Development and validation of a reversed-phase HPLC method for determination of assay content of Teriflunomide by the aid of BOMD simulations

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CHRONICLE	ABSTRACT
	Due to the new hopes for treatment of multiple sclerosis (MS) diseases by Teriflunomide (TFN), in this project, a cheap, robust, and fully validated method has been developed both for determination of assay content in API (active pharmaceutical ingredient), and for related impurities analysis (RIA). To operate the method, a common C18, end-capped (250 × 4.6) mm, 5µm liquid chromatography column, was applied. The mobile phase A was prepared by dissolving 2.74 g (20mM) of PDP (potassium dihydrogen phosphate) and 3.72 g (50mM) of PC (potassium chloride) in water (1000 mL). Then, pH was adjusted to 3.0 by adding OPA (ortho-phosphoric acid) 85%; while, the mobile phase B was acetonitrile (ACN) (100%). In order to confirm the experimental data about the λmax of TFN, we have used the Born-Oppenheimer molecular dynamics (BOMD) simulations, quantum mechanics (QM), and TD-DFT calculations. According to the results, the method showed a high level of suitability, specificity, linearity, accuracy, precision, repeatability, robustness, and reliable detection limit.

1. Introduction

Multiple sclerosis (MS), the complexneurological immune-mediated disease has poorly been understood (especially in view of aetiology). Among all complex disorders, MS which is caused by the interaction of genetic and environmental factors, has endangered the human race.[1] For many decades, scientists tried to find an appropriate method for treating the MS patients. Due to this, different type of drugs, based on peptides,[2] heterocyclic compounds,[3] and other materials[4] were introduced as cures for this vital disease. Teriflunomide (TFN), along with, Glatiramer acetate, and Fingolimod are of the first line treatment of MS in the USA and Australia.[2-4] Also, TFN has been characterized by a once daily oral Prescription medication for the treatment, and a comparably well-established long-term safety profile.[5] TFN is the active metabolite of leflunomide, and it had been investigated in the Phase III clinical trial TEMSO as a medication for MS disease. In 2012, the study was completed with positive results; while, the subsequent comparison of trial reported that permanent discontinuations were

substantially less common among MS patients who received teriflunomide compared with interferon beta-1a.[6] Finally, in 2012, the drug was approved by the FDA, and subsequently some of the other pharmacopeias permitted the use of that.[7] Then, pharmaceutical companies considered the use of TFN, and industrial researchers began to find suitable methods for analyzing this drug.

In 2010, Sobhani and colleagues designed a HPLC method containing 10 mM PDP and 100 mM PC in aqueous 25% ACN, acidified to pH 3 with o-phosphoric acid, for assay analysis of the leflunomide metabolite, teriflunomide.[8] In 2017, Mehta et al, developed a HPLC separation method for estimation of TFN in API by using an Agilent-1260 Infinity series instrument with Eclipse XBD C18 (Agilent) column (150X4.6 mm, 5μm). The mobile phase was ACN and PDP buffer solution (40:60; v/v) containing TEA (triethyl amine) to pH 7, and the flow rate was 1.0 ml/min. The method had been validated for specificity, linearity, precision, accuracy, robustness and ruggedness.[9] In one of the other reports, Rakhila and co-workers developed a method composed of 0.5mM ammonium acetate in a mixture of water, ACN, and formic acid with a volume ratio of 95:5:0.02, respectively. The flow rate was 0.5 mL/min and the temperature of the column oven was 25°C.[10] It should be noted that such accurate heterocyclic synthesis of such bioactive drugs is the result of years of attempts in different types of methodologies[11,12] and heterocyclic[13-15] organic synthesis. Also, the theoretical chemical calculations used in this project, to confirm the experimental results, have been widely applied[16-18] to give more trustable results.

