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HPTLC- Densitometric method for assay of chlorthalidone, metoprolol succinate and telmisartan in combined pharmaceutical formulation

Avani Chokshi [1*], Rohit Prajapati [1], Pritesh Desai [2] and Niraj Vyas [1]

[1]*Department of Pharmaceutical Chemistry, Ramanbhai Patel College of Pharmacy (RPCP), Charotar University of Science and Technology (CHARUSAT), CHARUSAT Campus, Changa - 388421, India*

[2]*Department of Analytical Research, PTC-Moraiya, Zydus Cadila Healthcare Limited, NH 8A, Moraiya-382213, Gujarat, India*

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Abstract: The present study shows the compilation of the results obtained for a very simple, fast and precise high-performance thin-layer chromatography (HPTLC) - densitometric determination of chlorthalidone (CHL), metoprolol succinate (MET) and telmisartan (TEL) in bulk drugs as well as the commercially available formulation. The chromatographic separation of samples was performed on Silica Gel 60 F254 aluminum sheets using Toluene: Methanol: Ethyl acetate: Tri-ethylamine as the mobile phase in the volume ratio 4:0.8:1: 1.2. The densitometric scanning was performed at 225 nm wavelength using CAMAG TLC Scanner- IV. The mentioned chromatographic system showed the compact band and symmetrical peaks of CHL, MET and TEL with 0.40 (±0.2), 0.69 (±0.2) and 0.27 (±0.2) retardation factor (Rf) respectively. The reported method is linear in the concentration range of 500-2000 ng/band, 1000-4000 ng/band and 1600-6400 ng/band while the recovery was found in the range of 98.94-99.62%, 98.26-98.41% and 99.86-100.28% for CHL, MET and TEL respectively. The method assayed the marketed formulation with 99.89 (±0.91) for CHL, 98.92 (±1.07) for MET and 100.12 (±0.65) for TEL concerning the label claim. All the results suggested the agreement of the developed method to the ICH Q2(R1) guidelines and its applicability for day-to-day analysis of these drugs in combined pharmaceutical formulations.

Keywords: Analytical method; chlorthalidone; densitometry; HPTLC; metoprolol succinate; telmisartan.

1. Introduction

One of the leading risk factors for cardiovascular diseases and death worldwide is hypertension and its prevalence is rising globally, regularly, due to many factors [1,2]. These factors range from the age of the population to lifestyle selection including unhealthy dietary habits, smoking and alcohol consumption and lack of physical activities [3]. Timely access to healthcare and medications, pollution, psychological

* Corresponding author E-Mail: avanichokshi.ph@charusat.ac.in

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stress and sleep disorders are also influencing factors, especially in the low and middle-income countries [3]. In fact, the etiological studies worldwide suggest a modest reduction in the prevalence of hypertension within high-income countries while a significant rise in low and middle-income countries during the past couple of decades [3]. During randomized clinical studies, lifestyle changes and antihypertensive medications are reported to be effective in lowering blood pressure and cardiovascular risk [4-7]. When lifestyle and dietary changes are not sufficient to lower blood pressure, medication interventions become necessary. The drug treatment options may include either a single drug or a combination therapy. The advantages associated with combination therapy are low dose and lowering of blood pressure by diverse mechanisms [8]. By combining two or more drugs, antihypertensive action has been reported to be increased by two to five times [8,9]. Also, while with monotherapy, the coronary risk is reduced by 29% and cerebrovascular risk is reduced by 40%, the same with a combination drug therapy is lowered by 40% and 54% respectively [10]. One such combination that has been approved for the hypertension treatment includes CHL, MET and TEL in a fixed dose and it is generally prescribed for patients with uncontrolled high blood pressure even with the dual drug combination. CHL is a thiazide-like diuretic that lowers blood volume and hence blood pressure by removing excess water and a few of the electrolytes in the form of urine [11]. MET slows down heart rate by blocking cardiac beta-adrenergic receptors [12]. TEL reverses the vaso-constricting and aldosterone-secreting effects of angiotensin-II by blocking the AT1 receptors present in vascular smooth muscles and the adrenal gland [13].

