RESEARCH PAPER

Analytical method development and validation of Resmetirom by Reverse Phase High Pressure Liquid chromatography

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ABSTRACT

A precise and accurate analytical method for estimation of Resmetirom was developed and validated as per ICH guidelines. The mobile phase consisted of Methanol: 0.1% Formic acid in Water in the ratio 68:32 v/v by using Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ) as stationary phase. The wavelength was set at 252 nm with 10 µl injection volume. The samples were allowed to run for 10 minutes. The Resmetirom peak eluted at 3.69 minutes. The % RSD for instrument precision was found to be 0.07% for Resmetirom. The % RSD of accuracy for Resmetirom at 80%, 100% and 120% was found to be 0.03%, 0.04% and 0.06%, respectively. The coefficient correlation was 0.999 for Resmetirom. The % RSD for inter and intra-day precision for Resmetirom was found to be 0.18% and 0.29%, respectively. The LOD and LOQ for Resmetirom was below 0.53 µg/ml and 1.14 µg/ml. From the validation data can it be concluded that method can be used for estimation of Resmetirom.

Keywords: Resmetirom, RP-HPLC, Validation, Method development, LOD and LOQ, Analytical estimation.

How to cite this article: Inamdar S, Kumari A, Chauhan K, Ingle SS, Deshmukh PS, Bhosale S, Sanmukhrao SR, Magarumkhane SS. Analytical method development and validation of Resmetirom by Reverse Phase High Pressure Liquid chromatography. Int J Drug Deliv Technol. 2026;16(47s): 266-271. DOI: 10.25258/ijddt.16.47s.33

1. INTRODUCTION

Resmetirom is an oral, first-in-class thyroid hormone receptor-beta (THR-). It is used to treat adults with non-cirrhotic non-alcoholic steatohepatitis (NASH) (now commonly called MASH) and moderate-to-advanced liver fibrosis. It acts by acting upon the liver to decrease fat accumulation, decrease inflammation and enhance liver fibrosis in association with diet and exercise.

Thyroid hormones, including free triiodothyronine (FT3) and FT4, are important modulators of the liver's lipid metabolism. The primary form of the thyroid hormone receptor in the liver is called thyroid hormone receptor-beta (THR-β), and activation of this receptor lowers intrahepatic triglycerides.

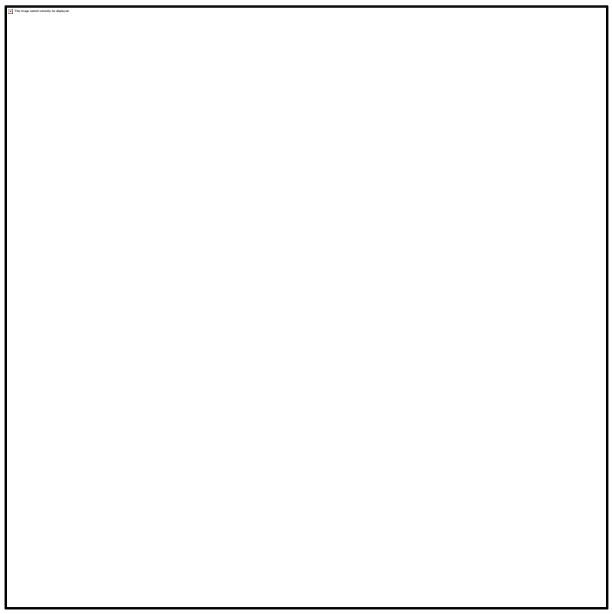

Figure 1: Chemical structure for Resmetirom

Thyroid dysfunction, including hypothyroidism, is a major risk factor for non-alcoholic fatty liver disease (NAFLD) and is present in many NAFLD patients.1,2,3 Additionally, dysregulated adipose tissue lipolysis and increased release of free fatty acids from the adipose to the liver have been associated with hypothyroidism, which has been shown to promote hepatic insulin resistance.1 There may also be elevated levels of proinflammatory adipokines in the blood, which are linked to hepatic fibrosis and inflammation.

The table given below shows the Limits as per ICH guidelines.

