Journal of Chromatography, 420 (1987) 189–196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3727

Note

High-performance liquid chromatographic determination of ribavirin in serum and urine and of its urinary metabolite 1,2,4-triazole-3-carboxamide

RITA PARONI

Department of Medical Chemistry and Biochemistry, University of Milan, Milan (Italy)

CESARE R. SIRTORI

Institute of Pharmacological Sciences, University of Milan, Milan (Italy)

CRISTINA BORGHI

Ciba-Geigy Medical Department, Origgio (Italy)

and

MARZIA GALLI KIENLE*

Department of Medical Chemistry and Biochemistry, University of Milan, Via Saldini 50, 20133 Milan (Italy)

(First received December 24th, 1986; revised manuscript received March 30th, 1987)

Only a few substances are known to demonstrate a broad spectrum of antiviral activity against DNA and RNA viruses both in vitro and in vivo. Among these agents, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a well known synthetic compound, structurally related to the naturally occurring ribonucleoside guanosine [1]. Its synthesis and antiviral activity were first described in 1972 by Witkowski et al. [2], and its in vitro and in vivo antiviral spectrum was later reported by other authors [3–7]. The toxicology and pharmacology of ribavirin were assessed in experimental animals [8] as well as in humans, where its effects on the immune system [9] and blood compartment were investigated [10].

Ribavirin demonstrated clinical effects in the treatment of influenza, viral pneumonia and respiratory syncytial viral infections [11–13] when administered as a small particle aerosol, and was effective in the treatment of type A hepatitis [14] and acute lassa fever [15] after oral administration. In view of the growing interest in the use of ribavirin in clinical practice, a simple and sensitive method

0378-4347/87/$03.50

is required to evaluate the plasma levels of the unmodified drug in order to correlate this parameter with therapeutic efficacy or toxic effects during clinical trials.

We have developed a specific and sensitive high-performance liquid chromatographic (HPLC) method for the measurement of ribavirin in serum at therapeutic levels. The method also allows the evaluation of the urine concentrations of the carboxamide produced by hydrolysis of the ribosylic moiety of the molecule, which is the main urinary metabolite of ribavirin [16]. Results are reported on the serum decay of ribavirin and on the urinary excretion in a healthy volunteer after an oral administration of 1 g of ribavirin.

EXPERIMENTAL

Reagents and chemicals

Pure ribavirin, 1,2,4-triazole-3-carboxamide, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxylic acid, 1,2,4-triazole-3-carboxylic acid and ribavirin 5′-mono- and triphosphates were obtained from ICN Pharmaceuticals (Irvine, U.S.A.). Uridine, which was used as the internal standard, was purchased from Sigma (St. Louis, MO, U.S.A.). All solvents and other chemicals were from Merck (Darmstadt, F.R.G.) and were of the highest commercially available purity. Dowex 1X4-100 (50–100 mesh) anion-exchange resin was from Sigma and Lewatit S-1080 (60–150 mesh) cation-exchange resin was from Merck. Centricon 30 membranes were supplied by Amicon (Danvers, MA, U.S.A.). All the HPLC eluents were filtered through 0.45-μm Millipore membrane filters (Molsheim, France) before analysis.

Apparatus

A Gilson liquid chromatograph was used (pump Model 302, flow-rate controller Model 802, gradient mixer Model 811) equipped with a LiChrosorb C_{18} reversed-phase column (25 cm$\times$0.5 cm I.D., 7-μm packing, Merck), an RP-18 Guard-Pak column (P/N 080040, Waters Assoc., Milford, MA, U.S.A.), a variable-wavelength UV detector (Gilson Model 440), an injector valve (Model 7125 Rheodyne, Berkeley, CA, U.S.A.) and a Baush & Lomb recorder (Houston Instruments, Austin, TX, U.S.A.).