Analytical methods are used for qualitative, quantitative or structural evaluation of any sample or mixture thereof. Instrumental analytical methods provide signals which are in proportion to the sample concentration and from the intensity of the signals, the samples are estimated. Chromatographic methods separate the sample or sample mixture based on the physicochemical properties between the two phases and the separated components are estimated. Spectroscopic methods involve the measurement of the intensity of absorbed or emitted electromagnetic radiation from the sample and based on that, the sample is quantified [14].

A number of publications are available presenting the spectroscopic and chromatographic analysis of CHL, MET and TEL in sample mixture as well as dosage forms either individually, in two-drug combinations or in combination with other antihypertensive agents [15-32]. Previously, we have reported the RP-HPLC determination of the same drug combination [33] but apart from it, not a single analytical method been reported for the estimation of CHL, MET and TEL in a combined formulation. Though the analysis of pharmaceuticals by the HPLC method is quick, precise and reproducible, it is very much expensive; requires a large amount of solvents and columns are very sensitive to the extreme pH of the mobile phase [34]. Due to the drawbacks associated with the liquid chromatographic analysis, we had aimed to develop an HPTLC method as well. A few of the benefits associated with this method are simplicity of the method; low cost; low solvent consumption and solvents do not need any prior treatments; no interference from the previous analysis as every time, fresh mobile phase and stationary phases are used and no interference of solvents during detection as solvents are first evaporated before analysis [35]. The developed plates were analyzed through densitometry and the method was validated as per International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) for specificity, linearity, sensitivity, precision, accuracy and robustness [36]. Also, the validated method was applied for the determination of CHL, MET and TEL in a combined marketed tablet formulation.

2. Experimental

2.1. Standard Drugs

CHL (99.4%w/w), MET (99.1%w/w) and TEL (99.4%w/w) were provided as the gratis samples by Sun Pharmaceutical Industries Ltd. and were of pharmaceutical-grade substances.

2.2. Sample (Formulation)

The Met XL 3D 50 tablets (CHL-12.5mg, MET-25 mg and TEL-40 mg) were purchased from the local medical store.

2.3. Solvents and Reagents

Solvents and reagents including methanol, ethyl acetate, toluene and triethylamine used for the overall studies were of analytical grade and purchased from Merck (India) Ltd.

2.4. Chromatographic Plates

The Silica Gel 60 F254 aluminum plates (20 cm x 20 cm, 0.2 mm thickness) were purchased from Merck and were activated prior to use by heating at 110ºC for 20 minutes.

2.5. Chromatographic System

The bands of the sample solutions were applied on the chromatographic plates with the Hamilton Syringe of 100 µL capacity using the CAMAG Linomat V semiautomatic applicator. The plates were developed in the CAMAG Twin Trough chamber (10×10 cm and 20×10 cm). The Deuterium lamp was used as a radiation source. The densitometric scanning of the developed plates was performed in the CAMAG TLC Scanner- IV at a speed up to 20 mm/s, in the spectrum range of 190-800 nm and with the slit dimension of 4.00 mm × 0.10 mm. The chromatograms were analyzed at 225 nm wavelength.

2.6. Standard Solution Preparation

The standard stock solution of CHL (0.125 mg/mL), MET (0.25 mg/mL) and TEL (0.40 mg/mL) were prepared using methanol as a solvent. The resulting solution was diluted appropriately to prepare the working standard solution containing 12.5 µg/mL CHL, 25 µg/mL MET and 40 µg/mL TEL. 10 µL of the solution was spotted on the previously activated plate (band width 6.0 mm and space between two bands 6.0 mm).