Table 1: Quality Target Profile for HPLC Method development

Parameters	Limits
Theoretical Plates	Not less than 2000
Asymmetry	Not More than 2.0 (Fairly at 1.0)
Tailing Factor	Not More than 2.0 (Fairly at 1.0)
Run time	Not More than 20 minutes
Resolution	Not Less than 2.0

2. EXPERIMENTAL

2.1. Chemicals and Reagents:

A Sample of Resmetirom was taken from Aadhaar life sciences, Solapur, Maharashtra. HPLC-grade Formic acid (Merck) Mumbai, India and Methanol (Molychem, Mumbai) were purchased from a local supplier. HPLC grade RO water was provided via the internal Milli-Q system.

All of the weighing was carried out on scales that had been calibrated by NABL. The production of the samples was carried out with the use of the analytical balance and Type A glassware along with the 0.45 µm Millipore syringe filters (Ultipor®N_{66}®Nylon Membrane).

2.2. Instrumentation:

The instrument used for development and validation was an Agilent 1260 Infinity II equipped with a quaternary pump and DAD detector. Software from Agilent called Open lab Ezchrom was used. Wet chemistry was conducted using the labman ultra-sonicator and the aczet analytical balance.

2.3 Selection of Mobile phase:

Based on literature review the mobile phase of Methanol: 0.1% Formic acid in Water was chosen for the HPLC analysis of Resmetirom in order to gain better results, various ratio trials like 50:50, 45:55, 70:30, 68:32 v/v were performed with gradient type of elution technique.

2.4 Selection of Wavelength:

Standards were scanned from 190-400 nm on HPLC with DAD detector.

2.5 HPLC Method Development:

Multiple trials were done performed to achieve the system suitability criteria as per ICH guidelines as mentioned in table1.

2.5.1 Preparation of Mobile Phase:

Mobile Phase: Methanol: 0.1% Formic acid in Water

In 1000 ml HPLC water, 0.1% formic acid was added and

mixed well with methanol and filtered through 0.45-micron membrane filter and sonicated to degas for 10 minutes.

2.5.2 Preparation of Diluent:
Mixed separately measured 800 ml of methanol and 200 ml of 0.1% Formic Acid into a suitable container. Mixture was filtered through 0.45µm nylon membrane filter and briefly sonicated to degas.

2.5.3 Preparation of Standard Solution:
Prepare a Standard Stock Solution of Resmetirom by adding 3mg in 10 ml volumetric flask & add 5 ml diluent and Mix and sonicate for 5 minutes. Make up the volume to 10 ml with diluent. (Conc. = 300µg/ml).

2.6 Method validation:
2.6.1 Specificity:
Resmetirom was injected individually and in mixture at 100 µg/ml concentration each and peaks were identified by retention time. Blank injection ensured that the blank peak would not interfere with the major analyte peaks.

2.6.2 System Suitability:
The system was tested to for its suitability. The theoretical plate count, tailing factor, and resolution should meet ICH guidelines as mentioned in table 1.

2.6.3 Accuracy:
A technique's accuracy is determined by how closely its test results match the actual value. Three concentration levels were tested in recovery trials. Three replicate injections were performed at each level to calculate drug concentration, recovery percentage, and standard deviation.

2.6.4 Repeatability:
Analytical Instrument precision depends on test concordance. Multiple uniform samples were examined. After following the instructions, six injections were made from a single sample to test the system. Instrument precision was measured (how well the instrument replicates injections of equal concentration).

2.6.5 Linearity:
Methodological linearity is an analytical method's ability to give results proportional to analyte concentrations within a certain range. Five standard solutions were used to determine linearity. Plotting peak area against standard solution concentration on the calibration curve yielded the regression equation. This enabled equation development. For slope, intercept, and correlation coefficient, the least-squares approach was used.