Extraction procedure

Ribavirin was extracted from serum essentially as described by Roboz and Suzuki [17]. To 1 ml of serum, uridine (5 μg) was added as the internal standard. After vortex-mixing, samples were diluted with 1 ml of doubly distilled water and vortexed again. The diluted serum was deproteinized by molecular filtration through membranes with a 30 000 exclusion limit. After centrifugation for 2 h at room temperature at 2750 *g*, ca. 80% of the diluted serum was filtered. The clear filtrate was then extracted with 1.5 ml of dichloromethane to remove residual non-polar compounds. Samples were further purified by filtration through a double-bed column (1 cm I.D.) packed with 2.3 ml of wet Dowex 1X4-100 (chloride form) layered on 0.8 ml of wet Lewatit S-1080 (hydrogen form). The column

was washed with 3 ml of water, which was collected into the sample vials. The resulting solution was dried under vacuum at 30°C, the residue was dissolved in 0.6 ml of water, and aliquots (20–100 μl) were analysed by HPLC.

Purification of urine samples was carried out as described by Roboz and Suzuki [17]. After the addition of uridine as the internal standard (25 μg/ml), the aqueous solution obtained after urine treatment with methanol, centrifugation and washing with *n*-hexane was taken to dryness under nitrogen and vacuum. The residue was then dissolved in 3.0 ml of water and aliquots (100 μl) were analysed by HPLC.

Chromatography

Among the various conditions tested, elution using distilled water as the mobile phase at a flow-rate of 1 ml/min gave the best separation of ribavirin, uridine, carboxamide and the endogenous interfering peaks of serum and urine. Peaks were detected at 207 nm.

Under these conditions retention times were 4, 6.9 and 13 min for the carboxamide, ribavirin and uridine, respectively. Other described metabolites of ribavirin, such as 1,2,4-triazole-3-carboxylic acid, its 1-β-D-ribofuranosyl derivative and ribavirin mono- and triphosphates [16] showed retention times shorter than 3 min; therefore, even if present, they were not detectable in serum samples owing to the presence of several interfering peaks at the corresponding retention times.

Preparation of standards

Stock solutions of ribavirin, 1,2,4-triazole-3-carboxamide and uridine were prepared in distilled water at 1 mg/ml and 1 μg/ml and stored at −30°C. Under these conditions standard samples were stable for several months.

To standardize the extraction procedure, and to find optimal analytical conditions, calibration curves were prepared with normal pooled human serum and urine specimens spiked with known amounts of ribavirin and/or carboxamide and of the internal standard. The concentrations of the standards were in the range expected in the samples (20 ng to 1.0 μg) for ribavirin in serum, and 5–50 and 50–500 μg/ml for ribavirin and carboxamide, respectively, in urine. A blank sample and a sample with only uridine added were also included in the calibration curves. The concentration of ribavirin in serum and urine was computed from ribavirin/uridine peak-height ratios on the basis of the regression line calculated for the calibration curves.

Ribavirin serum and urine levels

Blood samples were withdrawn at different times after oral administration of 1 g of ribavirin to a healthy volunteer. Blood was left at room temperature for 25 min and serum was obtained by centrifugation at 2750 *g* for 10 min. Urine was collected for 24 h after administration, and the volume was measured. All the samples were stored at −80°C until used.

The stability of ribavirin in serum and of ribavirin and carboxamide in urine was checked over a period of two months, by analysis of extracts prepared from control serum and urine samples to which 1 μg/ml of the authentic compounds

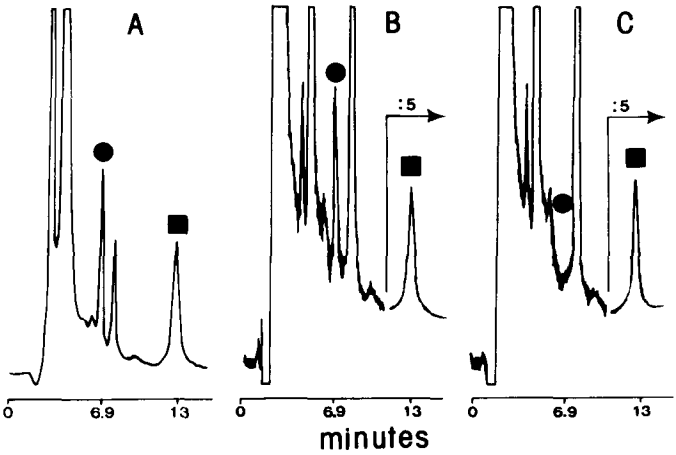

Fig. 1. Analysis of ribavirin in serum. (A) Normal pooled serum spiked with 5 μg/ml ribavirin; (B) serum sample collected 0.5 h after oral administration of 1 g ribavirin; (C) serum sample collected from the same subject before administration. Uridine (5 μg/ml) was added to serum samples B and C before extraction. Peaks: ● = ribavirin; ■ = uridine.

