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Validated Estimation of Temozolamide In Pure, Biological Sample By UV-Spectroscopic, RP-HPLC And HPTLC Methods

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ABSTRACT

Quantitative estimation of temozolamide and its pharmaceutical dosage form by UV spectroscopy, RP-HPLC, HPTLC methods was developed. In the UV method (geometric method), temozolamide was quantified at 309nm, 325nm, 340nm in serum and water. The corrected absorbance was calculated. The Recovery studies was found to be 95.5-96.9%. In RP-HPLC method, the drug was resolved using a mobile phase methanol: buffer (5.0ml glacial acetic acid in 1000ml water) (70:30%v/v) on C_{18} column in isocratic mode. The retention time of temozolamide was found to be 7.30 min. Recovery studies was found to be 99.55-100.98%. In HPTLC method, the chromatograms were developed by using a mobile phase Chloroform: glacial acetic acid: methanol (2:3:5% v/v) on precoated plate of silica gel $60F_{254}$ and quantified by densiometric absorbance mode at 254nm. The R_f value of Temozolamide was 0.47. Recovery studies of 98.99-100.6%, percentage relative standard deviation (%RSD less than 2%) and correlation coefficient (linearity range) that developed methods were accurate and precise. These methods can be employed for the routine analysis of capsules containing temozolamide.

Key words: Temozolamide, RP-HPLC, HPTLC, UV spectrophotometry, validation.

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INTRODUCTION

Temozolamide is chemically known as 3, 4-dihydro-3-methyl-4-oxoimidazo[5,1-d]-1,2,3,5 tetrazine-8-carboxamide[10].Temozolomide is an oral alkylating agent used for the treatment of Grade IV astrocytoma — an aggressive brain tumor, also known as glioblastoma multiforme — as well as for treating melanoma, a form of skin cancer. Temozolomide is also indicated for relapsed Grade III anaplastic astrocytoma and not indicated for, but as of 2011 used to treat oligodendroglioma brain tumors in some countries, replacing the older (and less well tolerated) PCV (Procarbazine-Lomustine-Vincristine) regimen. The agent was developed by Malcolm Stevens[2] and his team at Aston University in Birmingham,[3,4] A derivative of imidazotetrazine, temozolomide is the prodrug of MTIC (3-methyl-(triazen-1-yl)imidazole-4-carboxamide). It has been available in the US since August 1999, and in other countries since the early 2000s. Determination of Temozolamide in rat and dog plasma by LC/MS was reported[8]. No methods have been reported for determination of Temozolamide in capsule dosage form by UV, HPLC& HPTLC methods.

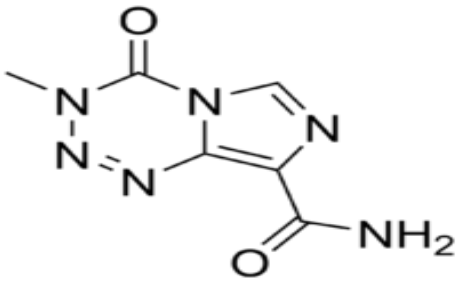


Figure1: Chemical structure of Temozolamide.

MATERIALS AND METHODS

Chemicals and Reagents

The bulk drug and capsule formulation of Temozolamide was gifted from Natco Pharma Ltd., Hyderabad. All solvents and reagents used were of HPLC (or) Analytical grade respectively. HPLC grade methanol and chloroform used were of HPLC grade (India). Fresh serum used in the method development was obtained from Singareni Collories Hospital, Kothagudem, Khammam District-507101.

Instrumentation

UV Spectral measurements were recorded in Scimadzu, UV- 1700, Pharmaspec , cuvetts with 1cm quartz cells were used. RP-HPLC was performed using water HPLC system equipped with double reciprocating pump, a 717 auto sampler and UV detector. The column was Kromasil 100 C_{18} (5µ, 4.6X150mm) used. The method was conducted using an isocratic technique by

Empower software. HPTLC was performed in Cagmag HPTLC (Cagmag, Muttenz, and Switzerland) system, equipped with linomat V sample applicator twin trough plate development chamber, TLC scanner II with WinCat soft ware.

