Determination of rifabutin by high-performance liquid chromatography using on-line concentration and column switching

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Abstract

A simple HPLC method has been developed that allows the sensitive determination of rifabutin (RBT) in human serum using on-line concentration and column switching. After pretreatment of the serum with acetonitrile and centrifugation, the samples were applied to a concentration column (CC) (Zorbax CN). Washing with phosphate buffer–methanol removed most of the contaminating substances. Via a six-port valve the CC was switched to the analytical mode. RBT was separated on a Chromspher RP 8 column (acetonitrile–phosphate buffer pH 7.4/sodium chloride) and determined photometrically at 278 nm. The lower limit of quantification for 200 μl serum precipitated with 200 μl acetonitrile and after injection of 2×150 μl was 33 μg/l and linearity was observed up to 27 mg/l. Different modes of sample application (single, repeated, and different injection volume portions), as well as washing time, cycle time and different CC materials were investigated.

Keywords: Rifabutin

1. Introduction

Rifabutin (RBT) is a potent spiropiperidyl-rifamycin derivative with broad spectrum antibacterial activity against *Mycobacterium tuberculosis*, including rifampicin-resistant strains, and atypical mycobacteria. The problem of RBT monitoring is its low concentration in human serum, normally <1 mg/l. Therefore analytical methods described in the literature [1–6] used different extraction procedures including several steps for mixing, evaporation, and resolution. These methods are time consuming and sometimes difficult to handle and reproduce. We developed a simple method using on-line concentration and column switching.

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2. Experimental

2.1. Materials

The chemicals used were of analytical-reagent grade, purchased from Merck (Darmstadt, Germany), as were Lichrosorb-CN (5 μm) and -RP-8 (5 μm). Acetonitrile (MeCN) and methanol (MeOH), HPLC grade, were from Baker (Gross Gerau, Germany). The analytical column (AC) (Chromspher RP8 column, 5 μm, 100×3 mm I.D.), guard column (Chromspher R2), and column holder were obtained from Chrompak (Frankfurt, Germany). Cartridges K1 (10×4 mm I.D.) filled with 5 μm material of Kromasil 100-C18, -C8, -C4, and -C1 or Zorbax CN were from Bischoff (Leonberg, Germany), as well as the cartridge holder. The HPLC equipment consisted of a WISP 712 autosampler from Waters (Eschborn,

Germany), a gradient former Spectroflow 430 combined with an HPLC pump Spectroflow 400 (Pump 1), a programmable absorbance detector Spectroflow 783, all from Kratos (Weiterstadt, Germany), a HPLC pump 420 (Pump 2) from Kontron Instruments (Eching, Germany), a Rheodyne 7710 motor driven valve, and the integration system EZchrom from Scientific Software (San Ramon, CA, USA). The centrifuge 5403 supplied with a rotor for 1.5-ml vials was from Eppendorf (Hamburg, Germany).

2.2. Mobile phases

A stock solution (PBS) of phosphate buffer pH 7.4 containing 2 g KH_2PO_4, 18.4 g K_2HPO_4 and 81.82 g NaCl per liter was used. The analytical mobile phase contained 133 ml PBS and 535 ml MeCN per liter. The mobile phase for the Zorbax CN CC consisted of 80% 10 mM phosphate buffer pH 7.4 (PB)+20% MeOH. The flow-rate for both columns was 0.6 ml/min.

2.3. Sequence of operations

The chromatographic system is shown in Fig. 3 of Ref. [7]. For a standard determination, 200 μl human serum was mixed with 200 μl MeCN and centrifuged for 20 min at 23 000 g, 4°C. A 330-μl sample of the clear supernatant was placed in a WISP micro vial. The CC (Zorbax CN) was linked with the autosampler and two parts of 150 μl each were injected with an interval of 1 min. During this time Pump 1 provided the AC with the analytical mobile phase. The valve was switched 2 min after the last injection. The CC was linked in back-flush mode with Pump 1/AC and the 12-min data acquisition of the AC effluent, monitored photometrically at 278 nm, was started. After 2 min the valve was switched back for re-equilibration of the CC. The whole cycle including the operation times of the autosampler needs about 19 min; therefore, 20 min after the first injection the next cycle was started.