2.7. Sample Solution Preparation

20 Met XL 3D 50 tablets were finely crushed and the tablet powder equivalent to 12.5 mg CHL, 25 mg MET and 40 mg TEL was sonicated with methanol in a 100 mL volumetric flask for 20 minutes. The resulting solution was filtered through a 0.45µ syringe filter and 2.5 mL of the solution was diluted in a 25 mL volumetric flask with methanol to prepare the sample solution. 10 µL of the solution was spotted on the previously activated plate (band width 6.0 mm and space between two bands 6.0 mm).

2.8. Method Validation

The HPTLC-densitometric method was validated as per the guidelines provided by ICH Q2(R1) for its specificity, linearity, sensitivity, precision, accuracy and robustness.

2.8.1. Specificity

The specificity of the developed method was established by determining the peak purity of active components in standard preparation, test preparation and spiked sample preparation at 225 nm. The Rf value of the bands and the spectra for each of the analytes for standard and test solutions were compared.

2.8.2. Linearity

Linearity was determined by applying the standard solutions of CHL, MET and TEL at seven concentration levels over the range of 500 - 2000 ng/band (500, 750, 1000, 1250, 1500, 1750 and 2000 ng/band) for CHL, 1000 - 4000 ng/band (1000, 1500, 2000, 2500, 3000, 3500 and 4000 ng/band) for MET and 1600 - 6400 ng/band (1600, 2400, 3200, 4000, 4800, 5600 and 6400 ng/band) for TEL. The chromatographic plates were developed at the optimized chromatographic conditions and the peak area for the analytes at each concentration level was obtained at 225 nm wavelength. Each analysis was repeated 6 times to obtain an average calibration plot. The mean area at each level was calculated and graphs of average area versus concentration in ng/band were plotted.

2.8.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Detection and quantification limits were calculated by the calibration curve method from the Standard Deviation of the Responses and the Slope using the following equations designated by ICH guidelines [36]:

Limit of Detection (LOD) is expressed as $LOD = 3.3 \times \sigma / S$

Limit of Quantification (LOQ) is expressed as $LOQ = 10 \times \sigma / S$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

2.8.4. Precision

Three concentration levels of each of the analytes- 750, 1250 and 1750 ng/band of CHL, 1500, 2500 and 3500 ng/band of MET and 2400, 4000 and 5600 ng/band of TEL- were selected for evaluating the precision of the newly developed method. Each analysis was performed in triplicate. The results of the precision were expressed as % relative standard deviation (%RSD) of the peak area at each concentration level. The repeatability (Intra-day precision) was checked by analyzing each solution on the same day while intermediate precision (Inter-day precision) was checked by analyzing each of the solutions on three different days.

2.8.5. Accuracy

The accuracy of the method was expressed as % recovery of CHL, MET and TEL in the sample solution at three levels- 80%, 100% and 120% of selected concentration levels (750, 1500 and 2400 ng/band of CHL, MET and TEL respectively) in triplicate. To the pre-analyzed sample solution, a known amount of standard solution of CHL, MET and TEL was added and % recovery and %RSD were calculated.

2.8.6. Robustness

Robustness studies of the developed method were performed by carrying out the small but deliberate changes in a few of the optimized chromatographic conditions and were reported in terms of %RSD of mean peak area of CHL, MET and telmisartan. The small changes selected to confirm the robustness of the developed method included changes in detection wavelength (± 2nm); development distance (±5 mm); chamber saturation time (±5 minutes) and mobile phase composition. The studies were performed in triplicate by applying 750, 1500 and 2400 ng/band of CHL, MET and TEL respectively on the chromatographic plates.

2.8.7. Analysis of Pharmaceutical Formulation

The validated HPTLC-densitometric method was effectively applied for the quantitative determination of CHL, MET and TEL in the Met XL 3D 50 tablets at a concentration of 12.5 µg/mL, 25 µg/mL and 40 µg/mL respectively. 10 µL bands were placed on the plate and the chromatograms were developed in the twin through chamber in the optimized solvent system- toluene: methanol: ethyl acetate: triethylamine at a volume ratio 4.0: 0.8: 1.0: 1.2. Once developed, the plates were dried properly and scanned at 225 nm wavelength. The amount of the individual analytes was determined corresponding to the measured peak area and percentage purity was calculated.