UV-method

In this method, three wavelengths were (309, 325 and 340) selected for Geometric correction method. The stock solution was prepared by dissolving 10mg of TMZ in 10ml of serum. The stock solution was further diluted with water to obtained final concentration range (3-15 µg/ml). The absorbance of different concentration solutions were measured at their selected wavelengths

Quantification with serum

Weighed twenty capsules of Temodal-100 mg and found average weight, the capsule powder equivalent to 10 mg of Temodal were weighed and transferred into a 10 mL volumetric flask and made up to volume with serum. The contents were ultra sonicated for 15 min. From the above solution containing 1 mg/ mL, 2.5 mL of solution was transferred into 25 mL standard flask, and made up to the volume with distilled water and filtered through whatmann filter paper no.41

The solution was further diluted with distilled water, to give concentration of 10 µg/ mL. Absorbances of these solutions were measured six times at their selected wavelengths (309 nm, 325 nm, 340 nm) using blank. Blank was prepared same as sample preparation excluding the drug.

Quantification without serum

The above same procedure was repeated without serum sample. The amount of present in formulation was calculated by using corrected absorbance from the slope and intercept of respective calibration curve.

Recovery studies [with Serum]

The recovery experiment was done by adding known concentrations (80%, 100% and 120%) of Temozolamide working standard to the pre-analyzed formulations. Standard stock solutions were prepared in serum.

Recovery studies [without Serum]

Similarly by using distilled water.

RP- HPLC

The buffer was prepared by dissolving 5.0 ml of glacial acetic acid in 1000ml distilled water. The drug was resolved using a mobile phase of buffer: methanol (30:70%v/v), filtered using whattman filter paper and degassed. The flow rate was 1.0ml/min and the effluent was monitored at 254nm. A stock solution was prepared by dissolving 10mg of Temozolamide in 10ml of

mobile phase. The stock solution was further diluted with the mobile phase to obtain final concentration range (20-60 µg/ml). These solutions were used to calculate the linearity and the relative quantification of the capsules. About 20 capsules were weighed and powder equivalent to 40mg was weighed accurately and transferred to 100ml volumetric flask. The capsule powder was dissolved in the mobile phase and filtered through a whattman filter paper. The sample was suitably diluted and used for the analysis. 10µl of standard and sample solutions were injected, under the specified conditions and the chromatogram was recorded. The amount of temozolamide present in capsule was calculated by using the linear regression equation.

Recovery Experiments

a) Preparation of raw material stock solution

An accurately weighed quantity of 40 mg of was transferred into a 100 mL volumetric flask and added sufficient mobile phase (HPLC grade) to dissolve the substance and made up to the mark with the same. This contains 400 µg/ mL concentration. Further 1 mL is diluted to 10 mL to get 40 µg/ mL concentration.

b) Procedure

To each 1 mL of pre-analysed sample solution (40 µg/ mL of) added 0.5 mL, 1 mL and 1.5 mL of working standard stock solutions into 10 mL volumetric flasks and made up to the mark with mobile phase and performed the recovery as described under assay. The quantity of drug recovered was calculated by using slope and intercept values from the calibration graph.

HPTLC Method

The drug was resolved using a mobile phase chloroform: glacial acetic acid: methanol (2:3:.5%v/v) Chamber and plate saturation time was 30min, migration distance was 90mm and UV detection was carried out 254nm. A stock solution was prepared by dissolving 10mg of Temozolamide in 10ml of mobile phase (1000µg/ml). The stock solution was further diluted with mobile phase to obtain final concentration range (100-600 ng/µl)(figures 6-12). The standard and sample solutions were applied on the TLC plate(figure13,14). The chromatograms were developed. The developed chromatograms were evaluated by scanning in densiometric mode at 254nm. The calibration curve was constructed by using peak area against concentration. The procedure was repeated for six times. The sample solution was prepared as that of HPLC method and filtered through whatmann filter paper. Finally the sample was diluted to get a concentration (150ng/µl).The 2µl of sample and standard was applied on the TLC plate. The chromatogram was developed. The developed chromatograms were evaluated

by scanning in densiometric mode at 254nm. The content of Temozolamide in capsule was calculated by using the linear regression equation.