2.4. Test solutions

Spiked serum samples were prepared from 90% serum and 10% of the corresponding 10-fold RBT concentration. Four samples per day of each final

concentration (9.1, 18.38, 36.75, 73.5, 91.88, 183.75, 367.5, 735, 1837.5, 2940 and 3675 μg/l) were prepared to determine accuracy and precision. For orienting investigations RBT was dissolved in 50% MeCN–H_2O (RBT1=525 μg/l, RBT2=262 μg/l).

3. Results and discussion

Based on our experience in the determination of rifampin, we established a preliminary analytical system for the quantification of RBT, using MeCN-precipitation, a Chromspher RP 8 column, MeCN–phosphate buffer pH 7.4/NaCl as mobile phase, and detection at 278 nm (the addition of NaCl improved the separation of unprecipitated serum components). However, after injection of 100 μl (50 μl sample+50 μl MeCN) the lower limit of quantification (LLQ) of this system was about 300 μg/l, near the normal serum level. Therefore, as the recovery of RBT requires the precipitation of serum proteins by addition of MeCN and as an increase of the injection volume was not suitable, a concentration step was necessary to enhance the sensitivity. Three techniques were feasible, liquid–liquid extraction [1–6], solid-phase extraction, or column switching. Recently Campins-Falcó et al. [7] reviewed the advantages of several column-switching techniques for the determination of drugs in biological samples. Based on this discussion we selected a system consisting of two pumps and a six-port valve in back-flush mode as shown in Fig. 3 of Ref. [7].

First of all we analyzed the retention behavior of different column materials, e.g. Lichrosorb CN and Lichrosorb RP8 in laboratory-prepared precolumns (50×4.6 mm I.D.) and commercially available K1 cartridges (10×4 mm I.D.) filled with Kromasil C18, C8, C4, C1 or Zorbax CN. Both CN materials followed by Kromasil C1 showed the lowest retention of unprecipitated serum components. The recovery of RBT from these three materials using 10 mM phosphate buffer pH 7.4 (PB) and different MeOH concentrations is demonstrated in Fig. 1. Whereas the recovery from the C1 material was constant up to 59% MeOH and for Zorbax CN up to 40%, the recovery from Lichrosorb CN decreased considerably already at 5% MeOH content. Therefore we selected Kromasil C1 with PB–40% MeOH
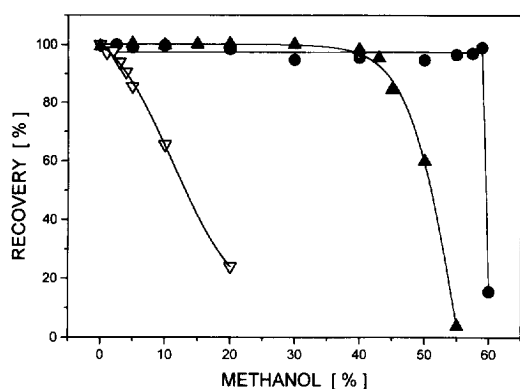
Fig. 1. Recovery of RBT vs. concentration of MeOH in 10 mM phosphate buffer pH 7.4; 90 μl of RBT1 were injected. Recovery from Lichrosorb CN (50×4.6 mm I.D.) (∇), Kromasil C1 (10×4 mm I.D.) ($\bullet$), and Zorbax CN (10×4 mm I.D.) ($\blacktriangle$). Mean values of two injections for each point.