3. Results and discussion

3.1. Optimized Chromatographic Conditions

The main target for the development of the analytical method is to separate all the analytes present in the bulk mixture as well as a formulation with good resolution. Several solvent mixtures were tried for the development of a suitable solvent system that can separate CHL, MET, and TEL on pre-coated Silica Gel 60 F254 aluminum plates. After the number of trials, toluene: methanol: ethyl acetate: triethylamine at a volume ratio of 4.0: 0.8: 1.0: 1.2 was considered the optimum as it produced a compact band with a symmetrical peak and acceptable Rf value at 0.40 (±0.2), 0.69 (±0.2) and 0.27 (±0.2). Methanol was selected as a solvent and diluent for the preparation of standard and test solutions. 10 µL solutions were applied in the form of a 6.0 mm band, by keeping a 6.0 mm distance between each band. The twin trough chamber was saturated with mobile phase for 20 minutes before placing the chromatographic plate for development. The solvent front was allowed to migrate at a distance of 80 mm on a plate and after that, the plates were removed and dried. As all three analytes showed maximum absorbance at 225 nm, it was selected as the detection wavelength for their simultaneous determination.

3.2. Method Validation

The optimized chromatographic method was validated in accordance with the ICH Q2(R1) guidelines provided for the validation of analytical procedures. The parameters selected for performing validation include specificity, linearity, sensitivity, precision, accuracy and robustness [36]. The validated method was applied for the assay of marketed tablet formulation containing CHL, MET, and TEL in combination.

3.2.1. Specificity

At the optimized chromatographic conditions, well-developed bends of analytes were observed with enough resolution between the individual peaks which shows the suitability of the method for simultaneous analysis of CHL, MET, and TEL. The specificity of the developed method was confirmed by comparing the Rf value and spectra of standard and sample solutions. A good agreement in the Rf values of the standard and sample of CHL, MET, and TEL was observed without the interference of excipients (No extra peaks were observed). This indicates that the developed method is specific for the determination of CHL, MET, and TEL in the provided formulation.

3.2.2. Linearity

The linearity of the developed method was evaluated at seven concentration levels over the range of 500 - 2000 ng/band for CHL, 1000 - 4000 ng/band for MET and 1600 - 6400 ng/band for TEL. The calibration plots were constructed from peak area and concentration range and linear regression analysis was performed. A good linear relation (correlation coefficient >0.9) was observed throughout the selected

range the data of which are shown in table 1. Also, an overlay spectrum of all selected concentration levels for the three drugs is shown in figure 1.

Table 1. Summary of validation parameters of HPTLC-densitometric analysis of CHL, MET and TEL

Validation Parameter	CHL	MET	TEL
Purity index	0.999933	0.999953	0.999910
Linearity (ng/spot)	500-2000	1000-4000	1600-6400
R^2	0.9992	0.9989	0.9997
Regression equation	y = 4.6564x + 2162	y=1.6323x + 1382.8	y=1.2869x + 1615.5
LOD (ng/spot)	42.53	280.52	308.05
LOQ (ng/spot)	128.89	850.07	933.49