Recovery

To the pre-analyzed formulation a known quantity of the standard drug solutions (80, 100, 120%) were added and the amount of drug recovered was calculated. The % RSD values were calculated.

LOD AND LOQ

The limit of detection (LOD) is defined as the lowest concentration of an analyte that an analytical process can reliably differentiate from back ground levels. The limit of quantification (LOQ) is defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability[5.]

The LOD& LOQ were calculated as LOD= 3.3σ/S and LOQ = 10σ/S Where σ is the standard deviation of the lowest standard concentration and S is the slope of standard curve.

Recovery Studies

Recovery studies were carried out by adding known quantities of standard at different levels to the pre analyzed sample to study the linearity, accuracy and precision of the proposed method. The recovery[6] studies also reveals whether there is positive or negative influence on the quantification parameters by the additive usually present on dosage forms. The recovery study data are present in table.

RESULT AND DISCUSSION

UV method

Geometric correction method

A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in samples of biological origin. The simplest of these procedures is the three-point geometric procedure, which may be applied if the irrelevant absorption is linear at the three wavelengths selected. In this method, three wavelengths 309, 325, and 340 nm were selected for the estimation of in Serum and without serum. Based on the absorbance the corrected absorbance was calculated by using the following equation

$$\text{Corrected absorbance, } D = \frac{y(A2 - A3) + z(A2 - A1)}{y(1 - w) + z(1 - v)}$$

$y = (\lambda_2 - \lambda_1)$

$z = (\lambda_3 - \lambda_2)$

$A_{1=}$ Absorbance of the sample solution at λ_1

$A_{2=}$ Absorbance of the sample solution at λ_2

$A_{3=}$ Absorbance of the sample solution at λ_3

v = vD/D [absorbance ratio of drug in methanol (without serum) at λ_1 and λ_2]

w = wD/D [absorbance ratio of drug in methanol (without serum) at λ_3 and λ_2]

The linearity of the drug was checked at 3-5µg/mL(figures 2). The percentage label claim present in capsules was found to be 96.93 ± 1.466, respectively. The percentage recovery was found to be in the range of 95.5 - 96.9%.