had been added before storing at −30°C. Differences between values determined in extracts prepared immediately after the addition of the standards and those prepared after two months of storage at −30°C were within the limits of variation of the analytical method.

Ribavirin and carboxamide in extracts obtained from serum and urine samples were stable at all the tested concentrations (20–1000 ng/ml) even if kept at room temperature for 8 h.

Stability in blood was checked by analysis of levels of ribavirin in serum obtained from control blood to which 1 μg/ml authentic ribavirin had been added immediately after blood collection from volunteers. Before extraction the blood samples were kept at room temperature for 20, 40 and 60 min and then centrifuged to obtain serum. Uridine was added to the serum (5 μg/ml) before the usual extraction. The results showed that under these conditions no degradation of ribavirin occurs during the tested time.

RESULTS AND DISCUSSION

The HPLC analysis of ribavirin showed a good reproducibility. Repeated injections of ribavirin, in a single serum extract (1 μg ribavirin per ml serum) showed a coefficient of variation (C.V.) of 6.2% when the sample was analysed over a period of six days (one analysis per day). The C.V. for the same serum extract analysed on one day was 2.8% ($n=4$). To check the linearity of the method, the ratios of the peak heights of uridine were plotted against the concentration (μg/ml) of ribavirin added to the calibration curve prepared in serum. A linear response was obtained for ribavirin concentrations from 20 to 1000 ng/ml, which were the expected serum levels after the therapeutic dosage. The equation of the line calculated by regression analysis was $y=0.294\ (\pm 0.012)x+0.005\ (\pm 0.006)$

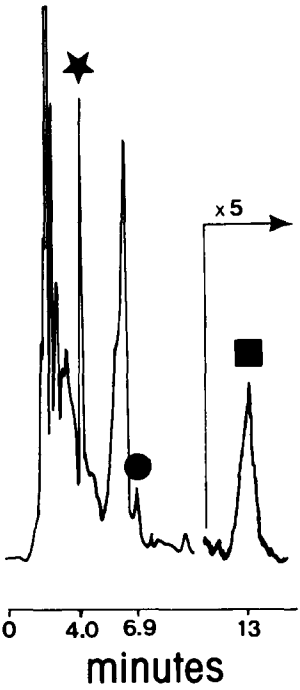

Fig. 2. Urinary excretion of ribavirin and 1,2,4-triazole-3-carboxamide. Urine collected during the 24 h following oral treatment of a healthy subject with 1 g of ribavirin was spiked with uridine (25 μg/ml), extracted and analysed by HPLC as described in the text. Uridine was detected at a sensitivity five-fold higher than that used to detect ribavirin and the carboxamide. Peaks: ★ = 1,2,4-triazole-3-carboxamide; ● = ribavirin; ■ = uridine.

(r=0.997). Since the internal standard was added before any manipulation of the sample, the linearity relates not only to the HPLC analysis but also to the extraction procedure. The recovery of ribavirin from serum was found to be

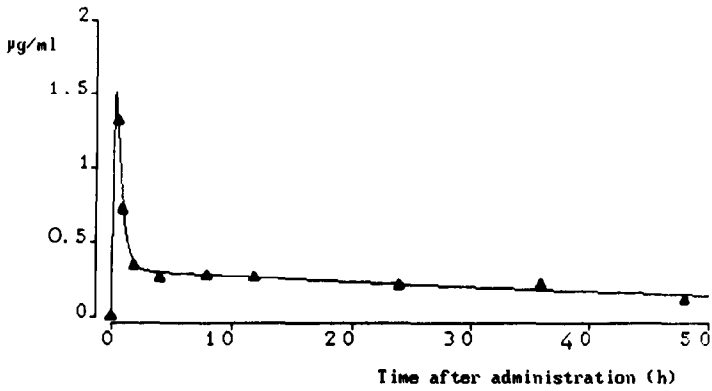

Fig. 3. Plasma decay of ribavirin. Triangles represent concentrations of ribavirin found in serum specimens of a subject after administration of 1 g of ribavirin. The curve was fitted with a three-compartment model by a computer program [20]; the correlation coefficient of the curve was 0.996.