Regarding these, in this project, a cheap, robust, and fully validated method has been developed both for determination of assay content in API, and for RIA test. Compared to the previous methods, the represent approach, which has used UV-Visible detector at 280 nm, shows a higher level of suitability, specificity, linearity, accuracy, precision, repeatability, and robustness, both for related separation, and assay analysis. Thus, it was suitable for use in pharmaceutical companies.
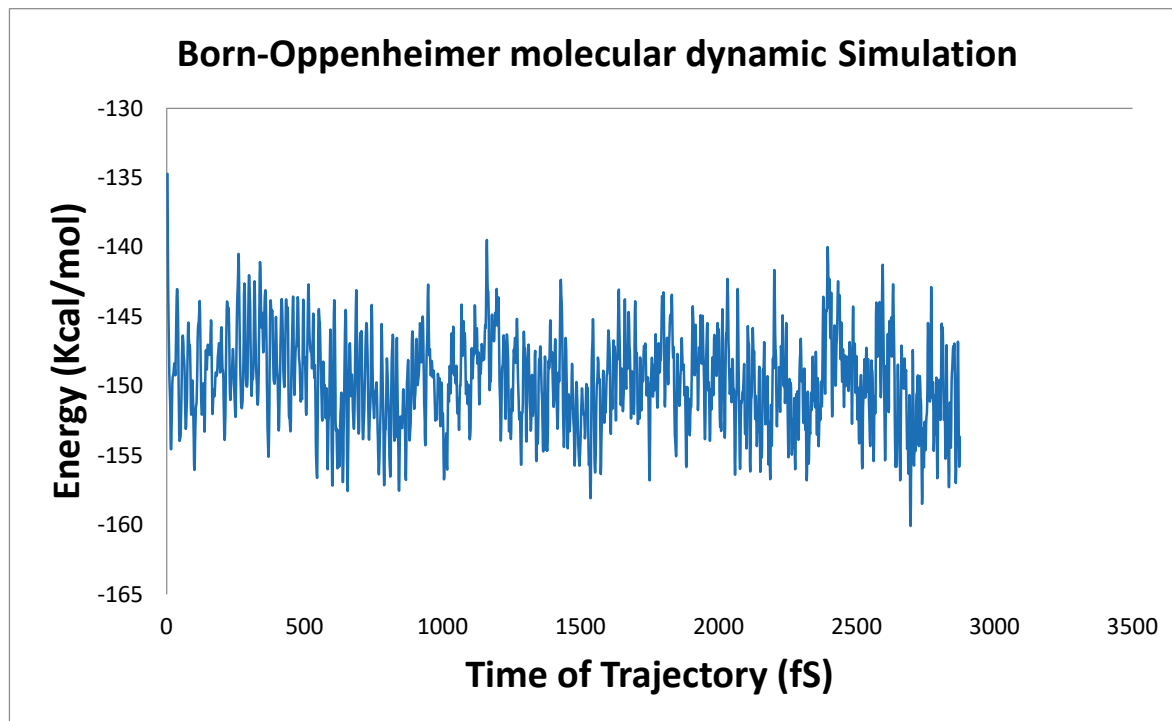
2. Results and Discussion

The present method showed a high level of validity using mobile phase A which was prepared by dissolving 2.74 g (20mM) of PDP and 3.72 g (50mM) of PC in water to pH 3, and the mobile phase B which was ACN (100%). In addition, the gradient elution program was (75%,0.00), (55%,30.00), and (55%,60.00) (mobile phase A%, time(min), respectively (**Table 1**). In the following sections, we have presented the suitability, specificity, linearity, accuracy, precision, repeatability, robustness, and reliable detection limit.

