Journal of Chromatography, 553 (1991) 35–40
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2351

High-performance liquid chromatographic determination of rifapentine and its metabolite in human plasma by direct injection into a shielded hydrophobic phase column

E. RIVA*, R. MERATI and L. CAVENAGHI
Marion Merrell Dow Research Institute, Lepetit Centre, Via R. Lepetit 38, 21040 Gerenzano (Italy)

ABSTRACT

A simple high-performance liquid chromatographic method for determination of rifapentine, a cyclopentyl semisynthetic analogue of rifamycin belonging to the class of piperazinyl hydrazone derivatives of 3-formylrifamycin SV, and its metabolite, 25-desacetylrifapentine, in human plasma was developed using direct injection of the sample onto a Supelco LC HISEP column. The mean recovery was 100.3% for rifapentine and 99.7% for the metabolite and the precision of the assays was 3% and 7%, respectively. The limit of determination was 0.2 μg/ml and the method was validated for concentrations up to 64 μg/ml for rifapentine and 32 μg/ml for the metabolite. The results correlated well with those of the microbiological assay with *Sarcina lutea* as test organism.

INTRODUCTION

Rifapentine (Fig. 1), an antibiotic currently used in clinical trials, has usually been assayed by a conventional microbiological assay with *Sarcina lutea* as test organism or using a multi-step extraction procedure with different solvents (ethyl acetate,

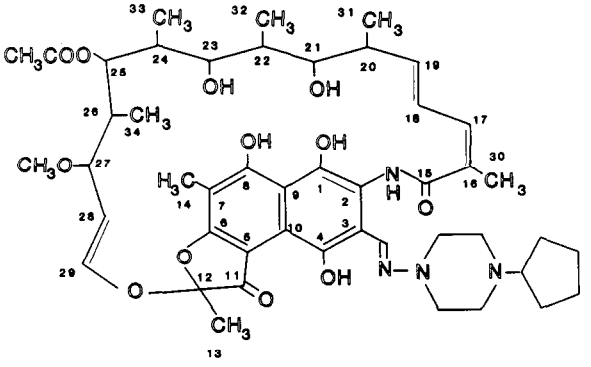

Fig. 1. Structure of rifapentine (MW 877.06).

0021-9673/91/$03.50

acetonitrile, *n*-heptane) followed by high-performance liquid chromatography (HPLC) [1–3].

We have recently developed an HPLC method for the determination of the antibiotic and its major metabolite in human plasma that uses direct injection of biological samples. It has the advantage over the previous HPLC method of eliminating several time-consuming steps, therefore increasing the accuracy, while retaining the advantage over the microbiological assay of permitting the determination, in the same assay, of rifapentine and its main metabolite. The shielded hydrophobic phase column used (Supelcosil LC HISEP) [4–6] represent a new concept for the separation of drugs because it is designed to avoid precipitation of the protein on the chromatographic support.

This paper describes a simple HPLC method for the determination of the level of rifapentine (rifap) and of its major metabolite, 25-desacetylrifapentine (desa), in human plasma that only requires filtration of the sample.

EXPERIMENTAL

Chemicals and reagents

Rifapentine, batch LCR-E/5/85, and 25-desacetylrifapentine, batch C/6/87, were internal standards produced by Lepetit. Analytical-reagent grade 2-(N-morpholino)ethanesulphonic acid (MES) was purchased from Sigma and trifluoroacetic acid and ethanolamine from Carlo Erba. Acetonitrile, methanol and tetrahydrofuran of HPLC grade were purchased from Carlo Erba or Rudi Pont.

Standard solutions

Stock standard solutions of rifap and desa were prepared by dissolution in methanol at a concentration of 2 mg/ml then diluted with distilled water, containing 1 mg/ml of ascorbic acid, to a final concentration of 1 mg/ml.

Spiked samples were prepared by adding increasing amounts of the stock standard solution to human plasma to obtain the following concentrations in μg/ml: sample 1, 0.94 rifap, 0.47 desa; sample 2, 3.78 rifap, 1.88 desa; sample 3, 15.13 rifap, 7.52 desa; and sample 4, 60.54 rifap, 30.08 desa. Each spiked sample was analysed five times on each of three different days and the analytical response obtained are summarized in Table I.

