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Short communication

Evaluation of the recently reported USP gradient HPLC method for analysis of anti-tuberculosis drugs for its ability to resolve degradation products of rifampicin

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Abstract

The recently notified USP gradient HPLC method for quantitative determination of rifampicin, isoniazid and pyrazinamide in fixed dose combination (FDC) formulations was evaluated to determine its ability to resolve major degradation products of rifampicin, viz. 3-formylrifamycin SV, rifampicin *N*-oxide, 25-desacetyl rifampicin, rifampicin quinone, and the newly reported isonicotinyl hydrazone, an interaction product of 3-formylrifamycin and isoniazid. The first observation was that the requirements of theoretical plates listed in the given method were met for rifampicin, but not for isoniazid and pyrazinamide, even on columns of different makes. The resolving power of the method was also dependent upon make of the column. On two of the three columns of the three tested, it was able to resolve most degradation products, except rifampicin *N*-oxide and 25-desacetylrifampicin, which were overlapping. The method was modified and an overall satisfactory resolution for all components was obtained by changing the buffer: organic modifier ratio of solution B in the gradient from 45:55 to 55:45 and decreasing the flow rate from 1.5 to 1.0 ml/min, keeping all other conditions constant.

Keywords: USP; Analysis; HPLC; Fixed dose combinations; Anti-tuberculosis drugs; Isoniazid; Pyrazinamide; Rifampicin; Degradation products; System suitability requirements

1. Introduction

There is a global concern on the quality of anti-tuberculosis fixed dose combination (FDC) products. Bioavailability of rifampicin has been found to reduce when the drug is combined with other anti-tubercular agents in single oral solid formulations. The matter is receiving intense attention from World Health Organization (WHO) and International Union against Tuberculosis and Lung Diseases (IUATLD) [1]. The pharmacopoeial authorities have also been concerned and as a sequel, USP has recently notified specifications for four-drug anti-tuberculosis FDC tablets

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containing rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E) [2]. The monograph was covered recently in a current interim revision announcement [3]. The monograph suggests two different methods for analysis of the four drugs. One assays R, H and Z and the other E alone. A separate method was necessitated for E because it lacks UV absorption.

Among the four drugs, R is known to be most unstable and recent studies have shown that this drug particularly undergoes rapid degradation in the presence of isoniazid in the formulations [4]. Accordingly, it is of interest to evaluate whether the USP method for combined assay of R, H and Z is able to simultaneously resolve the three drugs and known degradation products of rifampicin, including isonicotinyl hydrazone (HYD) of 3-formylrifamycin (3-FR) and, which has been found to be formed even in solid FDC formulations containing R and in the same dosage form [4]. It is also important to know whether separation and system suitability requirements of the official method are met during actual studies using different columns and instruments. Some users have reported problems with the method (personal communication). The results of studies on these aspects are covered in this communication.

2. Materials and methods

2.1. Materials

R, H, Z, 3-FR, rifampicin *N*-oxide (RNO), 25-desacetyl rifampicin (25-DR) and rifampicin quinone (RQ) were gift samples from Lupin Laboratories Ltd., Aurangabad, India. HYD was prepared according to the reported procedure [5]. HPLC grade acetonitrile was procured from J.T. Baker (USA) and same quality methanol from Mallinckrodt Baker Inc. (Paris, KY). All other chemicals used were of analytical grade. Ultra pure water was obtained from an Elga water-purification unit (Elga Ltd., Bucks, UK).

2.2. Equipment

pH recordings were made on a research pH meter (MA 235, Mettler Toledo, Switzerland). Other equipments used were sonicator (Branson, Germany), analytical balance (AG 135, Mettler Toledo, Switzerland) and autopipettes (Tripette, Merck, Germany).

Analytical separations were carried out on a HPLC system comprising of dual-piston reciprocating pump (LC-10ATVP), an online de-gasser (DGU-14AM), column oven (CTO-10ASVP), an auto injector (SIL-10ADVP), a UV–vis dual-wavelength detector (SPD-10AVP), and a CLASS-VP software (version 5.2) for data acquisition and processing (all from Shimadzu, Kyoto, Japan). Reversed-phase C18 columns of size 250×4.6 mm containing 5 μm particles of three different brands were used (column 1: Zorbax XDB, column 2: Shim-pack CLC ODS and column 3: Nucleosil EC 120-5).

2.3. Chromatographic conditions

The parameters specified by USP for gradient HPLC analysis of three anti-tuberculosis drugs in a four-drug combination product are given in Table 1. The prescribed gradient program for analysis is given in Table 2. The analysis was done maintaining all the specified conditions.