82.0 ± 6.1% both at 100 and 500 ng/ml. Because elution of standard ribavirin from the ion-exchange column gave a complete recovery of the compound, the result obtained is due to incomplete filtration of serum through the Centricon membrane.

The basal concentration of uridine in the serum of healthy volunteers, determined under the analytical conditions used for ribavirin, was 1.1 ± 0.1 μg/ml (mean ± S.D.; $n=5$), in good agreement with levels measured under different HPLC conditions [18, 19]. Since inter-individual variability was found to be low and 5 μg/ml internal standard was used, the presence of the endogenous compound did not significantly modify the slope of the standard curve determined in different serum specimens. To our knowledge, pathological conditions implying a severe modification of uridine serum levels were not reported. Nevertheless, as in the usual practice, analysis of blank serum of subjects to be included in the protocol of a pharmacokinetic study should be carried out to check for the presence of any compound that may give rise to erroneous evaluation of the peak of ribavirin and of the internal standard.

The chromatograms of a serum specimen spiked with a known amount of ribavirin (A), a serum from a subject 30 min after a normal oral dose of ribavirin (B) and a control serum (C), are shown in Fig. 1. An interfering peak due to endogenous component(s) was present at the retention time of ribavirin. Nevertheless, the ratio between this peak height and that of the internal standard, obtained in the analysis of various standard serum samples to which only uridine had been added (5 μg/ml), showed that the interference peak corresponded to 51 ± 11 ng/ml ribavirin (mean ± S.E; $n=4$). This concentration is less than half that observed in human serum (125 ng/ml) even 48 h after administration of a therapeutic dose.

Recovery of ribavirin and carboxamide from urine was complete, and a linear correlation was obtained for the calibration curve both for ribavirin ($r=0.988$) and the carboxamide ($r=0.983$). Extracts were also prepared from control urine avoiding the addition of the internal standard. No peak was detectable at the retention time of uridine at the sensitivity used to measure the internal standard signal.

As an application of the method we studied the pharmacokinetic profile of the unchanged ribavirin after oral administration of 1 g to a volunteer. In the chromatogram of urine samples collected for 24 h after administration, peaks of the carboxamide and ribavirin were clearly identifiable (Fig. 2) and corresponded to 27.5 and 128 μg/ml, respectively; the peaks were absent from extracts of urine collected before the treatment. From the peak heights in this sample it was calculated that urinary excretion of unmodified ribavirin accounted for only 3.3% of the oral dose; 32% was found as the carboxamide metabolite. Fig. 3 shows the serum decay curve of the intact compound. After very fast absorption and distribution phases, the decrease of the drug levels was then very slow, giving a long-lasting elimination phase (β-phase). The peak concentration was 1.50 μg/ml and occurred 0.30 h after administration. The half-life calculated for the β-phase was 42.7 h.

The method described here permits rapid, specific and sensitive quantification of ribavirin in serum and urine, and of the 1,2,4-triazole-3-carboxamide metab-

olite excreted in urine. Prior to the establishment of the described HPLC method we considered the possibility of using the previously described analysis of trimethylsilyl derivatives by mass fragmentography [17]. The derivatives of ribavirin, of the carboxamide and of uridine were easily obtained and separated using a capillary column. Multiple selected-ion monitoring analysis was found to be highly sensitive for the analysis of standard samples. However, in our hands, silylation of serum extracts purified as described [17] was not efficient enough.