and Zorbax CN with PB–20% MeOH for further studies. The retention behavior after injection of an increasing number of samples of the same volume (90 μl RBT1) was tested. The recovery from Kromasil C1 was linear at least up to 12 injections, whereas that of Zorbax CN showed a loss after the eighth injection. Fig. 2 demonstrates the influence of repeated injections on the CC (Zorbax CN) on the peak characteristics. The peak area showed a linear dependence on the injection number, whereas the peak height showed a deviation from the linearity. This difference indicates a slight peak broadening and is insignificant, at least up to 5 injections.
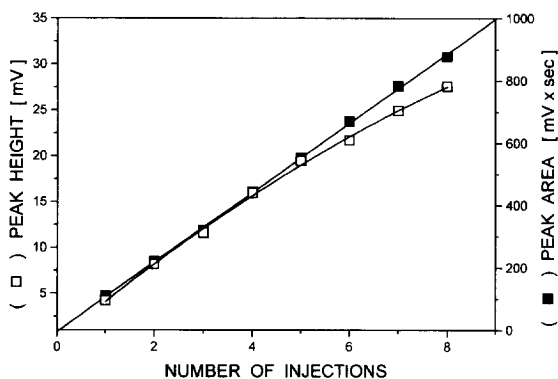
The loss of RBT during a washing cycle (time

between injection and valve switching) of 10–15 min was less than 1% and therefore is negligible for normal switching times of 2–6 min. The influence of the injection volume on recovery is shown in Fig. 3. A volume of 600 μl of RBT2 was applied in different portions. Whereas the C1 cartridges tolerated a lower number of injections with larger injection volumes, the recovery from the CN material decreased at volumes greater than 100 μl. Spiked serum samples, however, allowed injection volumes up to 150 μl on Zorbax CN without a loss of recovery. Probably the 50% MeCN solution of RBT in water disturbed the equilibration of the CN column at larger volumes and therefore the retention force decreased during the injection cycle, whereas the unprecipitated serum parts increased the adsorption of RBT. The recovery of RBT (525 μg/l) from the CC as compared with direct injection was 99.5% ($n=9$). According to these observations we chose an injection mode of 2×150 μl and Zorbax CN for analyzing RBT in serum samples. For validation the spiked samples described above were analyzed at four different days. The accuracy and precision data are shown in Table 1. Whereas the accuracy for the two lowest concentrations was unsatisfactory, the precision over the whole range was acceptable. The statistical estimation of the LLQ [8,9] using linear regression (Y=area units, X=RBT concentration) results in $Y=369.76(\pm3.55)X-1406.8(\pm2841.7)$ (95% confidence intervals in parenthesis) with a correlation coefficient of 0.9995 and a LLQ of 32.4
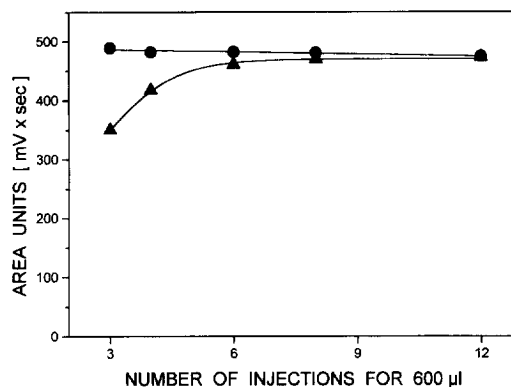

Fig. 2. Peak area ($\blacksquare$) and peak height ($\square$) of RBT vs. increasing number of injections of 90 μl RBT1 concentrated on Zorbax CN (10×4 mm I.D.).

Fig. 3. Peak area of RBT vs. different divisions of an injection volume of 600 μl RBT2. Recovery from Kromasil C1 (10×4 mm I.D.) ($\bullet$) and Zorbax CN (10×4 mm I.D.) ($\blacktriangle$).