Table 1
HPLC parameters for determination of R, H and Z by the proposed USP method

Parameter	Condition
Column	L1 (250×4.6 mm)
Particle size	5 μm
Mobile phase	Buffer solution: 1.4 g of dibasic sodium phosphate in 1 liter water (pH 6.8) Solution A = Buffer:ACN (96:4) Solution B = Buffer:ACN (45:55)
Flow rate	1.5 ml/min
Detection wavelength	238 nm
Column temperature	25 °C
Injection volume	20 μl

Table 2
Gradient program prescribed in the USP method

Time (min)	Solution A (%)	Solution B (%)	Elution
0	100	0	Equilibration
0–5	100	0	Isocratic
5–6	100 → 0	0 → 100	Linear gradient
6–15	0	100	Isocratic

The test mixtures containing drugs and degradation products were prepared in methanol and contained 0.16 mg/ml R, 0.08 mg/ml H, 0.43 mg/ml Z and 0.2 mg/ml each of 3-FR, 25-DR, RNO, HYD and RQ. The solutions were prepared daily or before the study.

3. Results and discussions

Fig. 1a shows the chromatographic behavior of R, H and Z, obtained in a study carried out to verify whether USP specified requirements of the separation behavior and system suitability parameters were met. It depicts that the three drugs were well separated. The pattern of resolution was similar in repeat studies on the same day and on different days. Table 3 lists the observed and required system suitability parameters. The data indicate that relative retention time value for H was close to that required but the same was much different in case of R. On the other hand, the relative resolution requirements were met for H and Z and the same was also the case for the column efficiency value for R. However, the column efficiency values were much less for H and Z. Because of the failure to match the pharmacopeial values in some cases, studies were repeated on columns of other makes. The theoretical plate values are given in Table 4. Evidently, the requirements of plate counts were not met for H and Z in either of the columns, though there was agreement with the required value of R on all columns. There was hence a generalized disagreement in the retention behavior of R, H and Z and plate counts for H and Z on all different columns.

Fig. 1b shows the separation behavior of a mixture of R, H, Z, 3-FR, RQ, RNO 25-DR and HYD. Here also the same pattern of resolution was observed on multiple injections on the same day and on different days. As evident, the resolution amongst some of the components was not sufficient. There was co-elution of two components, 25-DR and RNO. While the resolution was similar to Fig. 1b on HPLC columns 1 and 2, the same was drastically poor on column 3, when tested even on two different machines. On this column, there was co-elution of HYD with 25-DR and RNO and also the peak of 3-FR eluted before R, than after R on the other two columns.

Attempts were hence made to optimize the separation between 25-DR and RNO along with other components. Column 1 was used for this purpose. Different parameters like, flow rate, pH, buffer molarity, buffer components, type of organic modifier, gradient times, and buffer: organic modifier ratio were varied. The steady reduction of the flow rate from 1.5 to 1ml/min resulted in a general spread of various components, but the clustering of rifampicin and its degradation products between 11 and 13 min (Fig. 1b) was not removed. An increase in pH reduced overall resolution and the decrease in pH from 6.8 to 5.2 caused 3-FR to elute before rifampicin. It was also unfavorable for elution of RQ, resolution time of which increased and peak asymmetry obtained was not within the acceptable limits. The change in buffer molarity was again not helpful as it distorted the peak shapes. On using KH_2PO_4 instead of Na_2HPO_4, the solvent front merged with peak of H, which could not be separated by additional trials using varied compositions of the mobile phase. There was also no distinct improvement in resolution of other components. Another attempt was made, by substituting acetonitrile with methanol. The advantages observed were smoothening of baseline, well-resolved peaks and low column pressures. But the disadvantages were reduced sensitivity as compared to acetonitrile and prolonged run time. A trial was then made with incremental addition of acetonitrile along with methanol, but there was no improvement in peak shapes or other aspects. The shift in gradient program in step 3 (Table 2) from 5–6 to 3–4 min, 4–5 or 6–7 min did not change overall chromatographic resolution, though there was a

