N.J.). Phosphate-buffered saline with 1% bovine serum albumin was used in the RIA for all dilutions to keep the total assay volume at 500 μl. The procedure consisted of the sequential addition of 100 μl of diluted sample containing an unknown amount of ribavirin, 200 μl of RIA buffer, 100 μl of diluted rabbit antiribavirin serum, and 100 μl of [^{3}H]ribavirin (0.4 pmol; specific activity, 15 Ci/mmol). The tubes were then mixed on a vortex mixer. After overnight incubation at 4°C, 50 μl of bovine gamma globulin (10 mg/ml; Miles Laboratories, Inc.) and 550 μl of cold, saturated ammonium sulfate (pH 7.2) were added. The tubes were again mixed on a vortex mixer, incubated at 4°C for 2 h, and centrifuged at 4°C for 20 min at 1,400 × *g*. The supernatant was decanted, the remaining fluid was carefully aspirated, and the pellet was washed once with 1 ml of 50%-saturated ammonium sulfate (pH 7.2) at 4°C. After 10 min of incubation at 4°C, the tubes were centrifuged as before and the pellet was collected and dissolved in 100 μl of triple-distilled water. After the addition of 100 μl of 4 N HCl and 2 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.), the tubes were mixed on a vortex mixer. The RIA tubes were counted in a Beckman LS 250 scintillation counter.

With each RIA, a standard curve (0 to 100 pmol/100 μl) was included. These tubes were set up as described above, except that 100 μl of appropriately diluted normal sample (containing no ribavirin) replaced the 100 μl of clinical study sample. Total count tubes containing only 100 μl of [^{3}H]ribavirin and blank tubes containing all RIA constituents except antiserum were also included in each RIA. All assays were done in triplicate. From the scintillation counting data, the standard curve was obtained by plotting concentration versus percentage binding (B/B_o on Fig. 3), i.e., percent binding = (number of counts bound in the presence of unlabeled ribavirin or test sample/number of counts bound in the absence of unlabeled ribavirin or test sample) × 100, on log-logit paper, and the unknown concentrations were read directly from the graph. Each clinical sample was assayed at two dilutions, and the mean ribavirin concentration was reported.

For determination of antiserum titers, a binding assay consisted of tubes containing only 100 μl of antiserum, 100 μl of [^{3}H]ribavirin, and 300 μl of RIA buffer. These tubes were processed as per the same procedure for tubes for the RIA.

RESULTS

Characterization and protein coupling of succinylated ribavirin. After succinylation, ribavirin and its derivatives were eluted from QAE Sephadex A-25 (HCO_3^- form) columns in three peaks, as determined by radioactivity or UV spectroscopy (Fig. 1). Unreacted ribavirin produced a single peak that co-chromatographed with peak I. NMR revealed that succinyl groups were present in peak II, as evidenced by the signal centered at δ2.6. The integration ratios of the peaks agreed with the assumption that peak II contained monosuccinylated ribavirin. The spectra of material from peak III gave evidence of polysuccinylated ribavirin or succinic acid or both.

When the material from peak II was chromatographed by HPLC, a major peak appeared predominantly at 13.5 min, with smaller fused peaks at 11.8 and 12.0 min. When the material was assumed to have the same extinction coefficient as ribavirin, there appeared to be ca. 25-fold more succinylated ribavirin in the major peak as compared with the two smaller peaks. The material from peak I (i.e., non-succinylated ribavirin) was not retained on the column.

Before the monosuccinylated ribavirin was coupled to OVA, peak II, containing monosuccinylated ribavirin, was chromatographed on Ag-50W-X4 cation-exchange resin (Bio-Rad Laboratories, Richmond, Calif.) to remove ammonium bicarbonate. This material yielded a single peak (Fig. 2). Carbodiimide coupling resulted in ca. 8.5 mol of monosuccinylated ribavirin binding to each mol of OVA.

Rabbit antiribavirin serum. Of seven rabbits injected with ribavirin-succinyl-OVA conjugate, one responded by producing antibody with a high specific binding activity for ribavirin after six to seven booster injections, using different batches of conjugate, over a 15-month period. Two other rabbits initially developed antibody of low binding activity, but neither responded to further antigen stimulation.