Table 1. The gradient elution program of the analysis

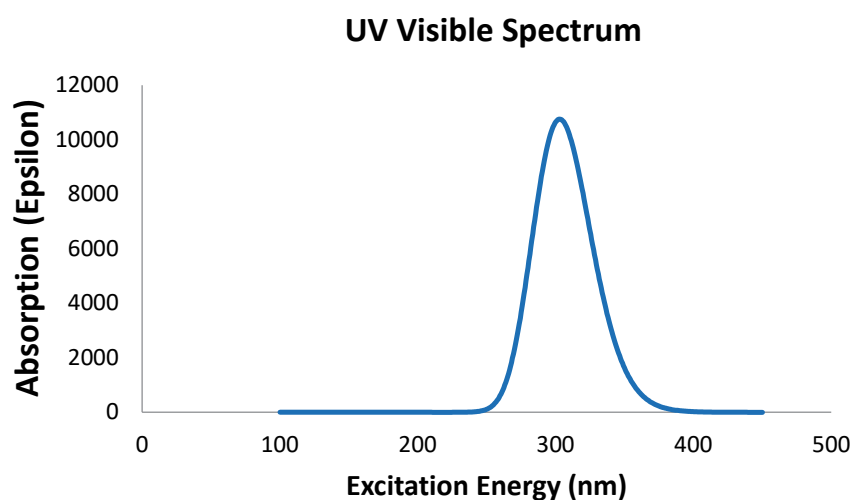| No.	Time (min)	Mobile phase A (percent V/V)	Mobile phase B (percent V/V)
1	0.00	75	25
2	30.00	55	45
3	60.00	55	45

2.1 Theoretical section

To find the best absorption area (λ_{max}) of TFN (and setting the detector in the method), first, we have used the Born-Oppenheimer molecular dynamic (BOMD) simulation (see Figure 1). This simulation method helps to find the more stable states of the molecular systems for further quantum mechanics optimizations.

Fig. 1. The Born-Oppenheimer molecular dynamic (BOMD) simulation spectrum for TFN molecular system

Then, some of the more stable states are being used as input files for optimization (in this case we have used the B3LYP/6311G(d,p) level of theory to reach more trustable data). Finally, the output geometrical system of the optimization process which shows one of the best orientations, would undergo the TD-DFT job to give the UV-Visible absorption spectrum of the molecule. As shown in **Fig. 2**, the best UV-visible absorption area for TFN molecules is about 300nm, which is in agreement with the experimental results. Indeed, in the present project, we have used the B3LYP/6311G(d,p) level of theory, both for optimization (to find the energy minima) and for TD-DFT calculation in order to reach the infrared spectrum.

Fig. 2. The TD-DFT UV-visible absorption spectrum of the optimized structure of TFN, calculated at B3LYP/6-311G(d,p) level of theory.

2.2 Method development and validation

Due to the fact that in pharmaceutical companies, any error may endanger the human lives, development and validation[19] of analytical methods (in any aspect) especially in the case of instrumental methods is very important. Thus, the present designed method was investigated in the case of validations.

2.2.1 Sample preparation

Stock solution was prepared by dissolving 0.01561 g of TFN standard in 50.0 mL of diluents. Then, solutions with different concentrations were prepared accordingly.

2.2.2 Equipment

A Shimadzu Prominence system (Shimadzu Corporation, Kyoto, Japan) Equipped with a LC-20AD pump, a DGU-20A degassing system, a CTO-20A column oven and a SPD-20A UV-Vis detector, was used for all analysis. The LabSolutions software version 5.51 was applied for processing and data analysis. Also, the chromatography column was a C18, end-capped with 250mm in length, 4.6 mm in internal diameter, and 5μm in pore size.

2.2.3 System suitability

In order to study the suitability of the method, the standard solution of TFN was spiked with the appropriate level of Leflunomide (as a known impurity of TFN) and then, the system suitability parameters were studied. As given in **Table 2**, the percentage of the RSD values for the area and RT of TFN peaks for five injections were not more than 2.0%. Moreover, the tailing factor (TF), resolution between peaks, and the number of theoretical plates (NTP), are less than 2.0, more than 2.0 and more than 2000, respectively, which show the suitability of the system (Table 2). The resolution between TFN and Leflunomide peaks should not be less than 20 (due to high resolutions, given in **Table 2**).

