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Development of a validated liquid chromatographic method for determination of related substances of telmisartan in bulk drugs and formulations

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ABSTRACT

A simple and rapid reversed phase liquid chromatographic method for separation and determination of the related substances of telmisartan (TLM) was developed and validated. The chromatographic separation was achieved on Lichrospher RP-18 column (250 × 4.6 mm, 5 μm), using 20 mM ammonium acetate containing 0.1% (v/v) triethylamine (pH adjusted to 3.0 with trifluoroacetic acid) and acetonitrile as mobile phase at 25 °C. The detection was performed at 254 nm. The method was validated and found to be robust, precise, specific and linear between 0.37 and 500 μg/mL. The limits of detection and quantification of telmisartan were 0.11 and 0.37 μg/mL, respectively. The method was successfully applied to quantify related substances and assay of TLM in bulk drugs and commercial tablets. The related substances relate to a novel synthetic route and different from those A-H impurities reported by European Pharmacopeia.

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1. Introduction

Telmisartan (TLM) (4-((2-*n*-propyl-4-methyl-6-(1-methylbenzimidazol-2-yl)-benzimidazol-1-yl)methyl)-biphenyl-2-carboxylic acid) is an angiotensin II type-I receptor antagonist blocker (Fig. 1), widely used in treatment of hypertension [1]. It is synthesized by multi step reactions during which a host of intermediates are produced in a laboratory [2,3]. It is likely that the small quantity of these unreacted intermediates may left over during the variety of reaction steps and finally decrease the yield and quality of the finished products. Recently, in an attempt to produce impurity free TLM, the original process was modified and the number of steps were reduced [4]. Monitoring of reactions as well as the yield and purity is very important to assess the viability of such processes for commercial production of TLM. Thus, there is a great need for development of analytical methods for separation and determination of related substances for process development and quality control of TLM.

A thorough literature search revealed that LC with different detection systems such as spectrofluorimetric, photodiode array, mass spectrometric was used for quantitative determination of TLM in biological fluids [5–17]. Several other approaches like cell based assay [18], immune assay [19,20], electrophoretic [21,22], polarographic [23,24] and high performance thin layer chromatography (HPTLC) [25] have been tried to analyse TLM in various matrices. European Pharmacopeia (EP) monograph [26] adopted a gradient HPLC method for impurity profiling of A-H impurities of TLM. The EP method uses sodium pentanesulfonate monohydrate an ion pairing reagent as a mobile phase additive phase which decreases the column life and needs a long time for equilibration. To the best of our knowledge no analytical method for monitoring of related substances of TLM in a manufacturing unit is available in the literature.

The present paper describes a reversed-phase gradient HPLC for simultaneous separation and determination of TLM, and related impurities on C_{18} column using 20 mM ammonium acetate (NH_4OAc) containing 0.1% triethylamine (pH adjusted to 3.0 with trifluoroacetic acid) and acetonitrile as a mobile phase. The method was validated and found to be suitable for the quality assessment of TLM in pharmaceutical formulations. The related substances studied in the present investigation are different from those A-H impurities reported by EP.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical reagent grade, unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA), HPLC grade methanol and acetonitrile were purchased from Merck, Mumbai, India. AR-grade triethylamine, trifluoroacetic acid and ammonium acetate were purchased from SD Fine Chemicals, Mumbai, India. TLM and its related substances I {7-methyl-2-propyl-3H-benzo[d]imidazole-5-carboxylic acid}, II {4-methyl-6-(1-methyl-1H-benzo[d]imidazol-

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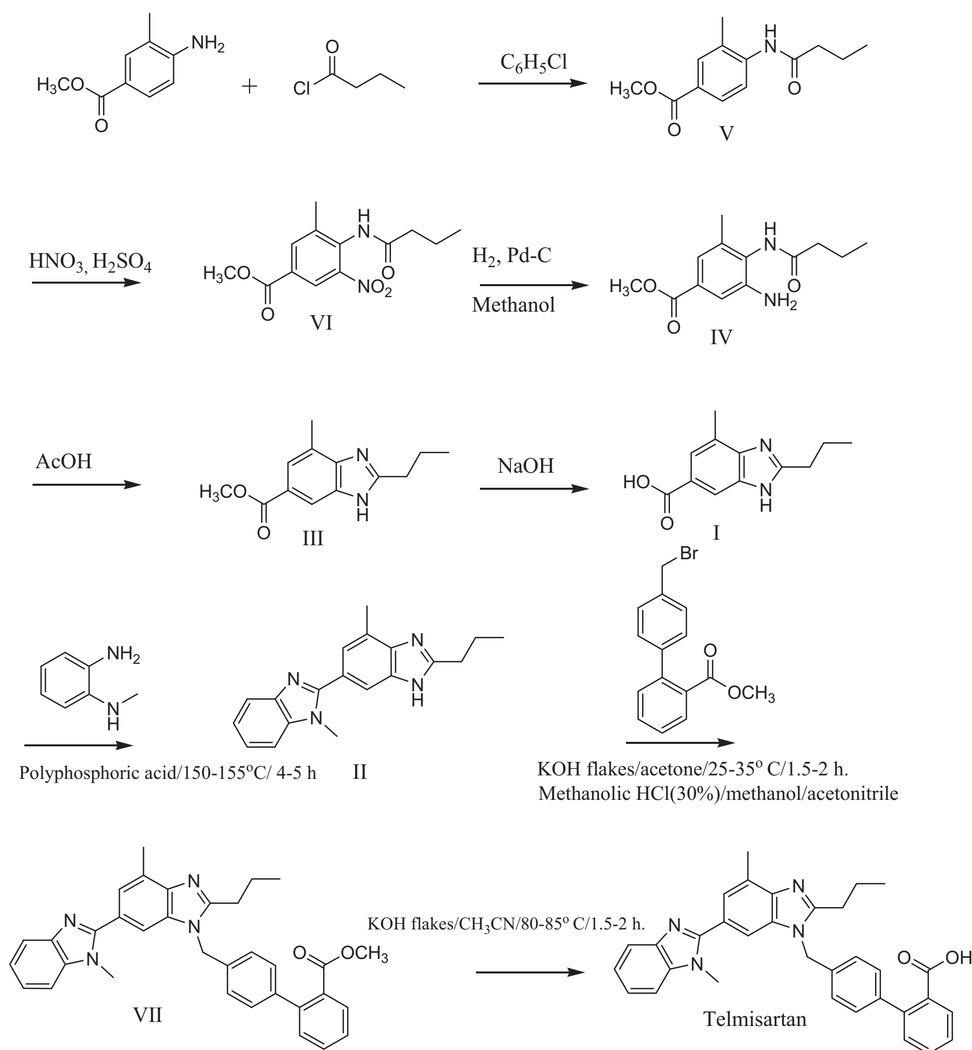