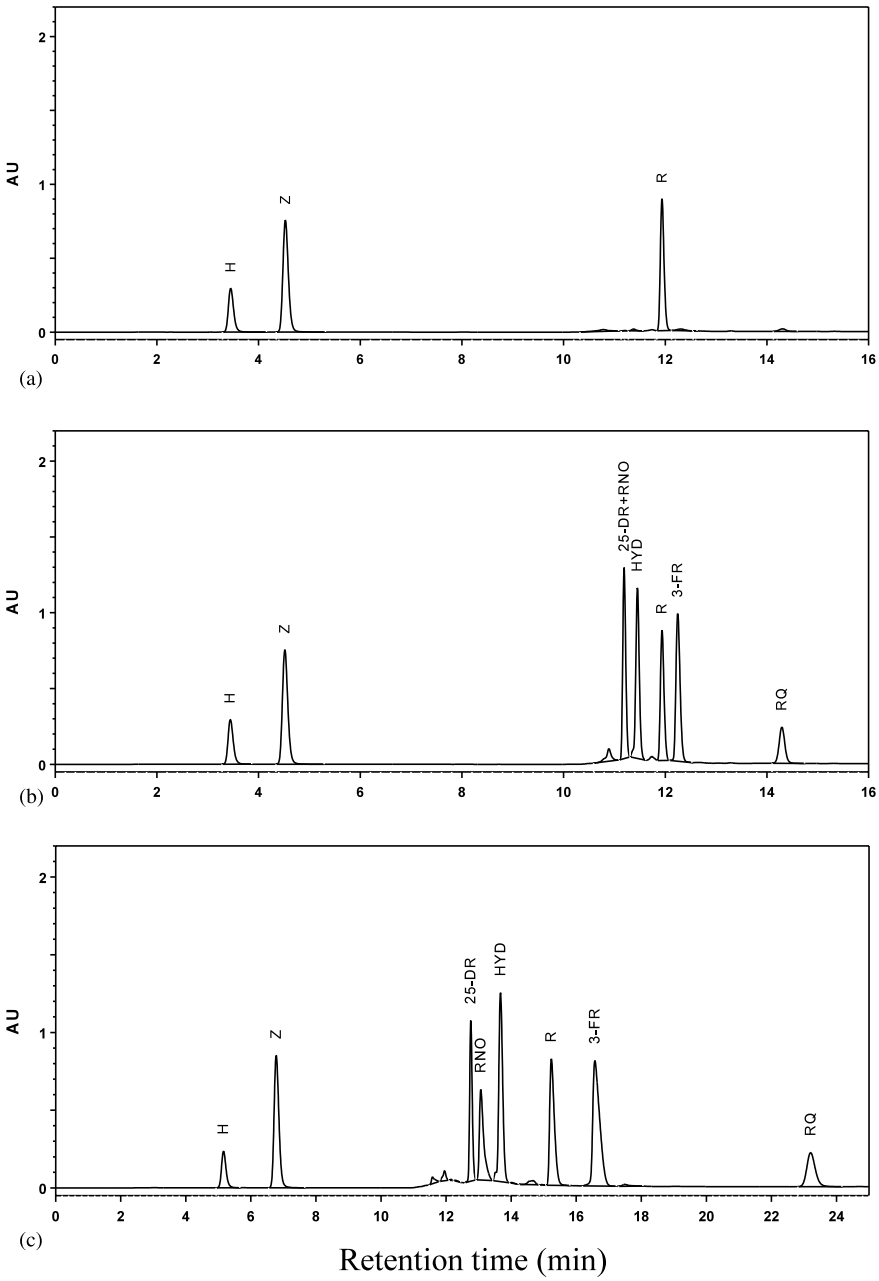

Fig. 1. Chromatograms showing resolution of drugs R, H and Z in the absence (a) and in the presence of degradation products 3-FR, HYD, 25-DR, RNO and RQ (b and c). Chromatograms a and b were obtained on use of the proposed USP method, while c was obtained by employing modifications proposed in this study.

Table 3
Obtained system suitability parameters for the three drugs R, H and Z on column 1

Parameters	H		Z		R	
	USP	Obtained	USP	Obtained	USP	Obtained
RRT, with respect to Z	0.7	0.76	–	–	1.8	3.01
Theoretical plates	12,000	2681	18,000	2129	100,000	107,762
Resolution with respect to Z, ≥ 4	–	Complied	–	–	–	Complied
Tailing, < 2	–	Complied	–	Complied	–	Complied

Table 4
Results of theoretical plates obtained for columns 2 and 3

Column	H	Z	R
2	5878	9556	112,696
3	4688	9181	124,116

decrease or increase in the run time, respectively. However, a fair amount of separation among closely resolving components was obtained on decreasing organic modifier ratio in solution B, but along with there was shift of RQ peak towards the right, resulting in prolongation of run time. In order to bring RQ peak closer, without disturbing resolution of rest of the components, another gradient step with higher organic modifier content was added. This resulted in strong gradient peaks in the same region where RQ was eluted.

Further trials were made by combining conditions where tendency existed towards separation of closely resolving components. R separation was achieved on changing the buffer: organic modifier ratio of solution B in the gradient from 45:55 to 55:45 and decreasing the flow rate from 1.5 to 1.0 ml/min, keeping all other conditions constant. The chromatogram showing separation of drugs and products under these conditions is given in Fig. 1c. The same separation was also achieved on column 2, but not on column 3, suggesting dependence of resolution on the brand of HPLC column.

4. Conclusions

A study was carried out to check whether the proposed USP method for assay of R, H and Z was able to simultaneously resolve the drugs and known degradation products of R and to evaluate whether separation and system suitability requirements were met in actual studies. It was found that:

(i) By and large the method was able to separate the three drugs from known and major degradation products of R, except 25-DR and RNO. Even these could be separated by introducing small modifications, as suggested in this study.

(ii) The nature of separation varied with the make of the columns. This means column specifications need to be fixed more exactly, whenever the objective is to apply the method to the analysis of stability samples.

(iii) The advised minimum theoretical plates for R were easily met on all different columns, but it was not the case with H and Z. The theoretical plate requirements for these two drugs were not met even on a single column, among the three different brands.

The study thus provides a good insight into the performance of the proposed USP method for assay of R, H and Z in pharmaceutical FDC formulations. It is hoped that the conclusions drawn would be useful to the manufacturers and all those involved in control of the quality of the anti-tuberculosis FDC products. This is likely keeping into view of the wide use of official methods.

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