2.6.6 LOD and LOQ:
Limits of detection (LOD) and quantification (LOQ) represent the method's capacity to detect and quantify the smallest analyte amounts. The following formulae determined the standard deviation and regression line slope needed to calculate LOD and LOQ.

$$LOD = \frac{3.3 \times Std.Error\ of\ Intercept}{Coefficients\ of\ X\ Variable\ 1}$$

$$LOQ = \frac{10 \times Std.Error\ of\ Intercept}{Coefficients\ of\ X\ Variable\ 1}$$

3. RESULTS AND DISCUSSION:

Using methanol and 0.1% formic acid in water as a mobile phase in HPLC is a common practice that offers several advantages. One of the primary benefits is the improvement in peak shape, as methanol helps in resulting improved peak shape and symmetry.
Additionally, the combination of methanol and 0.1% formic acid in water can provide high resolution and separation of closely related compounds, making it an ideal choice for analyzing complex mixtures. Furthermore, the use of methanol can also enhance the sensitivity of detection, especially for compounds that exhibit poor UV absorption, allowing for more accurate and reliable quantitation. Overall, the use of methanol and 0.1% formic acid in water as a mobile phase in HPLC is a valuable technique for achieving high-quality chromatographic separations and detections.
The trials were carried out by varying the ratio of methanol and 0.1% formic acid in water and the wavelength was finalised for analysis at 252 nm for appropriate intensity of Resmetirom peak based on the trials undertaken.

3.2 Final Chromatographic Conditions:
The Final chromatographic condition for estimation of Resmetirom was as follows (Table 2)**.**

Table 2: Final Chromatographic Conditions

Parameters	Condition
HPLC Instrument	Agilent 1260 Infinity II
Column	Agilent Zorbax Bonus RP (250 mm x 4.60 mm,5µm)
Wavelength	282 nm
Mobile Phase	Methanol: 0.1% Formic acid in Water (78:22)
Diluent	Methanol: 0.1% Formic acid in Water (80:20) v/v
Run time	10 minutes
Injection Volume	10 microliters
Flow Rate	1.0 ml/min
Column oven Temperature	30°C

3.3 Method Validation:

3.3.1 Specificity

Specificity was performed to check if there was any interaction between the peaks from blank or the API.

Table 3: Specificity results of Resmetirom

Sample ID	RT (min.)
Resmetirom	3.69

Based on specificity data, it was found that the Retention time of individual working standard was similar to that of mixture working standard and there is no interference of blank with main peak of Resmetirom.

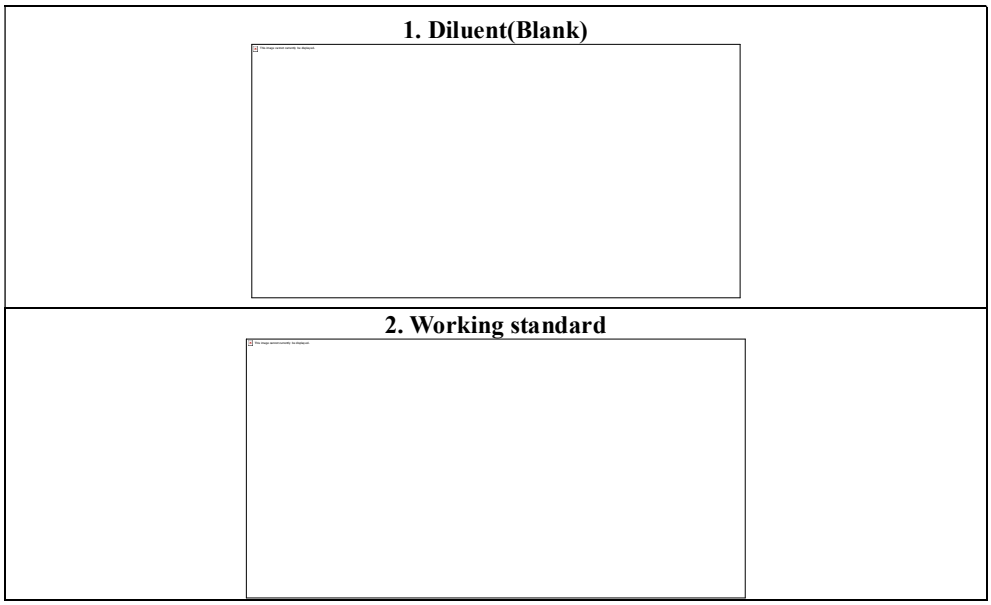

Figure 2: Chromatogram ID 1] Diluent, 2] Working Standard

3.3.2 Instrument Precision and System Suitability:

The appropriateness of the HPLC instrument was assessed for the purpose of validation. According to the specified limits in table 1, the equipment was deemed appropriate for carrying out the validations. The precision test was conducted following the assessment of system applicability. The data indicates that the % relative standard deviation for Instrument precision for Resmetirom are 0.07%. These % RSD demonstrates that the approach is highly precise and reliable when used by various analysts and for multiple sample preparations with the same concentration. (Table 4)