Fig. 1. Synthesis of telmisartan and its process related substances (I–VII in order of their chromatographic elution).

2-yl)-2-propyl-1H-benzo[d]imidazole}, III {methyl 7-methyl-2-propyl-3H-benzo[d]imidazole-5-carboxylate}, IV {methyl 3-amino-4-(butyramido)-5-methylbenzoate}, V {methyl 4-(butyramido)-3-methylbenzoate}, VI {methyl 4-(butyramido)-3-methyl-5-nitrobenzoate} and VII {methyl 4′-[[4-methyl-6-(1-methyl-1H-benzimidazol-2-yl)-2-propyl-1H-benzimidazol-1-yl]methyl]biphenyl-2-carboxylate)} (Fig. 1) were procured from local manufacturing companies in Hyderabad, India. Centrifuge and sample tubes supplied by Tarsons (Kolkata, India) were used. Nylon syringe filters (0.45 μm) purchased from Millipore (Banglore, India) were used.

2.2. Preparation of solutions

Stock solutions 1.0 mg/mL of TLM and related substances I–VII were prepared in methanol. The solutions were adequately diluted with methanol to study accuracy, precision, linearity, limit of detection and quantification. The specified concentration of TLM was taken as 500 μg/mL.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisting of a quaternary LC-20AD pump, a SPD-M20A diode array detector, a SIL-20AC autosampler, a DGU-$20A_5$ degasser and CBM-20A communications bus module (all from Shimadzu, Kyoto, Japan), was used. The pH measurements were carried out by Elico, model LI 120, pH meter equipped with a combined glass–calomel electrode calibrated using standard buffer solutions of pH 4.0, 7.0 and 9.2. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). Before the mobile phase was delivered into the system, mobile phase A consisting of 20 mM NH_4OAc containing 0.1% (v/v) triethylamine (pH adjusted to 3.0 with trifluoroacetic acid) and mobile phase B consisted of acetonitrile were filtered through 0.45 μm PTFE filters and degassed by sonication for half an hour. HPLC analysis was carried out on a Lichrospher RP-18 column (250 × 4.6 mm, 5 μm) (Merck, Darmstadt, Germany), at 25 °C. Mobile phases A & B were eluted in a gradient mode (Table 1) at a flow rate of 1.0 mL/min, the injection volume was 20 μL. The detection was performed at 254 nm.

2.4. Analysis of tablets

Ten tablets each one containing 40 mg of TLM were powdered and dissolved in 100 mL methanol in a volumetric flask and sonicated for half an hour. The solution was filtered through a 0.45 μm nylon filter before analysis. For assay, 12.5 mL of the filtered solution was diluted to 50.0 mL with methanol and 20 μL of the solution injected on to HPLC. To cross check the adsorption of drug on to the

Table 1
Gradient program.

Time (min)	Mobile phase A (%, v/v)	Mobile phase B (%, v/v)	Mode
0.01–7.00	65	35	Isocratic
7.00–8.00	65–50	35–50	Linear gradient
8.00–20.00	50–25	50–75	Linear gradient
20.00–22.00	25–15	75–85	Linear gradient
22.00–30.00	65	35	Back to initial condition

A: 20 mM ammonium acetate containing 0.1% (v/v) triethylamine (pH adjusted to 3.0 with trifluoroacetic acid).
B: acetonitrile.

filter paper, a standard 20.0 μg/mL TLM solution in methanol was injected with and without filtration. No significant adsorption of the drug was noticed.