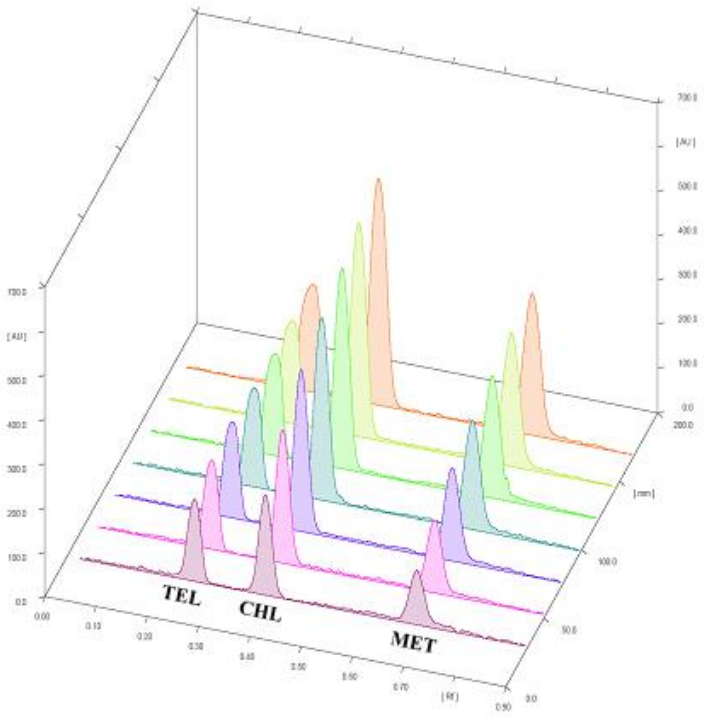

Figure 1. Overlay spectra of CHL, MET and TEL

3.2.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The detection and quantification limit of the analytical method indicates the sensitivity of the one. For the present work, they were calculated from the formulas provided by ICH guidelines, using the slope of the calibration plot for the selected range of CHL, MET and TEL and the standard deviation of the peak areas. The LOD and LOQ values for all analytes are included in table 1. The low detection and quantification limits of the present HPTLC method confirm that the method is sensitive enough to identify and estimate the analytes in its pharmaceutical formulations.

3.2.4. Precision

The precision of the developed method is expressed in terms of repeatability (Intra-day precision) and intermediate precision (Inter-day precision). Table 2 shows the results for the precision studies of the newly developed analytical method which are denoted in terms of %RSD of peak area at the selected three concentration levels. At each level, for all analytes, the %RSD values are less than 2 which implicates no significant deviation in the analysis of CHL, MET and TEL at selected concentration levels and hence the method is precise when applied for the estimation of analytes in bulk mixture or formulation.

Table 2. Precision of HPTLC-densitometric analysis of CHL, MET and TEL (n=3)

Drug	Concentration (ng/band)	Repeatability		Intermediate Precision	
		Mean Peak area ± SD	%RSD	Mean Peak area ± SD	%RSD
CHL	750	5621.15±72.11	1.28	5626.97±100.95	1.79
	1250	7903.67±83.47	1.05	7905.02±92.95	1.17
	1750	10340.54±109.86	1.06	10339.81±130.28	1.26
MET	1500	3788.16±40.45	1.06	3797.44±52.06	1.37
	2500	5402.08±54.60	1.01	5399.80±71.51	1.32
	3500	7049.13±53.46	0.75	7053.38±93.95	1.33
TEL	2400	4713.20±54.15	1.14	4710.42±81.54	1.73
	4000	6760.47±82.72	1.22	6761.58±95.22	1.40
	5600	8868.53±84.15	0.94	8870.75±110.37	1.24

3.2.5. Accuracy

The accuracy studies were carried out by standard addition method where the known concentration of a standard solution of CHL, MET and TEL was added to the selected test concentration and % recovery was calculated. Table 3 reports the % recovery of analytes at all concentration levels and as they fall into the prescribed limit of 98-103%, the method is said to be accurate and can measure the concentration of drugs without the interference of excipients in a formulation containing a mixture of CHL, MET and TEL.