Competitive binding RIA for ribavirin. RIA results were optimum when the assay was run at pH 7.6 with overnight incubation at 4°C, using 0.4 pmol of [^{3}H]ribavirin per 100 μl (i.e., 25,000

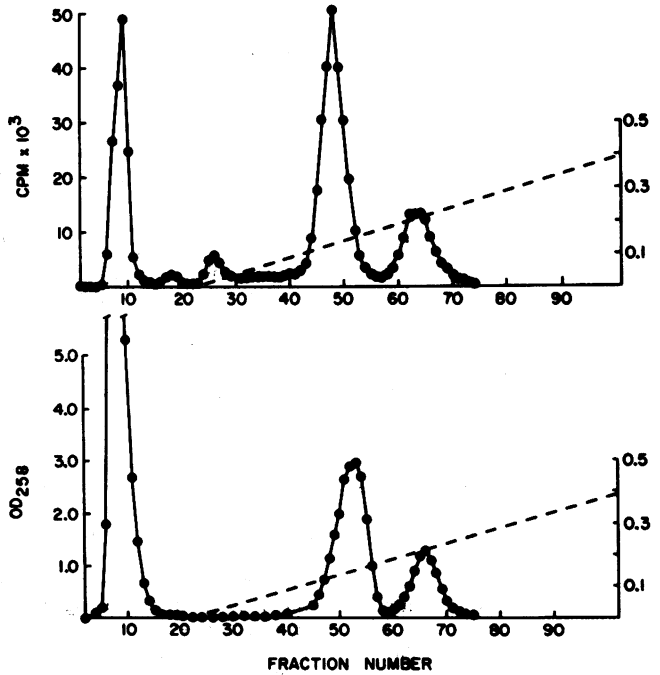

FIG. 1. Separation of succinylated and non-succinylated ribavirin by chromatography on a QAE Sephadex A-25 anion-exchange (HCO_3^- form) column. Column characteristics are as described in the text. The three major peaks monitored by radioactivity or by optical density at 258 nm represent free ribavirin (peak I), monosuccinylated ribavirin (peak II), and polysuccinylated ribavirin or succinic acid or both (peak III). Fraction volumes were 2 ml.

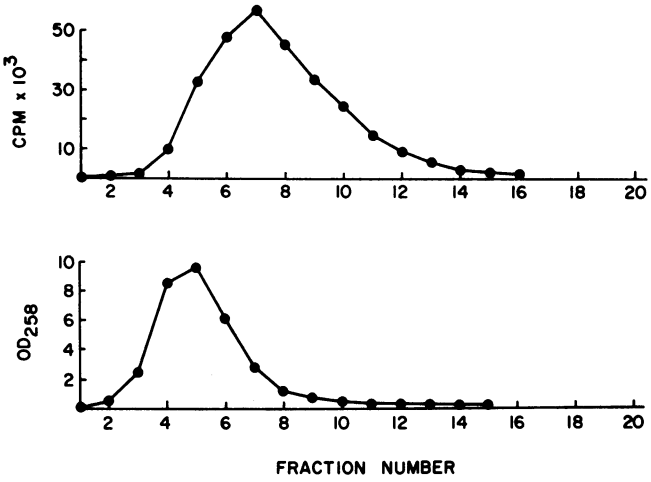

FIG. 2. Elution of monosuccinylated ribavirin from AG-50W-X4 cation-exchange resin. Monosuccinylated ribavirin is peak II material from QAE Sephadex A-25 (HCO_3^- form), chromatographed on two 5-ml columns of AG-50W-X4 cation-exchange resin to remove ammonium bicarbonate; one column was monitored by radioactivity and one was monitored by optical density at 258 nm. Fraction volumes were 1 ml.

cpm). The standard curve was linear over the range of ribavirin concentrations of 0.19 to 100 pmol per 100 μl (Fig. 3), but the shorter range of 1 to 10 pmol per 100 μl was routinely used for the determination of ribavirin levels in clinical samples. After 23 consecutive RIAs, the standard curve I_{50} values ranged from 2 to 4 pmol of ribavirin per 100 μl, with a mean of 3.2 ± 0.5 and a coefficient of variation of 16%. The assay was sensitive to 1 pmol of ribavirin per 100 μl in RIA buffer, plasma, or urine.