3. Results and discussion

3.1. Characterization of telmisartan and its related substances

Fig. 1 shows the modified synthetic process of TLM followed in the present investigation. It could be seen from Fig. 1 that there are 8 steps and 7 intermediates are produced during the process. Thus the 7 intermediates except starting material were considered for method development. TLM and the related substances were obtained from a local manufacturing unit and characterized by ^{1}H NMR and mass spectrometry. TLM, MS *m*/*z* 515 (M^++H); ^{1}H NMR ($CDCl_3$) δ 12.8 (s, 1H), 7.05–7.5 (m, 14H), 5.60 (s, 2H), 3.82 (s, 3H), 2.97 (t, 2H), 2.63 (s, 3H), 1.88 (q, 2H), 1.04 (t, 3H). Impurity I, MS *m*/*z* 219 (M^++H); ^{1}H NMR ($CDCl_3$) δ 8.1 (s, 1H), 7.6–7.8 (m, 2H), 2.95 (t, 2H), 2.90 (s, 3H), 1.90 (q, 2H), 1.02 (t, 3H). Impurity II, MS *m*/*z* 305 (M^++H); ^{1}H NMR ($CDCl_3$) δ 7.8 (s, 1H), 7.2–7.7 (m, 6H), 3.89 (s, 3H), 2.80 (t, 2H), 2.05 (s, 3H), 1.86 (q, 2H), 0.99 (t, 3H). Impurity III, MS *m*/*z* 233 (M^++H); ^{1}H NMR ($CDCl_3$) δ 8.1 (s, 1H), 7.6–7.9 (m, 2H), 3.93 (s, 3H), 2.81 (t, 2H), 2.2 (s, 3H), 1.81 (q, 2H), 1.01 (t, 3H). Impurity IV, MS *m*/*z* 251 (M^++H); ^{1}H NMR ($CDCl_3$) δ 8.0 (s, 1H) 7.28 (m, 2H), 3.86 (s, 2H), 3.82 (s, 3H), 2.39 (t, 2H), 1.81(q, 2H), 1.16 (t, 3H). Impurity V, MS *m*/*z* 236 (M^++H); ^{1}H NMR ($CDCl_3$) δ 8.12 (s, 1H), 7.71–7.79 (m, 2H), 7.01 (s, 1H), 3.89 (s, 3H), 2.41 (t, 2H), 2.31 (s, 3H), 1.8 (q, 2H), 1.08 (t, 3H). Impurity VI, MS *m*/*z* 281 (M^++H); ^{1}H NMR ($CDCl_3$) δ 8.65 (s, 1H), 8.15–8.51 (d, 2H), 3.98 (s, 3H), 2.45 (t, 2H), 2.34 (s, 3H), 1.82 (q, 2H), 1.13 (t, 3H). Impurity VII, MS *m*/*z* 529 (M^++H); ^{1}H NMR ($CDCl_3$) δ 7.2–7.9 (m, 14H), 5.7 (s, 2H), 4.10 (s, 3H), 3.59 (s, 3H), 3.28 (t, 2H), 2.88 (s, 3H), 2.01 (q, 2H), 1.14 (t, 3H).

3.2. Method development

TLM and its related substances are polar as well as basic in nature. Adsorption or normal phase chromatography although offers good selectivity, but rarely used for analysis of basic compounds. It could be due to the strong interaction between amino groups and the surface hydroxyls on the silica used as stationary phase which makes difficult to elute the compounds from the column. Therefore a reverse-phase chromatographic system was selected. This was based on the fact that most of the reported methods for analysis of basic drugs were based on reverse-phase chromatography. In the present study preliminary experiments were carried out by subjecting TLM and all the related substances on various commercial C_{18} columns (Table 2) and finally Lichrospher RP-18 column (250 × 4.6 mm, 5 μm, Merck, Darmstadt, Germany) was used. Initially, buffers such as 20 mM NH_4OAc (pH adjusted to 3.0 with trifluoroacetic acid) and 20 mM potassium dihydrogen ortho-phosphate (pH 3.0 adjusted with phosphoric acid) and an organic modifiers, viz; methanol and acetonitrile were tried. Methanol resulted in broad peaks, so it was discarded as an organic

Table 2
Column selectivity.

Column	Rs_I	Rs_{II}	Rs_{III}	Rs_{IV}	Rs_V	Rs_{VI}	Rs_{TLM}	Rs_{VII}
Lichrospher	2.43	2.16	2.34	3.26	17.86	2.01	2.61	6.64
Inertsil ODS	2.31	2.12	2.24	3.12	17.27	1.96	2.57	6.58
Hypersil C_{18}	2.22	2.08	2.30	3.04	17.65	1.92	2.42	6.42
t_{cal1}	0.84	1.80	0.64	1.49	0.21	0.62	1.02	0.19
t_{cal2}	1.12	2.34	0.82	0.29	2.20	0.96	0.37	0.34
t_{tab}	2.44	2.44	2.44	2.44	2.44	2.44	2.44	2.44

t = Student's *t*-value; *n* (no. of observations) = 7; (Rs_I, Rs_{II}, Rs_{III}, Rs_{IV}, Rs_V, Rs_{VI}, Rs_{TLM} and Rs_{VII} are resolutions of impurities I–VII and telmisartan). $t_{cal1} = t_{calculated}$ (Lichrospher vs. Inertsil ODS); $t_{cal2} = t_{calculated}$ (Lichrospher vs. Hypersil C_{18}); $t_{tab} = t_{tabulated}$ at 95% confidence interval.
$t_{calculated} < t_{tabulated}$, this indicate resolutions on compared columns are insignificant. It shows others column may be used for analysis.

modifier and acetonitrile was used for method optimization. In case of potassium dihydrogen ortho-phosphate, the separation of V, TLM and VI was poor, so ammonium acetate was selected as a buffer. All the peaks showed significant tailing under these conditions. To improve peak shapes, triethylamine (TEA) was added to the mobile phase. As TEA leads to column swelling due to hydrolysis of silica the mobile phase was acidified with trifluoroacetic acid. TEA competes with the analytes to interact with the further residual silanols, due to which the tailing was reduced significantly. Since the related substances of TLM have different polarities a gradient mode of elution was tried for optimum separation. The optimized chromatogram is shown in Fig. 2.