Chromatographic conditions

The HPLC system consisted in two Gilson Model 305 pumps, a dynamic mixer, a Gilson Model 805 manometric module and a Gilson Model 116 UV detector operating at 254 nm, controlled by an IBM PS/2 computer through the Gilson 714 program. Collection and analysis of the detector output were performed with Hewlett-Packard A Series computer using the Hewlett-Packard LDS software. Samples (50 μl) were injected with Gilson ASPEC autosampler onto a guard column (20 × 4.6 mm I.D.) of Supelcosil LC HISEP in series with a Supelcosil LC HISEP column (150 × 4.6 mm I.D.). Gradient elution was performed at room temperature at a flow-rate of 1.4 ml/min. The two mobile phases were (A) 0.0026 *M* MES–acetonitrile (95:5) containing trifluoroacetic acid (1‰) and adjusted to pH 6.5 with ethanolamine and (B) 0.0026 *M* MES–acetonitrile–tetrahydrofuran (7:2:1) containing trifluoroacetic acid

TABLE I

RESULTS FOR FIVE REPLICATES AT FOUR CONCENTRATIONS ANALYSED ON EACH OF THREE SUCCESSIVE DAYS

In the concentration ranges 0.94–60.54 μg/ml for rifapentine and 0.47–30.08 μg/ml for the metabolite the within-day precisions (means of the daily R.S.D. were 1.58%, 1.44%, 1.05% and 1.05% for rifapentine and 5.83%, 3.71%, 2.34% and 0.27% for metabolite, respectively. The between-day precision (R.S.D. of the daily mean) were 2.06%, 0.71%, 0.27% and 0.01% for rifapentine and 2.57%, 2.33%, 0.68% and 2.69% for the metabolite, respectively. The mean accuracies were 100.29% for rifapentine and 99.53% for the metabolite.

Parameter	Rifapentine				25-Desacetylrifapentine			
	Sample (μg/ml) (n = 5)	Day 1	Day 2	Day 3	Sample (μg/ml) (n = 5)	Day 1	Day 2	Day 3
Mean (μg/ml)	0.94	0.96	0.93	0.95	0.47	0.47	0.46	0.45
R.S.D. (%)		1.65	2.23	0.82		6.05	7.03	4.57
Accuracy (%)		102.60	98.58	101.50		100.14	98.48	94.97
Mean (μg/ml)	3.784	3.83	3.78	3.78	1.88	1.83	1.88	1.93
R.S.D. (%)		1.36	1.54	1.40		2.35	2.41	6.39
Accuracy (%)		101.10	99.93	99.80		97.46	99.02	102.46
Mean (μg/ml)	15.13	15.11	15.12	15.19	7.52	7.39	7.66	7.70
R.S.D. (%)		0.79	1.26	1.09		1.48	2.93	2.64
Accuracy (%)		99.87	99.96	100.38		98.08	101.86	102.38
Mean (μg/ml)	60.54	60.49	60.56	60.48	30.08	30.17	30.08	29.88
R.S.D. (%)		1.58	0.93	0.62		0.21	0.38	0.21
Accuracy (%)		99.92	100.04	99.90		100.63	99.62	99.33

(1‰) and adjusted to pH 6.5 with ethanolamine. Elution was carried out with a linear gradient from 30% to 70% B in 24 min after a 4-min run to permit the flow-through of plasma proteins. The column was then conditioned with 30% B for 6 min.

Microbiological assay

The microbiological assay was carried out with the parallel lines design. We used 120-mm diameter Petri dishes filled with 14 ml of antibiotic medium No.1 (pH 6) and inoculated with 0.5% of *Sarcina lutea* ATCC 9341. Three standard solutions were prepared in bovine serum at concentrations of 0.1, 0.2 and 0.4 μg/ml. The samples were preliminarily tested to assess the range of concentrations, and then diluted with bovine serum to obtain concentrations close to those of the standard.

Nine 6-mm diameter wells were made in each of six dishes used for every assay. Two samples were run on each set of dishes against the standard by filling the wells with 0.02 ml of the appropriate solution with an automatic pipette. After overnight incubation at 33°C the diameters of the inhibition zones obtained were measured to the nearest 0.1 mm. When plotted as log(concentration) *vs.* diameter of inhibition zone, the lines for the standard and for the samples were checked for linearity, parallelism and curvature and the potency of the sample was calculated as the mean difference between the two lines. The results were calculated with a computer program according to the statistical approach described in the British Pharmacopoeia 1980 (Appendix XIV A).