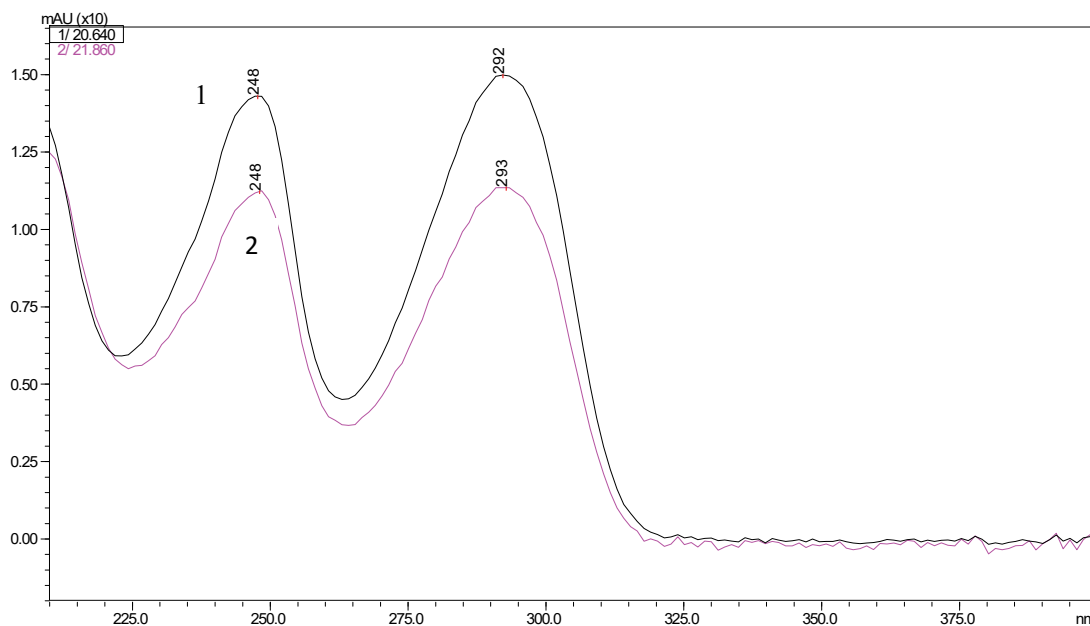**Table 2.** System suitability parameters for the developed method of TFN

Run#	RT	Area	Tailing factor	NTP	*Resolution
1	22.390	73733	1.166	15301	23.042
2	22.390	73997	1.153	14658	22.534
3	22.388	73891	1.161	15128	22.787
4	22.388	73434	1.139	14718	22.407
5	22.380	73824	1.144	14907	22.571
Average	22.387	73775.800	1.153	14942.400	22.668
% RSD	0.019	0.290			

*Resolution between TFN and Leflunomide peaks.

2.2.4 Specificity

ICH documents[20] define the specificity parameter as the ability to assess unequivocally the analyte in the presence of components, which could be presented. Typically, these might include impurities, degradants, matrix, etc. in this regard, the peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (like diode array, or mass spectrometry). Herein, the peak purity was studied by using a PDA detector, and the absorbance spectrums of TFN in the upslope, apex and downslope of the chromatographic peak were compared

accordingly. As could be observed, there is not a significant difference between the spectrums. Also, the standard solution of TFN was spiked with the appropriate level of known Leflunomide and the resolution between those two was presented in Table 3. The results revealed no peak interference with the TFN peak, therefore the specificity of the method was confirmed (**Fig. 3**).

Fig. 3. Absorbance spectrums of TFN at RT=20.64 min (upslope) and RT=21.86 min (downslope)

2.2.5 Linearity

To investigate the linearity of the method, the solutions of TFN standard were injected according to **Table 4** (each solution was injected three times). A linear calibration curve (**Fig. 4**) was achieved by plotting the average analytical signal versus the concentration of TFN standard, and then, the regression line was calculated by the method of least squares. Data presented in **Table 3**, and **Fig. 4** show that the coefficient of determination (R^2) is about 0.9991, which indicates the high level of linearity of the method (limit: the coefficient of determination (R^2) should not be less than 0.998).