By the method reported here, quantification of authentic ribavirin can be carried out at a lowest limit of 0.5 ng injected, with a C.V.of 2.7% ($n=5$). However, the limit is higher if quantification in serum extracts is considered, owing to the above-mentioned basal value of absorption corresponding to 51 ng/ml. Under the described conditions, at most 17% of the extract obtained from 1 ml of serum was injected, so the basal value corresponds to 8.7 ng. The minimum serum concentration giving rise to a signal-to-noise ratio of 2 corresponds therefore to ca. 100 ng/ml (17 ng injected), a concentration that is only four-fold higher than that claimed to be quantified by the mass fragmentographic method [17], and is suitable for the detection of the levels expected after the therapeutic dosage. In addition, the method described here offers rapid sample preparation without derivatization and does not require sophisticated instruments. It appears, therefore, suitable for the study of the pharmacokinetics of ribavirin and for routine analysis to be carried out eventually during clinical trials. Other antiviral agents are nucleoside analogues as ribavirin. In this work no attempt was made to check possible interferences in the analysis of ribavirin and carboxamide derived from the presence in blood of any of these agents and (or) of their metabolites. Nevertheless, this kind of interference should not be a drawback for the method because it is usual in clinical practice to take care to avoid simultaneous administration of drugs with similar structural features when pharmacokinetics of a drug are evaluated.

REFERENCES

1 P. Prusiner and M. Sundaralingam, Nature New Biol., 244 (1973) 116.
2 J.T. Witkowski, R.K. Robins and R.W. Sidwell, Abstracts of the 163rd Meeting of the American Chemical Society, Boston, MA, 1972.
3 R.W. Sidwell, J.H. Huffman, G.P. Khare, L.B. Allen, J.T Witkowski and R.K. Robins, Science, 177 (1972) 705.
4 G.P. Khare, R.W. Sidwell, J.T. Witkowski, L.N. Simon and R.K. Robins, Antimicrob. Agents Chemother., 3 (1973) 517.
5 J.H. Huffman, R.W. Sidwell, G.P. Khane, J.T. Witkowski, L.B. Allen and R.K. Robins, Antimicrob. Agents Chemother., 3 (1973) 235.
6 R.W. Sidwell, in R.A. Smith and W. Kirkpatrick (Editors), Ribavirin: a Broad Spectrum Antiviral Agent, Academic Press, New York, 1980, p. 23.
7 J.T. Witkowski, R.K. Robins, R.W. Sidwell, and L.N. Simon, J. Med. Chem., 15 (1972) 1150.
8 P.G. Canonico, M. Kende and J.W. Huggins, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 65.
9 B.W. Jolley and C. Suchil, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 93.
10 N.R. Shulman, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 79.

11 B.E. Gilbert, S.Z. Wilson, V. Knight, R.B. Couch, T.L. Melhof, H.W. McClung, G.W. Divine, D.D. Bartlett, L.C. Cohan and T.L. Gallion, in R.A.Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 125.
12 E.W. Gelfand, D. McCurdy, C. Pando Rao and P.J. Middleton, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 155.
13 C.B. Hall, J.T. McBride, E.E. Walsh, R.F. Betts, D.M. Bell, W.J. Hall, C.L. Gala, S.W. Hildreth, L.G. Ten Eyck and J.F Hruska, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 165.
14 J.B. McCormick, P.A Webb, K.M. Johnson and C.S. Scribner, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 187.
15 F.S. Sanchez, E.A. Nunez, G.M. Vargas and I.R. Guevara Sosa, in R.A. Smith, V. Knight and J.A.D. Smith (Editors), Clinical Applications of Ribavirin, Academic Press, Orlando, FL, 1984, p. 203.
16 D.H. Catlin, R.A. Smith and A.I. Samuels, in R.A. Smith and W. Kirkpatrick (Editors), Ribavirin a Broad Spectrum Antiviral Agent, Academic Press, New York, 1980, p. 83.
17 J. Roboz and R. Suzuki, J. Chromatogr,. 160 (1978) 169.
18 J.M. Karle, L.W. Anderson, D.D. Dietrick and R.L. Cysyk, Anal. Biochem., 109 (1980) 41.
19 M. Zakaria, P.R. Brown, M.P. Farnes and B.E. Barker, Clin. Chim. Acta, 126 (1982) 69.
20 J.L. Valentine and S. Hunter, J. Pharm. Sci., 74 (1985) 113.