3.3. Method validation

3.3.1. Robustness

According to the ICH (1994) guideline Q2A, 'robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [27–30]. Standardized Pareto charts are useful in evaluating robustness testing where variables are not interacting. With the help of scaled and centered coefficient plot obtained from design of experiments the impact of variables interacting with each other can be represented.

Three different types of method parameters exist: basic, internal and external parameters. The present robustness study was limited to investigating the influence of basic and internal parameters. External parameters, such as different laboratories, analysts and instruments, were not included in the study. An experimental design, the augmented Plackett–Burman, was applied to study the influence of the internal parameters, percentage concentration of NH_4OAc, percentage concentration of triethylamine, pH of buffer solution, column temperature and flow rate. A Plackett–Burman

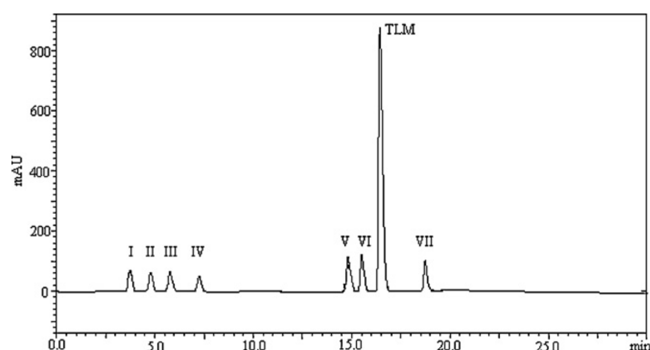

Fig. 2. A typical HPLC chromatogram of TLM spiked with 10% of related substances I–VII, obtained under optimized conditions.

Table 3
Selected parameters and their variations used to test robustness of the method by design of experiments.

Parameters and variation[a]	−1	0	+1
Conc. of NH_4OAc in mM (A)	15	20	25
% of Triethylamine (B)	0.09	0.10	0.11
pH (C)	2.8	3.0	3.2
Flow rate in ml/min (D)	0.9	1.0	1.1
Column temp. in °C (E)	20	25	30

[a] Dummy factor −1 indicates low level variance, 0 indicates middle level variance and +1 denotes upper level variance.

design is an orthogonal two-level experimental design which can be used to fit linear models. It has the advantage of a low number of runs combined with a high precision, but in the case of curvature of the resulting graph, it does not reveal the responsible factor. 'Augmented' refers to the fact that three central points were added in order to estimate the method's repeatability. The design matrix with the factor settings is shown in Tables 3 and 4.

The experiments were run randomly with sample spiked with 500 μg/mL of TLM and 50 μg/mL of related substances I–VII. The selected responses were Rs_x where x = I–VII and TLM (separation between x and $x+1$ peaks). In a separate design with same variables selected responses were T_I–T_{VII} (asymmetries for peak I to VII) and T_{TLM} (asymmetry for TLM peak). Plotting the scaled and centered coefficient plots for selected responses Rs_I–Rs_{VII}, T_I–T_{VII} and T_{TLM} (Fig. 3a and b) revealed that logarithmic transformations were necessary for optimizing the responses. The respective 95% confidence intervals are shown as error bars. Coefficients with 95% confidence intervals including zero were statistically insignificant.

It was observed that percentage concentration of triethylamine, buffer concentration and pH of buffer solution were significant factors in this design. A significant decrease in response of Rs_{VI} in experiment 18 with increase in buffer concentration was observed. Even in this case resolution was near to 2, which was under the limits of system suitability test hence did not effect the robustness of the system. In conclusion the developed method was robust and satisfies system suitability parameters.

Table 5
System suitability test (SST) data.

Analyte	t_R (min) (±SD)[a]	RSD%	RRT	R_s	T_f	k'	RRF
I	3.73 ± 0.03	0.80	0.23	2.43	1.43	2.11	0.96
II	4.78 ± 0.04	0.84	0.29	2.16	1.64	2.98	0.84
III	5.77 ± 0.05	0.87	0.35	2.34	1.72	3.81	0.86
IV	7.25 ± 0.04	0.55	0.44	3.26	1.52	5.04	0.80
V	14.79 ± 0.11	0.74	0.90	17.86	1.57	11.32	1.02
VI	15.48 ± 0.09	0.58	0.94	2.01	1.50	11.90	1.05
TLM	16.42 ± 0.16	0.97	1.00	2.61	1.43	12.68	1.00
VII	18.74 ± 0.17	0.91	1.14	6.64	1.29	14.62	0.93

[a] Average of 7 observations.

3.3.2. Quantitative aspects

3.3.2.1. System suitability test (SST). Parameters such as tailing factor (A_s), relative retention time (RRT), capacity factor (k'), resolution (R_s), relative retention factor (RRF) and reproducibility (RSD %) were determined and compared against the specifications set for the method (Table 5). These parameters were measured using the reference standards of TLM and related substances. SSTs were determined and compared with the recommended limits in British Pharmacopeia ($1 \le A_s \le 2$; $R_S > 2$; RSD < 1% for $n > 5$; and $k' > 2$).