Table 1
Data of precision and accuracy for RBT in human serum

Day	Statistical parameter	Theoretical concentration (μg/l)										
		9.1	18.38	36.75	73.5	91.88	183.77	367.5	735	1837.5	2940	3675
1	Mean (n=4)	13.42	21.64	38.72	71.51	90.04	183.48	377.9	748.5	1822.5	2848.5	3752
	% of Theory	147.5	117.8	105.4	97.3	98.0	99.9	102.8	101.8	99.2	96.9	102.1
	% of R.S.D.	7.73	5.55	2.53	3.54	1.96	3.04	0.38	0.89	0.58	0.67	0.20
2	Mean (n=4)	12.93	23.81	42.46	78.38	95.82	181.28	372.3	740.7	1824.2	2824	3773
	% of Theory	142.1	129.6	114.99	106.6	104.3	98.7	101.3	100.8	99.3	96.1	102.7
	% of R.S.D.	16.71	18.48	9.79	5.77	1.88	1.41	0.74	1.65	1.17	0.16	3.63
3	Mean (n=4)	12.83	20.6	37.98	69.55	87.27	181.49	379.2	750.7	1835	2847	3746
	% of Theory	141.0	112.1	103.3	95.6	95.0	98.8	103.2	102.1	99.9	96.9	101.9
	% of R.S.D.	4.05	10.23	3.80	4.20	2.22	2.81	1.86	0.69	0.31	0.42	0.28
4	Mean (n=4)	13.55	19.69	36.84	73.38	94.09	185.57	370.3	746.0	1825.1	2857	3727
	% of Theory	148.9	107.1	100.2	99.8	102.4	100.1	100.8	101.5	99.3	97.5	101.7
	% of R.S.d.	3.84	4.12	2.52	1.70	1.37	2.64	2.50	1.24	0.69	1.25	0.72
Overall	Mean (n=16)	13.18	21.44	38.95	73.21	91.8	182.92	374.9	746.5	1826.7	2847	3752
	% of Theory	144.88	116.63	105.99	99.6	99.6	99.6	102.0	101.6	99.41	96.8	102.1
	% of R.S.D.	9.29	13.17	7.55	5.87	4.13	2.44	1.79	1.17	0.73	0.85	1.70

For details of sample preparation see Section 2.3.

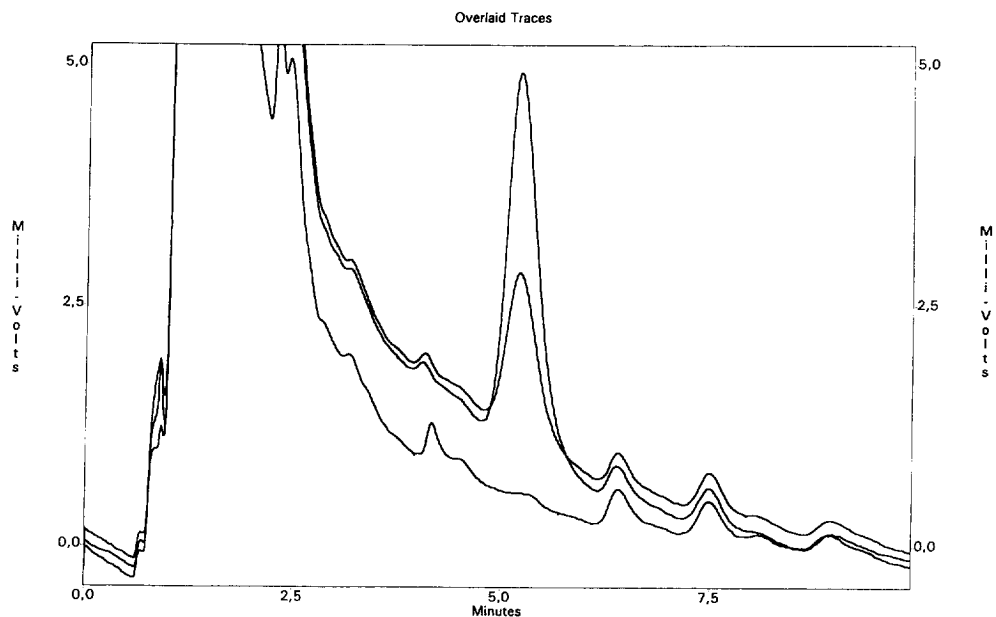Fig. 4. Overlaid chromatograms of blank and spiked serum samples. Samples spiked with 71.42 and 183.75 μg/l, respectively; 200 μl sample mixed with 200 μl MeCN, centrifuged (20 min, 23 000 g, 4°C), 2×150 μl injected and collected on Zorbax CN (10×4 mm I.D.) using 80% 10 mM PB+20% MeOH at 0.6 ml/min. Separated on Chromspher C8 (100×3 mm I.D.) using 46.5% PBS+53.5% MeCN at 0.6 ml/min.
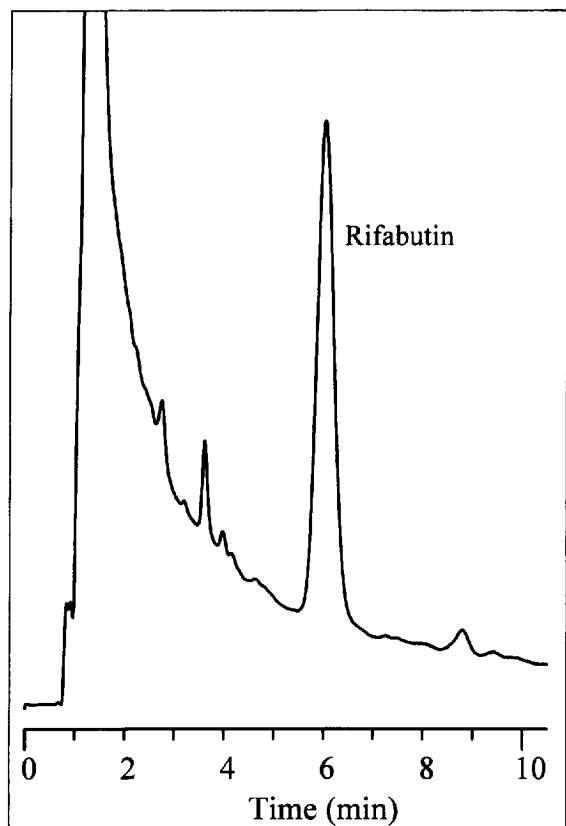
Fig. 5. Chromatogram of rabbit serum. 150 μl sample mixed with 150 μl MeCN, centrifuged (20 min, 23 000 g, 4°C), 2×110 μl injected. For further conditions see Fig. 4.