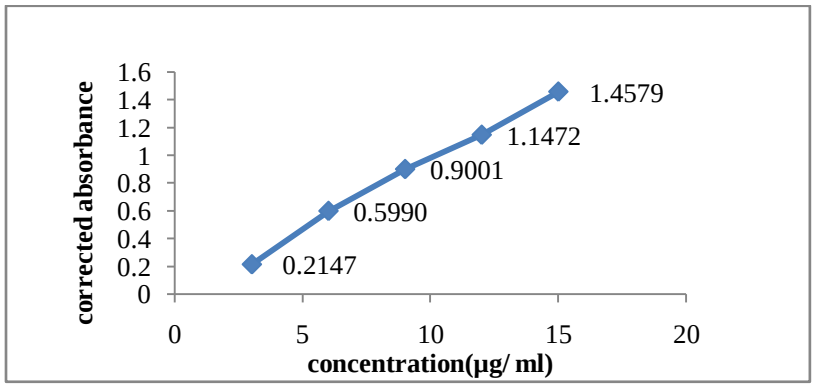


Figure 2: Calibration curve of temozolamide by geometric correction method

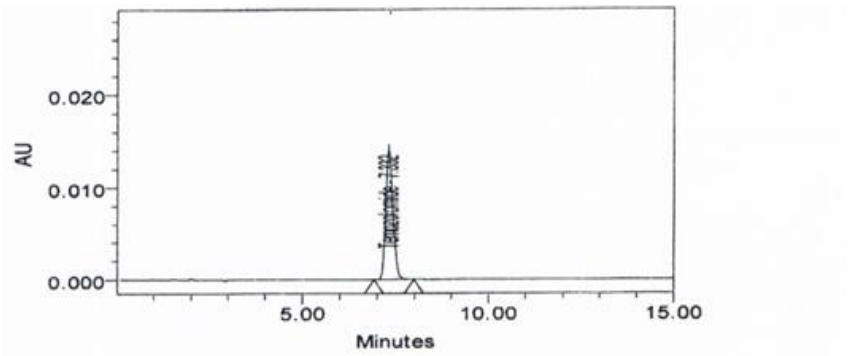
RP – HPLC Method

In RP-HPLC method, mobile phase used was methanol: buffer (5.0 mL in 1000 mL water) (70:30 V/V) with flow rate of 1.0 mL/min, the retention time of was found to be 7.31 minutes at 254 nm. The linearity of the drug was checked at 3-5µg/mL (figures 3-7). The standard and sample chromatograms were mentioned in figures 8 and 9. System suitability parameters for the optimized chromatogram by RP – HPLC method are shown in table:1

The percentage purity was found to be 99.86 ± 0.6786. The precision of the method was confirmed by repeatability of formulation for six times and further intra-day and inter-day studies were also done. The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range between 99.55 – 100.98 %. The low % RSD values for recovery indicated that the method was found to be accurate.

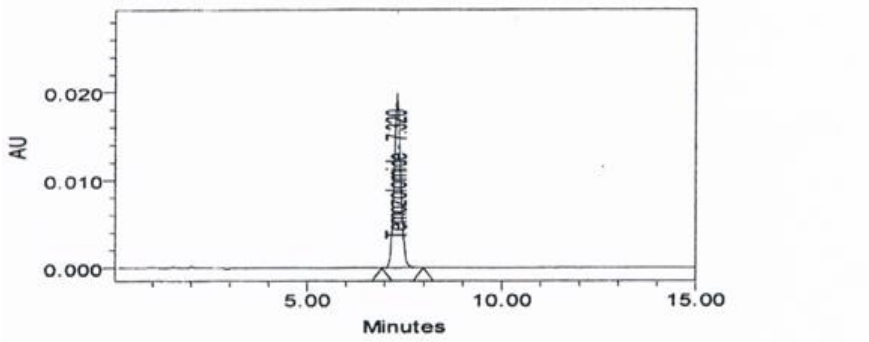
Table 1:System suitability parameters for optimized chromatogram by RP–HPLC method

Parameters	Temozolamide	Standard limit
Retention time	7.303	
USP Tailing	1.07	< 2
USP plate Count	9021	>2000
Theoretical plate per unit length	0.016627	



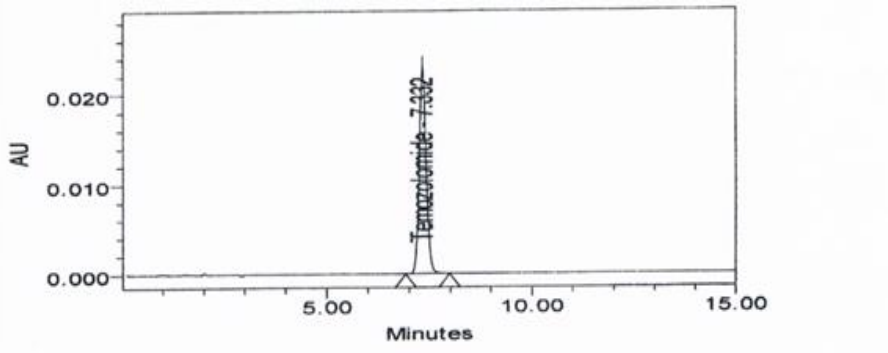
	Name	RT	Area	Height	% Area	USP Tailing	USP Plate count
1	Temozolamide	7.332	330183	26574	100.0	1.072	7646

Figure: 3 Linearity chromatogram of Temozolamide(20µg/mL)



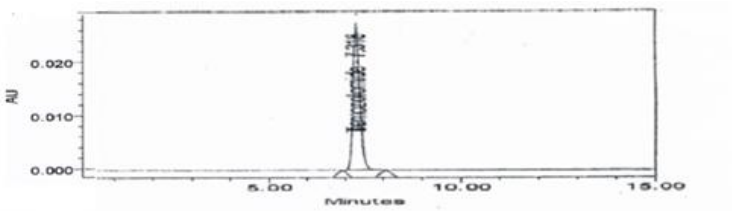
	Name	RT	Area	Height	% Area	USP Tailing	USP Plate count
1	Temozolamide	7.320	491238	31888	100.0	1.02	8881