Table 3. Calibration curve data to give the linearity parameters

Solution	Conc. (mg/L)	Run1	Run2	Run3	Average	% RSD
S1	0.311	21407	21742	21682	21610.333	0.827
S2	0.622	46572	46428	46417	46472.333	0.186
S3	0.777	59435	59561	59682	59559.333	0.207
S4	1.036	80253	79853	80570	80225.333	0.448
S5	1.244	93822	93512	93211	93515	0.327
S6	1.555	119603	119023	119954	119526.667	0.393

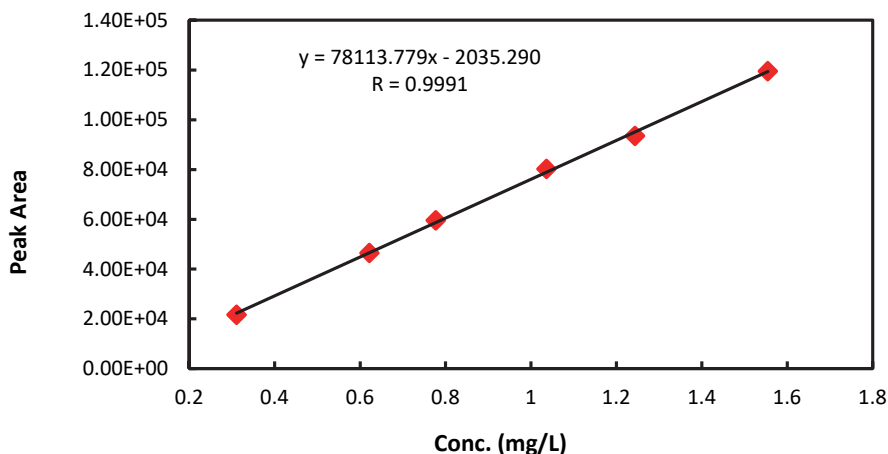|	
Regression equation	y = 78113.779x - 2035.290
Coefficient of determination (R^2)	0.9991
Lower concentration (mg/L)	0.311
Upper concentration (mg/L)	1.555

Figure 4. Calibration curve for the linearity analysis of the TFN method.

2.2.6 Detection Limit and Quantitation Limit

As given in literature, the detection limit (DL) of an individual analytical method is the lowest possible amount of analyte in a sample which could be detected; while, it is not necessarily quantitated as an exact value. Also, the quantitation limit (QL) of an individual analytical approach is the lowest amount of analyte in a sample which could be quantitatively determined with a suitable level of precision and accuracy. As shown in following, in this study, DL and QL were determined based on the standard deviation of the y-intercept and the slope of the calibration curve.[21]
The DL and QL were expressed as (Eq. (1) and Eq. (2)):

$$LOD = \frac{3.3\,\sigma}{S} \tag{1}$$

$$LOQ = \frac{10\,\sigma}{S} \tag{2}$$

where, σ is the standard deviation of y-intercept, and S is the slope of the calibration curve.
The DL and QL values were presented in **Table 4.**

Table 4. DL and QL values calculated for examining the validity of the method

Slope	78113.779
Standard deviation of y-intercept	1179.141
DL (mg/L)	0.050
QL (mg/L)	0.151

As presented in **Table 5**, the sample known to be near the DL, was reliably detected. Also, the sample known to be near the QL, was quantitatively determined with suitable precision and accuracy. The results show that the amounts of DL, and QL are 0.052 ppm, and 0.155 ppm, respectively, which indicate that the method is pretty sensitive. The DL, and QL parameters should not be more than 0.06, and 0.2, respectively. In addition, the recovery % should be between 98% to 102%.