3.3.2.2. Limits of detection (LOD) and quantification (LOQ). Limit of detection (LOD) was measured as the lowest amount of the analyte that could be detected to produce a significant response. It was approved by calculations based on the standard deviation of the response (σ) and the slope (S) of the calibration curve at the levels approaching the limits according to equation LOD = 3.3 (σ/S) and LOQ = 10 (σ/S). The LOD and LOQ values of TLM and related substances I–VII are given in Table 6.

Table 4
Fractional factorial experimental design and responses developed to test the robustness of the method.

E.N.	R.O.	A	B	C	D	E	Rs_I	Rs_{II}	Rs_{III}	Rs_{IV}	Rs_V	Rs_{VI}	Rs_{VII}	T_I	T_{II}	T_{III}	T_{IV}	T_V	T_{VI}	T_{TLM}	T_{VII}
1	22	−1	−1	−1	−1	+1	1.583	3.839	3.181	20.371	2.119	1.929	8.616	1.47	1.68	1.74	1.56	1.58	1.53	1.48	1.35
2	4	+1	−1	−1	−1	−1	0.996	4.678	3.126	22.559	1.515	1.031	7.481	1.45	1.65	1.73	1.55	1.56	1.51	1.45	1.34
3	5	−1	+1	−1	−1	−1	0.080	2.587	3.815	22.080	1.007	2.473	4.907	1.38	1.60	1.62	1.43	1.52	1.46	1.37	1.26
4	6	+1	+1	−1	−1	+1	1.473	3.982	4.481	21.331	2.303	0.76	8.761	1.37	1.56	1.70	1.46	1.48	1.43	1.34	1.24
5	21	−1	−1	+1	−1	−1	5.516	0.720	2.145	19.614	2.498	7.995	7.502	1.49	1.69	1.74	1.54	1.57	1.53	1.49	1.36
6	20	+1	−1	+1	−1	+1	4.144	1.873	3.469	20.893	2.256	5.981	8.441	1.46	1.66	1.72	1.57	1.55	1.54	1.47	1.35
7	18	−1	+1	+1	−1	+1	4.215	1.724	4.266	19.599	2.156	5.196	8.503	1.40	1.58	1.63	1.43	1.56	1.48	1.38	1.26
8	1	+1	+1	+1	−1	−1	5.842	1.005	3.373	20.367	2.571	7.321	7.307	1.38	1.56	1.71	1.45	1.49	1.45	1.34	1.27
9	13	−1	−1	−1	+1	−1	1.115	4.031	2.599	20.912	2.527	1.282	8.015	1.50	1.69	1.74	1.54	1.57	1.55	1.49	1.33
10	28	+1	−1	−1	+1	+1	1.218	4.142	3.389	22.471	0.627	0.137	7.829	1.50	1.67	1.74	1.56	1.54	1.55	1.50	1.31
11	23	−1	+1	−1	+1	+1	0.268	2.276	4.138	17.987	0.456	1.685	6.750	1.33	1.58	1.63	1.44	1.51	1.43	1.33	1.23
12	29	+1	+1	−1	+1	−1	0.946	4.101	3.911	22.444	1.742	0.383	5.855	1.36	1.55	1.67	1.44	1.46	1.45	1.36	1.27
13	16	−1	−1	+1	+1	+1	5.175	2.014	0.710	19.729	2.161	8.783	7.992	1.49	1.68	1.74	1.56	1.56	1.51	1.50	1.34
14	11	+1	−1	+1	+1	−1	3.362	2.284	3.112	21.816	2.704	5.212	8.096	1.50	1.65	1.75	1.55	1.55	1.53	1.48	1.32
15	3	−1	+1	+1	+1	−1	3.500	2.129	4.034	21.025	2.615	4.271	8.063	1.37	1.57	1.62	1.46	1.52	1.43	1.33	1.26
16	17	+1	+1	+1	+1	+1	4.807	0.330	2.303	21.014	2.324	8.952	7.804	1.37	1.58	1.69	1.48	1.44	1.45	1.37	1.28
17	10	−1	0	0	0	0	2.926	2.847	3.890	21.595	2.496	4.102	8.747	1.43	1.65	1.72	1.51	1.57	1.48	1.44	1.30
18	24	+1	0	0	0	0	2.011	3.674	4.008	21.832	2.512	1.879	9.155	1.41	1.61	1.69	1.49	1.55	1.50	1.44	1.29
19	2	0	−1	0	0	0	2.475	3.476	3.405	21.987	2.472	3.471	8.509	1.46	1.68	1.72	1.54	1.59	1.52	1.45	1.31
20	27	0	+1	0	0	0	2.514	2.586	3.930	18.399	2.124	2.610	8.180	1.39	1.58	1.70	1.48	1.50	1.46	1.38	1.26
21	25	0	0	−1	0	0	1.545	3.972	3.986	22.157	2.437	0.891	8.685	1.42	1.63	1.70	1.51	1.55	1.47	1.40	1.29
22	19	0	0	+1	0	0	5.655	0.800	3.070	21.382	2.497	7.937	7.753	1.43	1.65	1.71	1.52	1.56	1.51	1.42	1.28
23	15	0	0	0	−1	0	2.479	2.984	4.015	20.793	2.373	2.756	8.699	1.45	1.63	1.72	1.53	1.56	1.53	1.44	1.30
24	8	0	0	0	+1	0	2.301	3.043	3.938	21.282	2.414	3.133	8.439	1.44	1.64	1.70	1.52	1.54	1.50	1.43	1.30
25	7	0	0	0	0	−1	2.177	3.355	3.896	21.143	2.546	2.319	8.331	1.43	1.64	1.71	1.52	1.56	1.49	1.45	1.31
26	14	0	0	0	0	+1	4.039	1.695	3.506	20.338	2.216	6.119	8.349	1.46	1.66	1.73	1.54	1.57	1.50	1.43	1.29
27	9	0	0	0	0	0	2.421	3.167	4.088	21.275	2.407	2.983	8.587	1.43	1.65	1.72	1.52	1.56	1.49	1.42	1.29
28	12	0	0	0	0	0	2.432	3.173	4.089	21.285	2.412	2.989	8.589	1.42	1.64	1.72	1.53	1.57	1.50	1.42	1.29
29	26	0	0	0	0	0	2.430	3.169	4.082	21.270	2.416	2.989	8.595	1.42	1.64	1.71	1.51	1.55	1.48	1.41	1.31