Figure: 4 Linearity chromatogram of Temozolamide(30µg/mL)



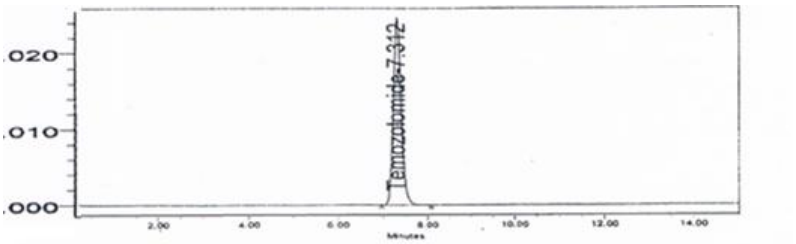
	Name	RT	Area	Height	% Area	USP Tailing	USP Plate count
1	Temozolamide	7.332	669808	59260	100.0	1.07	8880

Figure: 5 Linearity chromatogram of Temozolamide(40µg/mL)



	Name	RT	Area	Height	% Area	USP Tailing	USP Plate count
1	Temozolamide	7.318	834812	46574	100.0	1.12	7527

Figure: 6 Linearity chromatogram of Temozolamide(50µg/mL)



	Name	RT	Area	Height	% Area	USP Tailing	USP Plate count
1	Temozolamide	7.312	991237	46574	100.0	1.06	7527

Figure: 7 Linearity chromatogram of Temozolamide(60µg/mL)

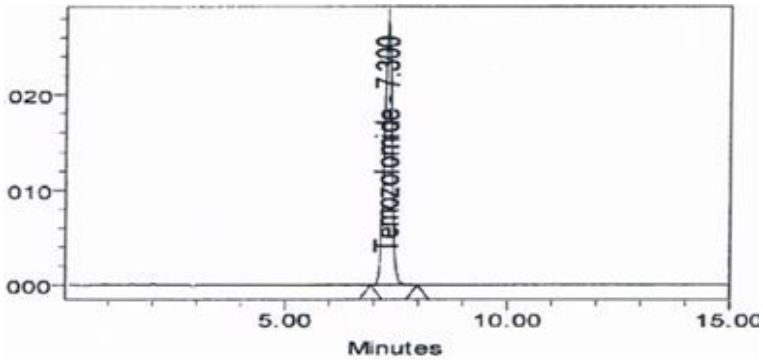
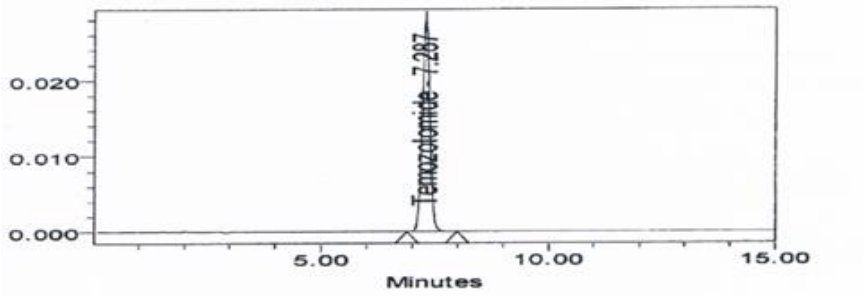


Figure 8: A Typical HPLC Standard Chromatogram of Temozolamide at 254nm

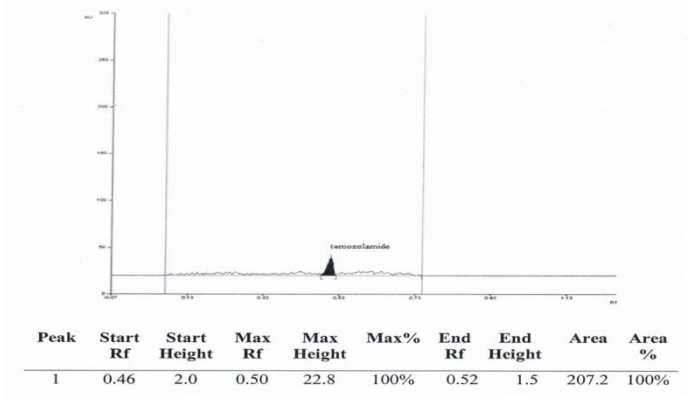


	Name	RT	Area	Height	% Area	USP Tailing	USP Plate count
1	Temozolamide	7.287	655311	35614	100.0	1.07	9041