RIA. Standards of nominal ribavirin concentrations of 10, 2, 1, and, after a 1/50 dilution, 2 pmol of ribavirin per 100 μl were included in 23 RIAs; the mean values ± standard deviation were 10.8 ± 1.0, 1.9 ± 0.3, 1.0 ± 0.2, and 1.9 ± 0.4 pmol of ribavirin per 100 μl, respectively. Two monkey serum samples collected after one 15-mg/kg intramuscular dose of ribavirin were also subjected to repeated analysis by the RIA; the mean values were 3.3 ± 0.4 and 1.4 ± 0.3 pmol of ribavirin per 100 μl.

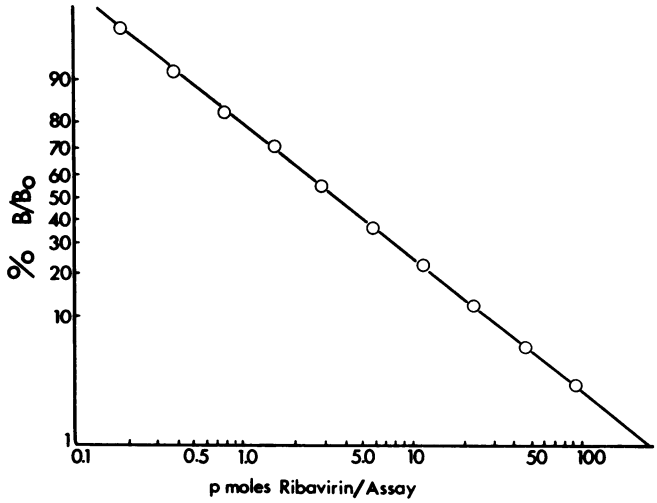

FIG. 3. Representative standard curve for ribavirin RIA. Parallel standard curves were obtained in multiple experiments yielding a mean I_{50} value ± standard deviation of 3.2 ± 0.5 pmol of ribavirin per 100 μl. The assay was run at pH 7.6 with 0.4 pmol of [^{3}H]ribavirin. The curve was plotted on log-logit paper.

Characterization of antiserum. The major degradative metabolites of ribavirin (i.e., 1,2,4-triazole-3-carboxamide [T-$CONH_2$], 1,2,4-triazole-3-carboxylic acid, and 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxylic acid; [14]) were tested for binding to the rabbit antibody over a range of 0.1 to 100 μM. Only T-$CONH_2$ reacted with the rabbit antiribavirin serum. When equimolar quantities of unlabeled ribavirin and T-$CONH_2$ were used in the competitive binding RIA, the rabbit antiserum detected only 2 to 5% as much T-$CONH_2$ as ribavirin. Although the data is not shown, ribose (5 μM), hypoxanthine (100 μM), uridine (100 μM), guanosine (100 μM), GMP (100 μM), thymidine (100 μM), and uric acid (100 μM) did not bind to the rabbit antibody.

Animal and human specimens assayed. RIA-coded specimens of murine plasma after intraperitoneal administration of ribavirin at a dosage of 40 mg/kg are shown in Table 1. Predose specimens had undetectable levels. Rising levels in plasma were seen as a result of absorption across the peritoneal membrane, and a decline in levels was noted 4 to 6 h later.

RIA results of coded sera from adults enrolled in a Sierra Leone Lassa fever study who received ribavirin orally or by intravenous infusion are shown in Table 2. Pretreatment samples had no detectable ribavirin. Sera from oral treatment (days 1 to 10) had concentrations of ribavirin ranging from 1.2 to 9.6 μM. Sera from patients receiving intravenous treatment at much higher doses contained higher concentrations of ribavirin, rising from pretreatment, undetectable levels to a range of 4.4 to 69 μM with treatment. The mean concentrations for 5 patients were 43.7, 33.4, 27.0, 23.1, and 33.2 μM during therapy.

Plasmas obtained at intervals from four children receiving ribavirin aerosol therapy for respiratory viral infections in San Diego, Calif., had concentrations that increased from undetectable pretherapy levels to peak levels ranging from 0.3 to 1.8 μM with a mean of 0.76 μM and estimated half-life ($t_{1/2}$) of 9.5 h (Table 2).

TABLE 1. Ribavirin levels in plasma of mice injected with ribavirin[a]

Sample time after dose (h)	Level of ribavirin (μM)
0	0
½	25
1	26
2	25
4	13
6	13

[a] Mice treated for influenza pneumonitis received 40 mg/kg intraperitoneally. Plasma specimens were supplied, courtesy of Fred Hayden, University of Virginia, Charlottesville.