E.N. = experiment number and R.O., run order.

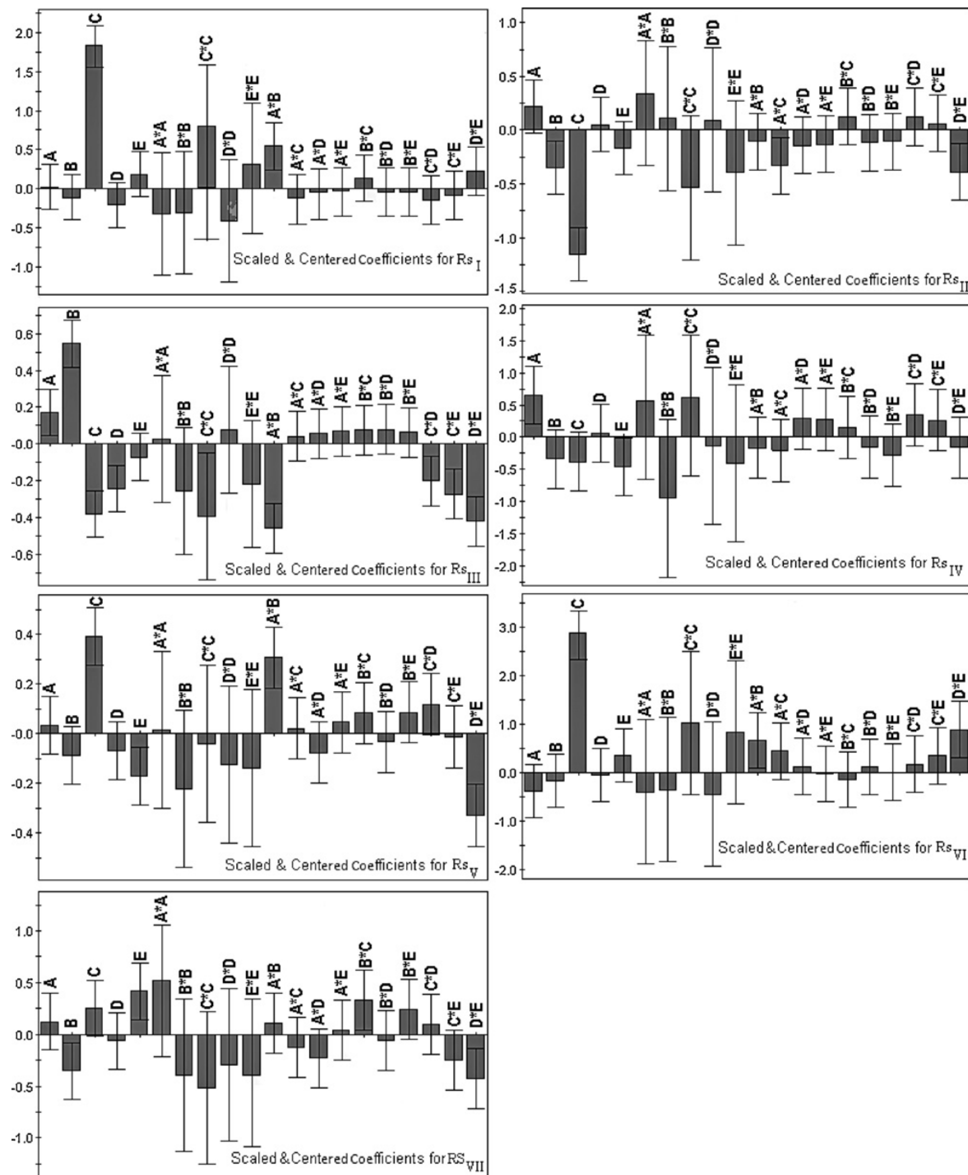

Fig. 3. (a) Scaled and centered coefficients of resolutions, Rs_I–Rs_{VII} [A: conc. of NH_4OAc; B: conc. of triethylamine; C: pH; D: flow rate; E: column temperature]. A*A, B*B, etc. represent interaction of variables A, B, C, D and E with their own. (b) Scaled and centered coefficients of asymmetric factors, T1–T8.

3.3.2.3. Linearity. Peak areas of each compound were measured and used for quantification. The peak areas of TLM and related substances I-VII versus concentration were plotted and found to be linear within the concentration range 0.37–500.00 μg/mL. Standard curves were constructed on three consecutive days and the evaluation parameters like slope, intercept and correlation coefficient were calculated. Calibration equation and correlation coefficients (r^2) of TLM and related substances I–VII are given in Table 6.

3.3.2.4. Specificity. Specificity of a method can be defined as absence of any interference at retention times of peak of interest, and was evaluated by observing the chromatograms of blank samples and samples spiked with TLM and all its related substances.