Figure 9: A Typical HPLC Working Chromatogram of Temozolamide at 254nm

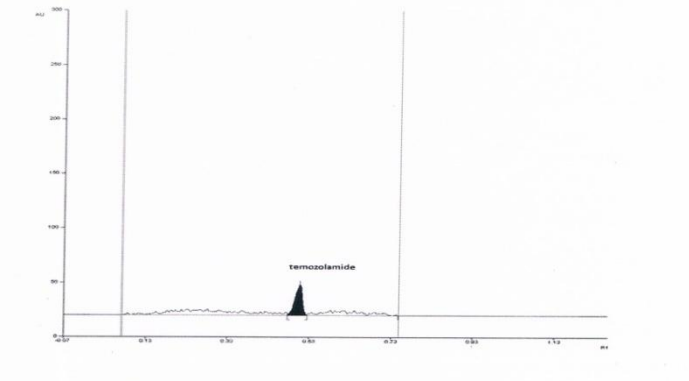
High Performance Thin Layer Liquid Chromatography

In HPTLC method, the mobile phase used was chloroform: glacial acetic acid: water (2:3:5% v/v) and the chromatogram was developed. The R_f value was found to be 0.48. The scanning of the developed plates shows a good peak shape. The linearity of the drug was checked at 100-600ng/mL(figures10-15). The standard and sample chromatograms are shown in figures 16 and 17.



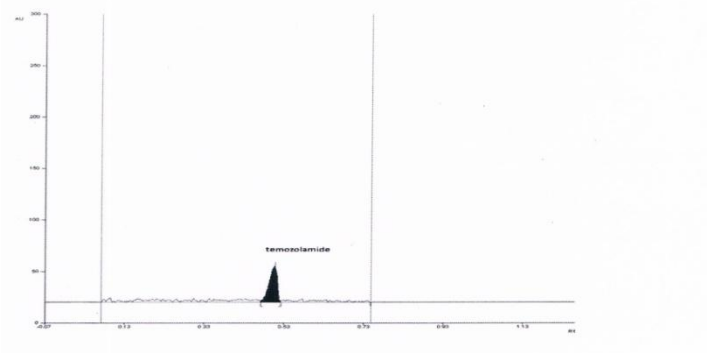
Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	2.0	0.50	22.8	100%	0.52	1.5	207.2	100%

Figure 10: Linearity chromatogram for temozolamide by HPTLC (100 ng/ µl**)**



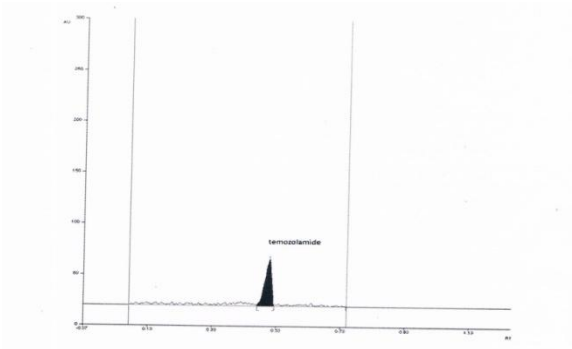
Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	1.6	0.51	29.2	100%	0.53	1.8	385.565	100%