Cross-validation

About 450 plasma samples collected from healthy volunteers in a study by the Clinical Research Department of the Lepetit Research Centre, were analysed with the microbiological assay and the results were compared those obtained with the direct injection HPLC method.

RESULTS AND DISCUSSION

HPLC

Direct injection of biological fluids into a liquid chromatograph is advantageous from the standpoints of speed, cost, safety, sample tracking and analytical recovery.

Chromatograms obtained with blank plasma and after spiking it with known amounts of drug and metabolite at concentrations of 10 μg/ml of rifap and 5 μg/ml of desa, and at the limit of determination for desa (0.4 μg/ml rifap and 0.2 μg/ml desa) are shown in Fig. 2. No interference from endogenous substances in the blank plasma sample was observed within the elution range of the analytes.

During a typical overnight run with 24 plasma samples and six spiked plasma standards, there was no change in the retention times of the drugs and a negligible increase in the pressure in the column. In fact, we found that it was sufficient simply to invert the analytical column between runs to restore the original pressure. We decided to filter each sample through a 0.45-μm acetate membrane (Acrodisk; Gelman) to prevent clogging of the guard column filter.

Assay linearity

Statistical evaluation was carried out following the procedure described by Cavenaghi *et al.* [7].

Calibration graphs were obtained using drug-free plasma spiked with known amounts of drug and metabolite. The standard daily responses were collected for each day and cumulated to calculate the respective daily cumulative least-squares linear regression of the peaks area.

Analysis of variance (ANOVA) with $p = 0.05$ was used to confirm the significance of the regression calculated from the respective daily cumulative calibration graphs.

The calculated day-to-day overall least-squares linear regression is reported in Table II.

Precision and accuracy

Table I summarizes the results obtained by the described method for the analysis of spiked standard samples. The mean accuracy ranged from 95.0% to 102.6%, whereas the within-day precision, indicated by the mean of the daily relative standard deviation (R.S.D.), varied from 0.27% to 5.88%. The reproducibility expressed as the between-day precision indicated by the R.S.D. of the daily means ranged from 0.1% to 2.7%.