Table 6
Linearity, limits of detection and quantification of standard curves.

Analyte	Calibration equation (y = area, x = μg/mL)	Correlation coefficients (r^2)	LOD (μg/mL)	LOQ (μg/mL)
I	$y = 8.17x + 0.996$	0.9918	0.14	0.47
II	$y = 11.04x - 4.43$	0.9976	0.17	0.57
III	$y = 10.16x + 6.04$	0.9945	0.16	0.53
IV	$y = 11.03x - 0.03$	0.9929	0.09	0.30
V	$y = 11.41x+3.62$	0.9936	0.12	0.40
VI	$y = 6.37x - 2.14$	0.9954	0.13	0.43
TLM	$y = 11.24x + 362.4$	0.9990	0.11	0.37
VII	$y = 5.98x - 4.58$	0.9987	0.16	0.53

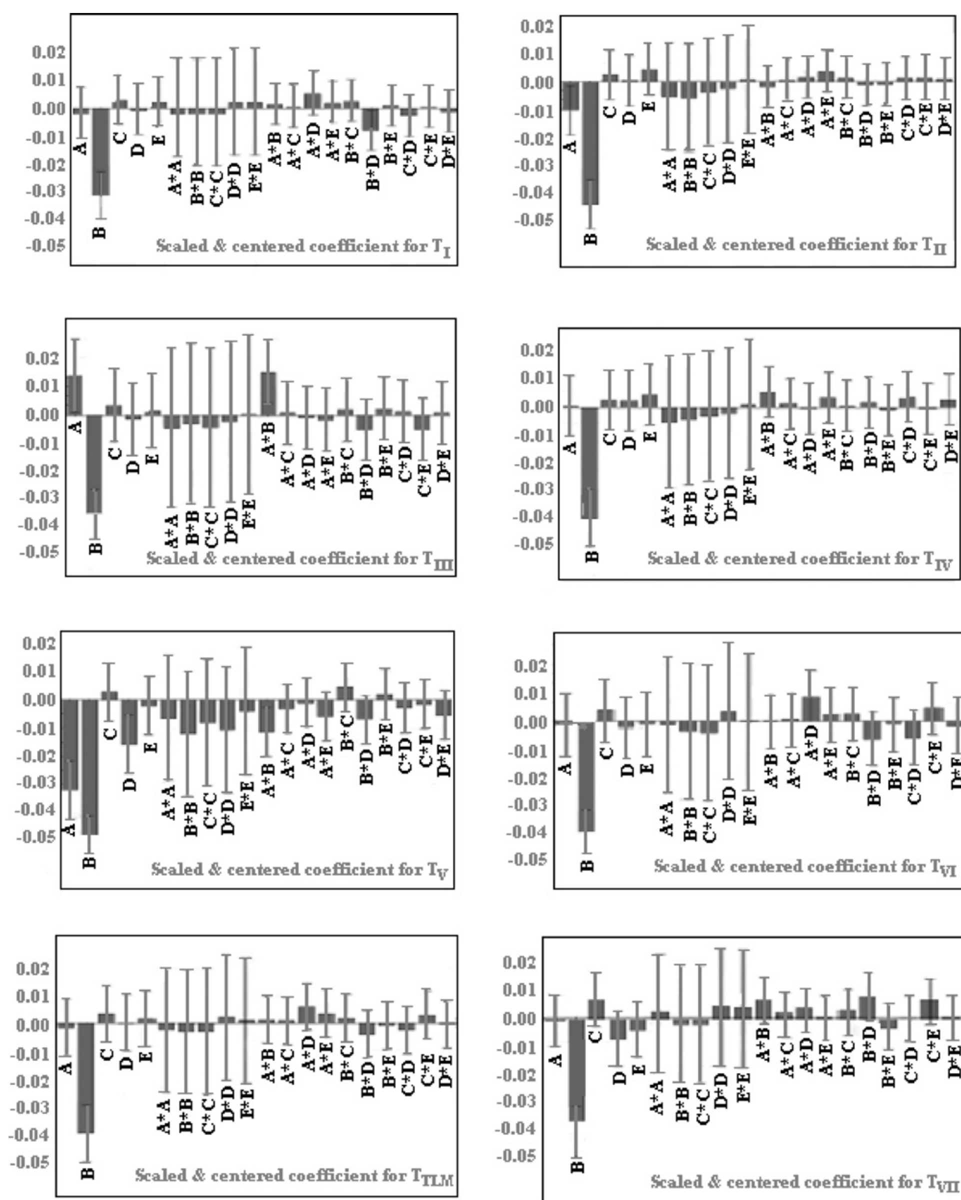

Fig. 3. (*Continued.*)

The elution peaks of each analytes were presented in representative chromatograms shown in Fig. 2. The retention times of TLM and I–VII is given in Table 5.

3.3.2.5. Accuracy and precision. The precision of the assay was studied with respect to both repeatability and intermediate precision. Repeatability was calculated from five replicate injections of freshly prepared solution in the same equipment on the same day. Repeatability for TLM was realized with a 500 μg/mL solution. Related substances were tested at a concentration of 50 μg/mL of each in a solution containing 500 μg/mL of TLM. The experiments were repeated by assaying freshly prepared solution at the same concentration on three consecutive days to determine intermediate precision. The intra- and interday precisions were in the range

Table 7
Intra-day and inter-day precision and accuracy.