Figure 11: Linearity chromatogram for Temozolamide by HPTLC(200 ng/ µl**)**



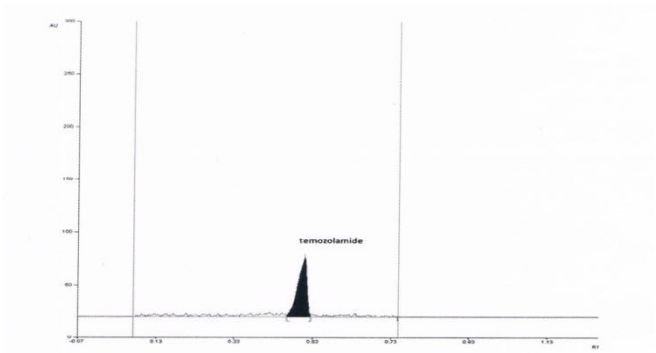
Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.2	0.50	37.7	100%	0.52	1.2	554.91	100%

Figure 12: Linearity chromatogram for Temozolamide by HPTLC(300 ng/ µl**)**



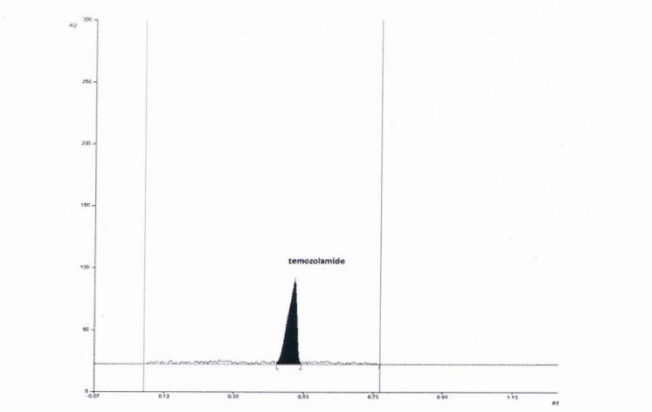
Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	1.6	0.51	46.2	100%	0.53	2.1	726.15	100%

Figure 13: Linearity chromatogram for Temozolamide by HPTLC(400 ng/ µl**)**



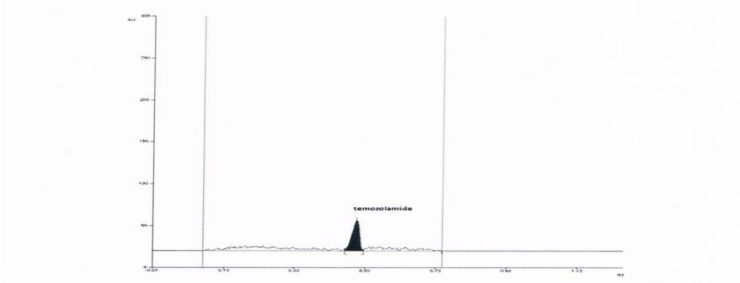
Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.8	0.49	56.8	100%	0.53	2.3	900.365	100%

Figure 14: Linearity chromatogram for Temozolamide by HPTLC(500 ng/ µl**)**



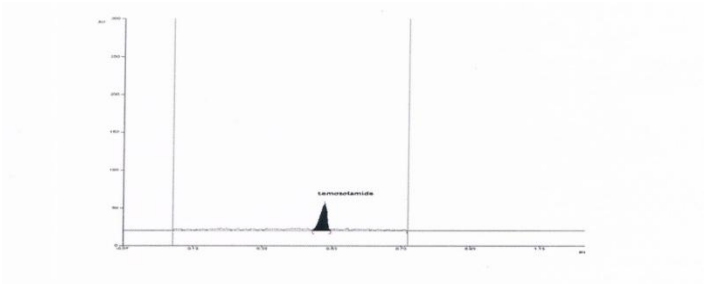
Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.7	0.49	67.1	100%	0.54	3.1	1073.667	100%

Figure 15: Linearity chromatogram for Temozolamide by HPTLC(600 ng/ µl**)**



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.47	1.9	0.51	38.3	100%	0.54	1.5	557.23	100%

Figure 16: **A Typical HPTLC Standard Chromatogram of Temozolamide at 254 nm**



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.47	0.8	0.51	36.9	100%	0.54	2.2	555.72	100%

Figure 17: **A Typical HPTLC working Chromatogram of Temozolamide at 254 nm**

Table 2: Summary of Validation Parameters for Temozolamide by the Proposed UV, RP-HPLC and HPTLC