μg/l for the described method. Linearity above the validated range was observed in single tests up to 27 mg/l. The intercept value (1406.8=3.4 μg/l) does not deviate significantly from zero and possibly indicates an interference from the matrix. Furthermore it should be noted in relation to the range of concentrations.

Fig. 4 shows the overlaid chromatograms of blank and spiked serum samples (0, 73.21 and 182.92 μg/l RBT). The stability and the retention capacity depends on the sample matrix. During the validation phase at least 200 injections were possible using the same cartridge. Strong peak tailing is an indication of soiled CCs, an exchange delivered normal peaks with the same analytical column. Preliminary experiences were obtained during the analysis of rabbit serum and liquor samples. Fig. 5 shows the analysis

of rabbit serum 14 h after an injection of 1.25 mg/kg. The method was adapted to the available sample volume. A serum sample of 150 μl was diluted with 150 μl MeCN, and after centrifugation 2×110 μl of the supernatant were injected on the CC. The peak area represents a RBT concentration of 1.29 mg/l. These experiments show the possibility of sample volume variation. However, a change of injection volume within the same analysis is not recommended.

4. Conclusion

The column-switching technique allows a very sensitive quantitative determination of RBT. RBT is extracted by the CC in the collection mode and then transferred in a concentrated form to the analytical column. Therefore an injection of larger volumes is possible without loss of separation power. The LLQ depends on the injected volume. However, the adsorption of unprecipitable matrix components to the CC and the eventually resulting insufficient separation of those components on the analytical column reduce the sensitivity. Therefore, the selection of the CC material in combination with the analytical column depends on the sample matrix and the sample volume. Furthermore, the maximal single injectable volume depends on the sample matrix. For the analysis of RBT in serum samples we focused our investigations on Zorbax CN as CC and a C8 phase as analytical column. The selected combination of materials provides a powerful system for the sensitive quantification of RBT in serum.

Acknowledgments

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