TABLE 2. Ribavirin levels in human sera and plasma

Route of administration	Dosage	Duration	Ribavirin concn (μM)	
			Mean	Range
Oral[a]	1,000 mg per day	10 days	3.1	1.2–9.6
Intravenous[b]	1,000 mg per dose	4 days	32.1	4.4–69.0
Aerosol[c]	2 mg/kg	3 days	0.8	0.3–1.8

[a] Serum samples were from four Sierra Leone patients, courtesy of Joseph McCormick, Special Pathogens Branch, Centers for Disease Control, Atlanta, Ga. Patients received 1,000 mg per day in three divided doses. Samples were collected ca. 2.5 h postdose.

[b] Serum samples were from five Sierra Leone patients, courtesy of Joseph McCormick, Special Pathogens Branch, Centers for Disease Control, Atlanta, Ga. Patients received 4,000 mg per day in four divided doses. Samples were collected ca. 2.5 h postdose.

[c] Plasma samples were from four patients enrolled in an aerosol clinical study at the University of California, San Diego. With a ribavirin concentration of 20 mg/ml in the reservoir of the aerosol generator, the aerosol delivers a dosage of 0.82 mg/kg per h. The dosage was equal to 2.0 mg/kg (0.82 mg/kg per h × 2.5 h).

DISCUSSION

As antiviral agents move into clinical use, it is important that pharmacologic and pharmacokinetic parameters for these compounds be defined. Until now, there has been no method to analyze the absorption, fate, and excretion of the broad-spectrum antiviral drug ribavirin in patient studies. HPLC has often been used to detect nucleoside antiviral agents. However, HPLC has not been useful in preclinical or field studies with ribavirin since the defined absorption spectra markedly limit the specific detection of this compound in the presence of other UV-absorbing compounds found in biological specimens. Further, mass spectrometry has not provided a reliable system for quantitating ribavirin. We have developed a sensitive and specific RIA to permit the measurement of ribavirin in clinical specimens.

The competitive binding RIA is specific for ribavirin and is capable of measuring to levels of 10 pmol/ml (0.01 μM). Of the metabolites of ribavirin, only the major metabolite, T-$CONH_2$, cross-reacted by 2 to 5% with the rabbit antibody.

The major metabolite, T-$CONH_2$, appears in plasma and other biological fluids, including

urine, and conceivably could interfere with the quantitation of ribavirin by the RIA. The relative concentration of T-$CONH_2$–ribavirin that must be present in a sample to interfere with the ribavirin RIA is 10:1 or a T-$CONH_2$ concentration of greater than 100 pmol per 100 μl. However, such high ratios of T-$CONH_2$–ribavirin are not known to be present in human biological fluids. Mock samples, prepared to contain T-$CONH_2$–ribavirin concentrations of ≤10:1, had no effect upon ribavirin quantitation. In animal studies, the highest ratio of T-$CONH_2$–ribavirin occurred in urine (6:1), with levels of T-$CONH_2$ exceeding 1,000 pmol per 100 μl (8), whereas in plasma, the T-$CONH_2$–ribavirin ratio was usually 1:1. As the excretion and metabolism of ribavirin occurred, the ratio exceeded 1:1, but the levels of T-$CONH_2$ were below the detection limits of the RIA (8). For these reasons, it is not necessary to pretreat plasma or serum samples, or other biological materials, except for urine, before assay. In the case of urine, T-$CONH_2$ can be removed by a simple procedure before ribavirin quantitation. Separation of T-$CONH_2$ from ribavirin is accomplished by liquid chromatography on columns of Sephadex G-25 layered over QAE Sephadex A-25 (HCO_3^- form). Sephadex G-25 retards the passage of salts, whereas QAE Sephadex A-25 (HCO_3^- form) retards the passage of T-$CONH_2$, and ribavirin, which is not retarded, can be quantitatively recovered in the column effluent.

We have used the ribavirin RIA to measure ribavirin in animal and human plasma and sera after therapy with ribavirin. Levels varied according to dose and route of administration of ribavirin but, in every case, rose from pretreatment undetectable concentrations to levels which would be predicted to have substantial antiviral activity against a variety of susceptible viral agents.

The RIA can now be employed to examine the pharmacokinetics of ribavirin as dose tolerance and therapeutic studies are done. The ability to monitor ribavirin concentrations should help in understanding the relationships between dose, distribution, and toxicity of this antiviral agent.

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