Table 5. Validation of the DL and QL values for the developed TFN method

Conc. (mg/L)	Run1	Run2	Run3	Average	% RSD	Found Conc. (mg/L)	Recovery%
0.155	10382	10257	10220	10286.333	0.825	0.158	101.466
0.052	3091	3075	3098	3088	0.382	--	--

2.2.7 Accuracy

The accuracy of the present developed method has been evaluated by means of analyzing standard samples of TFN at three concentration levels with three independent sample preparations (Table 6). The recovery percentages were calculated regarding the regression equation. The results show that the recovery data are in the acceptable range of 98.0-102.0%.

Table 6. The results of the Accuracy calculations of the developed TFN method

Sample	Conc. (mg/L)	Run1	Run2	Run3	Average	Found Conc. (mg/L)	% Recovery
1	0.681	51455	51655	51789	51633.000	0.687	100.860
2	0.680	50740	51007	50945	50897.333	0.678	99.623
3	0.679	50646	50936	50842	50808.000	0.677	99.674
1	0.908	68052	68562	68699	68437.667	0.902	99.331
2	0.907	68721	69000	68743	68821.333	0.907	100.018
3	0.905	68278	68278	67825	68127.000	0.898	99.256
1	1.362	104025	104790	104017	104277.333	1.361	99.898
2	1.360	103912	104224	104729	104288.333	1.361	100.054
3	1.357	103422	103880	103891	103731.000	1.354	99.749

2.2.8 Precision

Due to the literature, the precision of an analytical method is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the defined conditions. Thus, the precision is the standard deviation, variance, or coefficient of variation of a series of measurements.[22]

2.2.9 Repeatability (Intra-day precision)

Repeatability of the method was assessed via analyzing the standard samples of TFN at 100% of the test concentration with six independent sample preparations. As given in Table 6, the % RSD values for TFN responses are less than 0.2% which could be acceptable (**Table 7**).

Table 7. Intra-day precision of the method on the first day

Sample#	Conc. (mg/L)	Run1	Run2	Run3	Average	% RSD
1	1.012	76720	76669	76723	76704	0.040
2	1.014	77754	77723	77854	77777	0.088
3	1.013	77657	77554	77820	77677	0.173
4	1.012	76750	76996	76740	76828.667	0.189
5	1.013	77509	77517	77439	77488.333	0.055
6	1.014	77083	77331	77255	77223	0.165

Table 8. Intra-day precision of the method on the second day.

Sample#	Conc. (mg/L)	Run1	Run2	Run3	Average	% RSD
1	1.013	77627	77517	77481	77541.667	0.098
2	1.013	78097	78036	78044	78059	0.042
3	1.014	78409	78451	78213	78357.667	0.162
4	1.012	76948	76849	76878	76891.667	0.066
5	1.014	77896	77768	77855	77839.667	0.084
6	1.013	77325	77246	77190	77253.667	0.088

As could be observed in Table 7 and Table 8, the intraday precision of the developed method does not significantly change between different days, and different instrument.

2.2.10 Intermediate precision

To investigate the intermediate precision, the standard samples of TFN at 100% of the test concentration (with six independent sample preparations on each day) were prepared by two different analysts and were analyzed by using two different columns. The results showed that the % RSD values for TFN responses are about 0.551, 0.656, and 0.947 for the first, second, and third days, respectively, which are less than 2.0% showing to be acceptable (the related tables are presented in the supplementary data).

2.2.11 Robustness study

To perform the robustness study on the developed method, a standard solution of TFN (which was spiked with the appropriate level of Leflunomide (its known impurity)) was used. During the study, the flow rate, column temperature and pH of the mobile phase A (buffer) were changed and the amount of change of the response was calculated, accordingly. As given in Tables 9, 10, and 11, by small changes in column temperature (-0.201%, and 0.159% changes in area for 33°C, and 37°C, respectively) and in pH (-0.759%, and -0.298% changes in area for pHs 2.8, and 3.2, respectively), the amount of change of the peak area was less than ± 2.0%. However, the results presented in Table 7 revealed that small changes in the mobile phase flow rate (4.933%, and -4.206% changes in area for 0.95ml/min, and 1.05 ml/min respectively) can significantly affect the peak response. Thus, it is necessary to ensure that the HPLC pump operates properly and correctly.