Table 3. Accuracy of HPTLC-densitometric analysis of CHL, MET and TEL (n=3)

Recovery Level	% Recovery (Mean ± RSD%; n = 3)		
	CHL	MET	TEL
80 %	99.11 ± 0.74	98.26 ± 0.39	99.86 ± 0.93
100 %	98.94 ± 0.52	98.41 ± 0.35	100.28 ± 0.64
120 %	99.62 ± 0.57	98.32 ± 0.39	100.01 ± 0.16

3.2.6. Robustness

In a number of the optimized chromatographic conditions, small deliberate changes were made and the effect of those changes in the peak area of analytes at selected concentration levels was calculated. The results or robustness studies are shown in table 4 in terms of %RSD of analyte peak area, the value of which is less than 2, indicating the robustness of the method as it is not affected by small changes in the chromatographic conditions.

Table 4. Robustness of HPTLC-densitometric analysis of CHL, MET and TEL (n=3)

Chromatographic Condition		CHL		MET		TEL	
		Mean Peak Area ± SD	%RSD	Mean Peak Area ± SD	%RSD	Mean Peak Area ± SD	%RSD
Wavelength (225 nm)	223 nm	8063.53 ± 83.19	1.03	5421.20 ± 52.08	0.96	6700.72 ± 76.40	1.14
	227 nm	7861.25 ± 64.46	0.82	5355.16 ± 51.18	0.95	6732.45 ± 86.88	1.29
Development distance (80 mm)	75 mm	7869.96 ± 81.80	1.03	5365.16 ± 60.41	1.12	6699.12 ± 61.41	0.91
	85 mm	7896.63 ± 110.20	1.39	5431.83 ± 50.50	0.92	6792.45 ± 71.94	1.05
Chamber saturation Time (20 min)	15 min	7870.19 ± 88.38	1.12	5387.87 ± 44.36	0.82	6700.72 ± 90.28	1.34
	25 min	7916.86 ± 68.02	0.85	5404.54 ± 70.52	1.30	6800.72 ± 85.99	1.26
Mobile phase (Toluene: Methanol: Ethyl acetate: Tri-ethylamine 4.0:0.8:1.0:1.2 v/v/v/v/)	**3.8**:0.8:1.0:1.2	7929.96 ± 60.32	0.76	5415.16 ± 71.81	1.32	6759.12 ± 43.98	0.65
	4.2:0.8:1.0:1.2	7883.29 ± 69.48	0.88	5395.16 ± 73.45	1.36	6725.79 ± 73.21	1.08
	4.0:**0.7**:1.0:1.2	7866.63 ± 70.81	0.90	5355.16 ± 50.65	0.94	6692.45 ± 79.87	1.19
	4.0:**0.9**:1.0:1.2	7913.29 ± 74.27	0.93	5418.5 ± 37.36	0.68	6792.45 ± 30.27	0.44
	4.0:0.8:**0.8**:1.2	7898.63 ± 78.30	0.99	5395.16 ± 70.34	1.30	6745.79 ± 77.13	1.14
	4.0:0.8:**1.2**:1.2	7905.29 ± 94.14	1.19	5401.83 ± 42.11	0.77	6759.12 ± 93.77	1.38
	4.0:0.8:1.2:**1.0**	7885.29 ± 67.87	0.86	5391.83 ± 50.38	0.93	6752.45 ± 75.39	1.11
	4.0:0.8:1.2:**1.4**	7908.63 ± 43.69	0.55	5405.16 ± 55.81	1.03	6769.12 ± 62.07	0.91

3.2.7. Analysis of Pharmaceutical Formulation

The validated HPTLC-densitometric method was applied for the assay of the commercially available formulation Met XL 3D 50 tablets containing 12.5mg CHL, 25 mg MET and 40 mg TEL per tablet. The average amount of each of the active constituents in a tablet was determined based on the peak areas. The results of the assay reported in table 5 show % purity of CHL, MET and TEL in the range of 98-103% of the label claim which is in accordance with the ICH guidelines as well as the pharmacopoeial requirements in terms of average drug content in any pharmaceutical formulation.