Table 4: Results of Repeatability

Sample ID	DFZ Peak Area
Replicate 1	3481717
Replicate 2	3483920
Replicate 3	3486356
Replicate 4	3480383
Replicate 5	3482396
Average	3482954
STDEV	2289.01
% RSD	**0.07**

3.3.3 Accuracy:

Accuracy for Resmetirom was performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120%. The relative standard deviation for 80%, 100% and 120% were 0.03%, 0.04% and 0.06% respectively. The accuracy determined the methods ability to analyses different concentration of drug in solution accurately.

Table 5: Results of Accuracy

Sample ID	Reps	Spiked Conc. (ug/ml)	Area	Amt Recovered (ug/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	239.74	2798513	240.78	100.44	100.42	0.025403	0.03
	Rep 2	239.74	2797512	240.70	100.40			
100%	Rep 1	299.67	3481717	299.56	99.96	100.00	0.044725	0.04
	Rep 2	299.67	3483920	299.75	100.03			
120%	Rep 1	359.60	4167911	358.60	99.72	99.76	0.055813	0.06
	Rep 2	359.60	4171210	358.89	99.80			

3.3.4 Linearity:

Linearity was performed at different levels. The graph plotted between peak area and concentration showed linearity with correlation coefficient as shown in table below. The linearity data in shown in graph in figure 3.

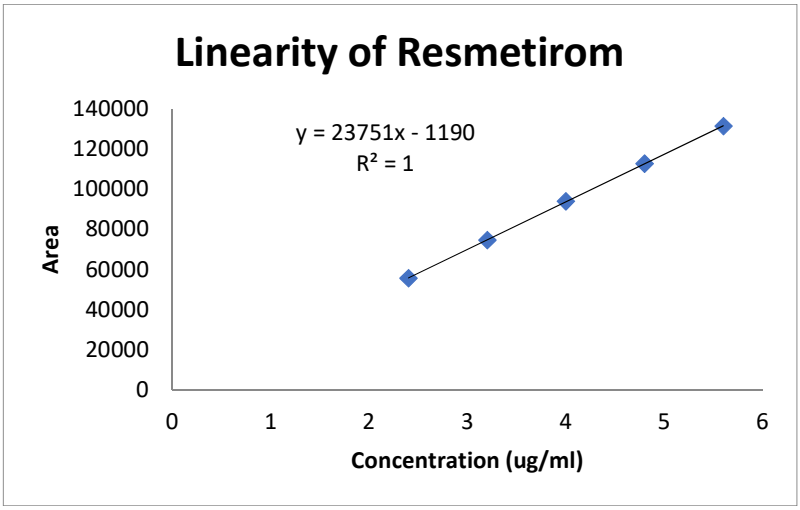

Figure 3: Linearity of Resmetirom

Linearity was performed for Resmetirom with concentration ranging from 80-120 µg/ml for Resmetirom and the Correlation Coefficient was found to be 1 for the API.

3.3.5 LOD and LOQ:

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for Resmetirom. The results of analysis are shown in table 6.

Table 6: Results of LOD and LOQ for Resmetirom

Name	LOD (µg/ml)	LOQ (µg/ml)
Deflazacort	0.53	1.14

The LOD and LOQ were significantly low, implying the method to be very efficient in determining low concentration of drug. This value of LOD and LOQ can be used during cleaning validation in industry which can help companies know if the manufactured vessel or equipment is free from APIs stains.

4. CONCLUSION

In this research article, a precise and accurate method was developed for estimation of Resmetiromin bulk drugs by RP-HPLC technique. The developed method was validated for accuracy and precision as per ICH guidelines. The proposed method was found to be appropriate due to its simplicity, reliability, sensitivity, rapidness and selectivity for detection at very low concentrations of drug biomarkers. Based on validation data it is demonstrates and concluded that this method could be the highly sensitive and accurate method to be used in the routine analysis of Resmetirom for estimation with varied concentration in different formulations.

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