Table 9. The effect of column temperature on the peak response of the developed method

	TFN (0.993 mg/L)					
	Column temperature					
Run	T= 33°C		T= 35°C		T=37°C	
	RT	Area	RT	Area	RT	Area
1	22.715	73651	22.390	73733	22.024	73733
2	22.769	73756	22.388	73434	22.046	73690
3	22.688	73140	22.380	73824	22.046	73919
Average	22.724	73515.667	22.386	73663.667	22.039	73780.667
%RSD	0.181	0.448	0.019	0.290	0.058	0.165
Average of TF	1.156		1.153		1.145	
Average of NTP	15283.333		14942.400		15222.333	
Average of *Resolution	23.339		22.668		23.956	
% Change of peak area	-0.201		---		0.159	

*Resolution between TFN and Leflunomide peaks.

Table 10. The effect of pH of the mobile phase A on the peak response of the developed method.

	TFN (0.993 mg/L)					
	pH of mobile phase A					
Run#	**pH= 2.8**		**pH= 3.0**		**pH=3.2**	
	RT	**Area**	**RT**	**Area**	**RT**	**Area**
1	22.899	73078	22.390	73733	21.311	73548
2	22.839	73095	22.388	73434	21.309	73430
3	22.802	73141	22.380	73824	21.274	73355
Average	22.847	73104.667	22.386	73663.667	21.298	73444.333
%RSD	0.214	0.044	0.019	0.290	0.098	0.132
Average of TF	0.770		1.153		1.129	
Average of NTP	19399.333		14942.400		13698.667	
Average of *Resolution	28.005		22.668		24.724	
% Change of peak area	-0.759		---		-0.298	

*Resolution between TFN and Leflunomide peaks.

Table 11. The effect of the mobile phase flow rate on the peak response of the developed method

	TFN (0.993 mg/L)					
	Flow rate (mL/min)					
Run#	**F= 0.95**		**F= 1.0**		**F=1.05**	
	RT	**Area**	**RT**	**Area**	**RT**	**Area**
1	23.104	77178	22.390	73733	21.813	70721
2	23.114	77568	22.388	73434	21.819	70541
3	23.121	77147	22.380	73824	21.831	70434
Average	23.113	77297.667	22.386	73663.667	21.821	70565.333
%RSD	0.037	0.303	0.019	0.290	0.042	0.206
Average of TF	1.153		1.153		1.129	
Average of NTP	15138		14942.400		13842.667	
Average of *Resolution	22.411		22.668		23.047	
% Change of peak area	4.933		---		-4.206	

*Resolution between TFN and Leflunomide peaks.

According to the results, the method showed a high level of suitability, specificity, linearity, accuracy, precision, repeatability, robustness, and reliable detection limit.

3. Conclusions

In the case of specificity, the results show that there is not a significant difference between the spectrums of TFN. Also, the standard solution of TFN was spiked with the appropriate level of known Leflunomide and the resolution between those two was generally high. The results revealed no peak interference with the TFN peak, therefore the specificity of the method was confirmed. The Data, which were achieved by calibration curve, show that the coefficient of determination (R^2) is about 0.9991. It indicates the high level of linearity of the method. Also, in the case of the detection limits, the results showed that the amounts of DL, and QL were 0.052 ppm, and 0.155 ppm, respectively, which indicated that the method is pretty sensitive. Moreover, the results showed that the recovery data obtained within

the acceptable range of 98.0-102.0%. On the other hand, the repeatability of the method was studied via analyzing the standard samples of TFN at 100% of the test concentration with six independent sample preparations. The results showed that the percentage of the RSD values for TFN responses are less than 0.2% which could be acceptable. Finally, the results showed that by small changes in column temperature (-0.201%, and 0.159% changes in area for 33°C, and 37°C, respectively) and in pH (-0.759%, and -0.298% changes in area for pHs 2.8, and 3.2, respectively), the amount of change of the peak area was less than ± 2.0%. However, small changes in the mobile phase flow rate (4.933%, and -4.206% changes in area for 0.95ml/min, and 1.05 ml/min respectively) can significantly affect the peak response. Thus, it is necessary to ensure that the HPLC pump operates properly and correctly. According to the results, the method showed a high level of suitability, specificity, linearity, accuracy, precision, repeatability, robustness, and reliable detection limit.