Cross-validation

The linear regression determined by the method of least squares on the results

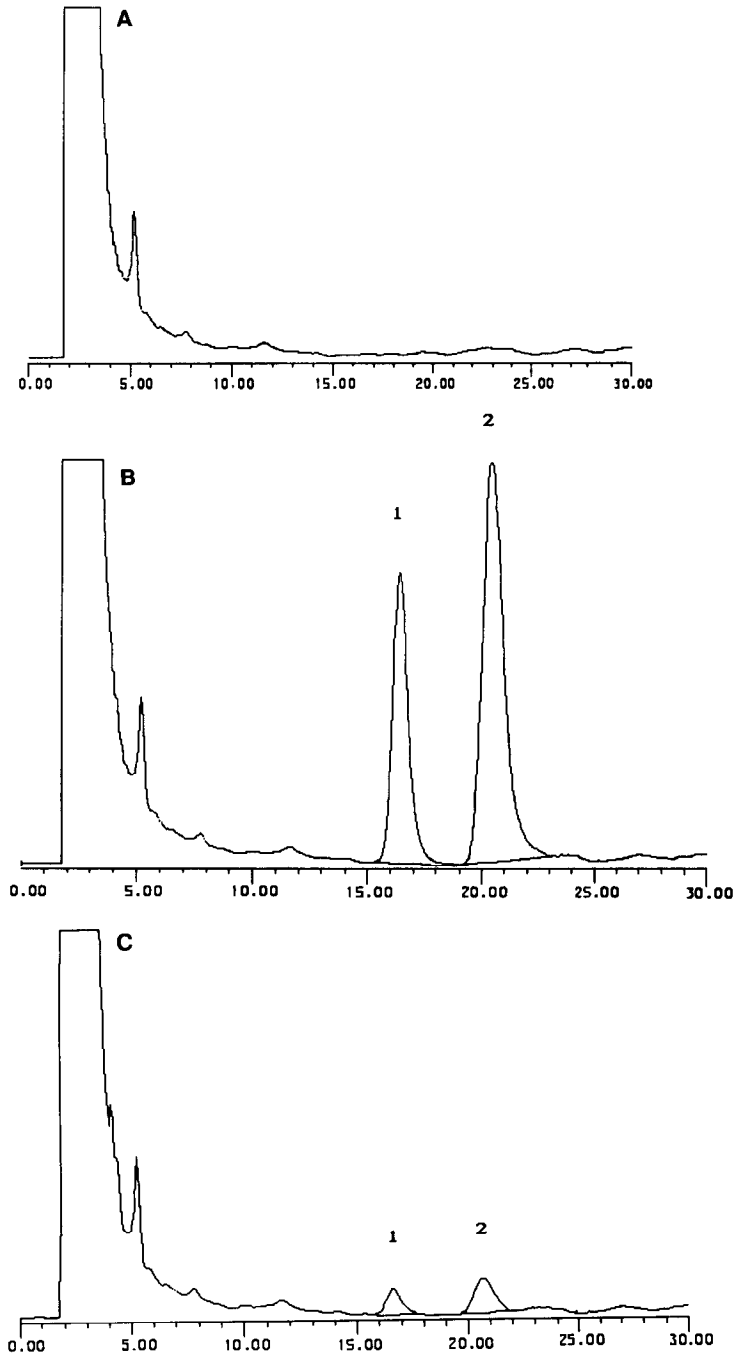

Fig. 2. Chromatograms obtained with (A) drug-free human plasma; (B) plasma spiked with 10 μg/ml of rifap and 5 μg/ml of desa; (C) plasma with drugs at the limit of determination of desa (0.4 μg/ml of rifap, 0.2 μg/ml of desa). Time scale in min.

TABLE II

DAY-TO-DAY CUMULATIVE REGRESSION LINE WITH THE RESPECTIVE ANALYSIS OF VARIANCE

Compound	Day	*n*	Slope	Intercept	Correlation coefficient	*F*-ratio	
						ANOVA	Lack of fit
Rifap	1	5	29693	760	0.99967	49133	0.0125
	2	10(5+5)	29971	512	0.99904	100335	0.0088
	3	15(10+5)	30033	412	0.99972	203199	0.0034
Desa	1	5	30387	−279	0.9999	428698	0.3193
	2	10(5+5)	30337	−164	0.99992	434348	0.1294
	3	15(10+5)	30272	−73	0.99988	454956	0.0145

obtained with the two methods had a slope of 1.006 and the correlation coefficient was 0.9077, indicating that antibiotic concentrations obtained by the HPLC method correlate well with those obtained by microbiological assay.

CONCLUSIONS

The applicability of the direct injection HPLC method to measure rifapentine and its metabolite in plasma was demonstrated. The method has adequate precision and accuracy at plasma levels from 0.94 to 60.54 μg/ml for rifapentine and from 0.47 to 30.08 μg/ml for its metabolite. The good correlation for about 450 samples between the results obtained with the HPLC method and a microbiological assay shows that either method can be used when assessing the total level of antibiotic in human plasma. However, under conditions that may interfere with a microbiological assay (such as the presence of other antibiotics) or when differentiation between the parent compound and the metabolite is necessary, the HPLC method is to be preferred.

REFERENCES

1 B. Ratti, A. Toselli and R. Rosina Parenti, *Farmaco*, 36 (1981)319–328.
2 A. Assandri, B. Ratti and T. Cristina, *J. Antibiot.* 37 (1984) 1066–1075.
3 K. Vekey, D. M. F. Edwards and L. F. Zerilli, *J. Chromatogr.*, 474 (1989) 317–327.
4 D. J. Gisch, B. Feibush, B. T. Hunter and T. L. Asceh, *Biochromatography*, 4 (1988) 206–215.
5 D. J. Gisch, B. T. Hunter and S. Feibush, *J. Chromatogr.*, 433 (1988) 264–268.
6 M. R. Lockemeyer and C. V. Smith, *J. Chromatogr.*, 532 (1990) 162–167.
7 L. Cavenaghi, G. G. Gallo, G. M. Leali and S. Vianello, *Drug Dev. Ind. Pharm.*, 13 (1987) 2571–2615.