Table 5. Assay of Met XL 3D 50 tablets by validated HPTLC-densitometric method (n=6)

Parameter	CHL	MET	TEL
Label claim (mg)	12.5	25	40
Mean peak area	7976.36	5419.48	6769.78
Average amount (mg)	12.49	24.73	40.04
%Recovery ± SD	99.89 ± 0.91	98.92 ± 1.07	100.12 ± 0.65
%RSD	0.91	1.07	0.65

3.2.7. Uncertainty Assessment

Errors arising from the different experimental conditions like instrumental calibration and operation, purity of chemicals and solvents, sampling procedures, standard and sample solution preparations, and environmental conditions including temperature, humidity, light etc. can be justified for the analytical methods by performing the uncertainty assessment along with the suggested validation parameters. The uncertainty assessment for the present method was based on the guiding documents provided by the EURACHEM/CITAC guide and the corresponding literatures [36-40]. We report here the combined uncertainty ($u_{combined}$) and expanded uncertainty ($U_{Expanded}$), calculated from the uncertainty in standard preparation ($u_{standard}$), uncertainty associated with the slope of calibration curve ($u_{calibration}$), uncertainty of recovery ($u_{recovery}$) and uncertainty of repeatability ($u_{repeatability}$) from equation 1.

$$u_{Combined} = \sqrt{(u_{standard})^2 + (u_{calibration})^2 + (u_{recovery})^2 + (u_{repeatability})^2} \quad (1)$$

The major contributing factor in the uncertainty associated with the standard preparation is the purity of the analyte. $U_{Standard}$ of each analytes was calculated from the %purity provided by the supplier using equation 2.

$$u_{standard} = \frac{100 - \%Purity}{\sqrt{3}} \quad (2)$$

$U_{Calibration}$ was calculated for each of the analytes from the standard error of slope and slope value for the calibration curve using equation 3.

$$u_{calibration} = \frac{(\text{Standard Error of Slope} * 100)}{\text{Slope}} (3)$$

The mean relative standard deviation (RSD) associated with the recovery studies was considered as $U_{Recovery}$ while that of repeatability studies was considered as $U_{Repeatability}$ for each of the analytes. Expanded uncertainty at a 95% confidence interval is calculated by multiplying combined uncertainty with the coverage factor (k = 2) The uncertainty profile for the present method is given in table 6.

Table 6. Uncertainty Assessment of HPTLC-densitometric method

Uncertainty (U)	CHL	MET	TEL
$u_{Standard}$	0.35	0.52	0.35
$u_{Calibration}$	0.06	0.55	0.47
$u_{Recovery}$	0.61	0.38	0.58
$u_{Repeatability}$	1.13	0.94	1.10
$u_{Combined}$	1.33	1.27	1.37
$U_{Expanded}$	2.66	2.54	2.74

$U_{Expanded:}$ k=2 95 % confidence level; U % values reported.

4. Conclusions

A very novel, simple, and economical HPTLC method with densitometric detection was developed for the estimation of chlorthalidone, metoprolol succinate and telmisartan in the bulk mixture as well as the commercially available tablet formulations containing these drugs in a combination. The method allows the direct determination of drugs of interest in UV light without the need for derivatization or any special reagent treatment. The developed method was validated for specificity, linearity, sensitivity, accuracy, precision and robustness as per the guidelines provided by ICH Q2(R1). All the statistical data satisfies the passing criteria according to the guidelines. Moreover, the validated method was applied for the determination of drug content in the tablet formulation and the results of the assay satisfy the %label claims as per the pharmacopoeial recommendations. The obtained results positively conclude the

applicability of the newly developed and validated method for routine simultaneous analysis of the formulations containing a combination of chlorthalidone, metoprolol succinate and telmisartan.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

ORCID

Avani Chokshi: 0000-0002-6229-4804
Rohit Prajapati: 0000-0002-5636-9128
Pritesh Desai: 0000-0001-7105-598X
Niraj Vyas: 0000-0001-9187-8510

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