Supplementary Data

Supplementary data are available at the Journal home page.

Acknowledgment

3. Materials and methods

3.1 Experimental

Chemicals containing potassium dihydrogen phosphate (PDP), ortho-phosphoric acid (OPA), and potassium chloride (PC), and acetonitrile (ACN) were prepared from Merck chemical company (Germany). Teriflunomide (TFN) was provided from the Chemical Synthesis Department of Tofigh Daru Research and Engineering Company (Tehran, Iran).

3.1.1 Instrumentation

The Shimadzu Prominence system (Shimadzu Corporation, Kyoto, Japan) Equipped with a LC-20AD pump, a DGU-20A degassing system, a CTO-20A column oven and a SPD-20A UV-Vis detector, was used for all analysis. Also, the LabSolutions software version 5.51 was applied for processing and data analysis.

3.1.2 Chromatographic conditions and sample preparation

A C18, end-capped (250 × 4.6) mm, 5µm liquid chromatography column was applied for both Assay and Related substance analysis. The mobile phase A was prepared by dissolving 2.74 g of PDP and 3.72 g of PC in water and further dilution to 1000 mL with water as solvent. Then, pH was adjust to 3.0 by adding OPA 85%. Also, the mobile phase B was ACN (100%). Also, a UV detector on the wavelength of 280 nm was used to record the chromatograms. The column temperature was maintained at 35°C and the injection volume was 20 µL. The sample solutions were prepared as following:

Test solution (a): Dissolve 10 mg of the substance to be examined in the diluent (sonicate the solution

if it is needed) and dilute to 10 mL with the same solvent (1000 mg/L).

Test solution (b): Dilute 1.0 mL of Test solution (a) to 50.0 mL with the diluent (20 mg/L).

Test solution (c): Dilute 1.0 mL of Test solution (b) to 20.0 mL with the diluent (1 mg/L).

The injections were 20 µL of the Blank solution, Test solution (c) and Test solution (a). To calculate the number of impurities, the peak areas of impurities in the chromatogram obtained with Test solution (a) and the peak area of principal peak in the chromatogram obtained with Test solution (c) were required. The concentration range of the solutions required for calibration curve preparation were 151.626 mg/L to 252.710 mg/L.An end-capped C18 (5 µm, 4.6 × 250 mm) Hector chromatographic column was applied for the present reverse-phase HPLC system. Also, the elution program for the process was: 0.00/75, 30.00/55, 60.00/55 (time (min)/A%) at the flow rate of 1.0 ml/min. The solvent selected for dissolving and diluting of the sample was ACN, and the chromatograms have been recorded at 280nm. The column temperature was 35°C, and volume of injection was 20 µL. The stock solution for the linearity study was prepared by dissolving the substance in acetonitrile on the concentration of 0.01561 g of TFN in 50.0 mL of diluents.

3.2 Theoretical

At the first step, a reasonable structure based on the usual predictions of the Gaussian 03 quantum chemical package[23] was developed and undergone BOMD simulations.[24,25] Then, some hypothetical structures were extracted from the output spectrum of the trajectory simulation. Then, those structures were designed as input files, and optimized to give the most stable system. Finally, the best system which was detected by optimization of the system at B3LYP/6-311G(d,p) level of theory,[26,27] was set for the TD-DFT calculations[28,29] to give the UV-visible spectrum at the mentioned level.

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