Analyte	Nominal concentration (in μg/mL)	Mean concentration (in μg/mL)	Intra-day precision (RSD%) ($n = 5$)	Inter-day precision (RSD%) ($n = 5$, 3 days)	Accuracy (% bias)
I	50.00	49.97	0.39	0.86	–0.06
II	50.00	50.22	0.83	0.98	+0.44
III	50.00	49.94	0.38	0.81	–0.12
IV	50.00	49.92	0.37	0.91	–0.16
V	50.00	49.76	0.87	0.65	–0.48
VI	50.00	49.79	0.43	0.92	–0.42
TLM	500.00	500.51	0.32	0.97	+0.10
VII	50.00	50.02	0.22	0.82	+0.04

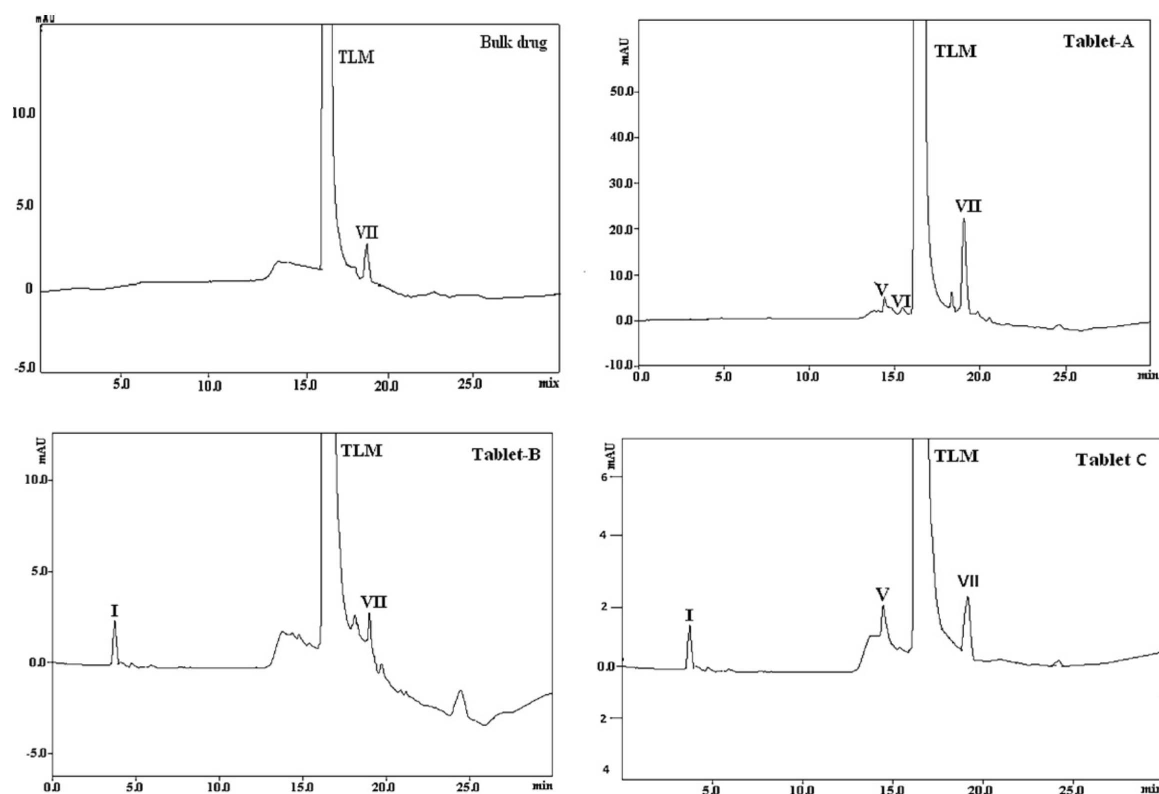

Fig. 4. Chromatograms of bulk drug and commercial tablets A, B and C of TLM (40 mg each).

Table 8
Assay of TLM and its process related substances in bulk drug and tablets by HPLC.

Analyte	Tablet A	Tablet B	Tablet C	Bulk drug
I	<LOD	0.021%	0.016%	<LOD
II	<LOD	<LOD	<LOD	<LOD
III	<LOD	<LOD	<LOD	<LOD
IV	<LOD	<LOD	<LOD	<LOD
V	0.021%	<LOD	0.009%	<LOD
VI	0.013%	<LOD	<LOD	<LOD
TLM	97.567%	100.312%	101.012%	99.12%
VII	0.045%	0.017%	0.026%	0.021%

Percentage of impurities was given by blank correction and comparison with chromatograms of standard peaks.

of 0.22–0.87% and 0.65–0.97% (% RSD), respectively (Table 7). The accuracy was expressed as bias, ranged from −0.48% to +0.44% (Table 7). These results were in accordance with acceptance criteria for accuracy (from −5% to +5%) and precision (<5%).

3.3.2.6. Analysis of bulk drugs and tablets. The developed method was used with blank correction for identifying impurity peaks from gradient humps in bulk drugs and tablets A, B and C (Fig. 4). The results obtained are recorded in Table 8. There were no interfering components from the tablet excipients indicating that the method can be used for the assay and related substances of TLM tablets.

4. Conclusions

A gradient LC method has been developed and validated for determining the related substances of TLM in bulk drugs and pharmaceuticals. The robustness of the method was studied using design of experiments. The developed method was found to be accurate, precise, specific and linear. Thus, the method can be used for quality assurance of TLM in bulk drugs as well as pharmaceutical formulations.

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