Validation Parameters	UV	HPLC	HPTLC
Beer's law limit	3-15µg/ml	20-60µg/ml	100-600 ng/µl
Correlation coefficients r^2	r^2=0.99	r^2=0.999	r^2 =0.999
Regression equation	Y=0.098X+0.032	Y=16656X-2817.2	Y=1.7420X+29.98
Slope	0.098	16656	1.7420
Intercept	0.032	-2817.2	29.98
LOD	1.0088 µg/ml	7.895 µg/m	4.6077 µg/ml
LOQ	3.0570 µg/ml	2.392 µg/ml	1.3962 µg/ml
Precision			
Interaday (% RSD)	0.3744	0.1101	0.4379
Intraday (% RSD)	0.1183	0.4916	0.0290
Accuracy (% RSD	0.7320	0.7429	0.4512

The percentage purity was found to be 99.41 ± 0.4354. The precision of the method was confirmed by repeatability of formulation for six times and further intra-day and inter-day studies were also done. The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range between 98.99 – 100.6 %. The low % RSD values for recovery indicated that the method was found to be accurate. Summary of validation

parameters for temozolamide by the proposed UV, RP-HPLC and HPTLC are mentioned in table 2. Recovery analysis for temozolamide by the proposed UV, RP-HPLC and HPTLC are mentioned in table 3.

Table 3: Recovery analysis for Temozolamide by the Proposed UV, RP-HPLC and HPTLC

Methods	Amount present µg/ml	Amount added µg/ml	Amount found µg/ml	Amount recovered µg/ml	%Recovery	SD	%RSD
UV	4.9	7.9	11.72	4.531	95.5	0.70946	0.732
	4.9	9.9	13.56	4.584	96.0		
	4.9	11.9	15.64	4.672	96.9		
HPLC	39.62	19.87	59.71	39.84	100.1	0.744401	0.742915
	39.62	39.43	79.186	39.40	99.55		
	39.62	59.70	99.89	40.17	100.98		
HPTLC	29.9	24.2	54.1	29.9	100.0	0.813654	0.8147
	29.9	30.0	60.2	29.6	98.99		
	29.9	35.7	65.8	30.1	100.6		

*Mean of six observations

Simple, rapid and accurate UV Spectroscopic (Geometric correction method),an isocratic RP – HPLC and HPTLC methods showed excellent sensitivity, reproducibility, accuracy, and repeatability, which was evidenced by low percentage relative standard deviation. The result obtained in recovery studies indicates that there was no interference from the excipients used in the formulation. Hence it was suggested that the proposed UV spectroscopic, an isocratic RP-HPLC and HPTLC methods can be effectively applied for routine analysis of in bulk, biological sample and in capsule formulation and the results will be presented elsewhere.

CONCLUSION

The UV, HPLC and HPTLC methods developed for Temozolamide shows good precision and accuracy. The low %RSD values in the recovery studies for these method shows that there is no interference due to excipients used in the formulation. Hence it was concluded that the developed methods are simple, precise, accurate and rapid for the analysis of Temozolamide in pure and in capsule dosage form. Thus the developed methods can be adopted for the routine analysis of Temozolamide in pure, Biological sample and capsule dosage form.

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REFERENCES

1. ICH Harmonized Tripartite Guidelines, Text on Validation of Analytical Procedures: Text and Methodology. Q2B. Geneva: 1996: 8.
2. Malcolm Stevens - interview, Cancer Research UK impact & achievements page
3. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C (January 1997). "Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials". *Cancer Treat. Rev.* **23** (1): 35–61. doi:10.1016/S0305-7372(97)90019-0. PMID 9189180.
4. Stevens MF, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, Slack JA, *et al.* (November 1987). "Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine.". *Cancer Res.* **47** (22): 5846–52.PMID 3664486.
5. Argekar AP, Sawant JG. Determination of Cisapride in pharmaceutical dosage forms by reversed-phase liquid chromatography. J Pharm Biomed Anal 1999; 21:104.
6. Varma MVS, Kapoor N, Sarkar M, Panchagnula R. Simultaneous determination of digoxin and permeability markers in rat in situ intestinal perfusion samples by RP-HPLC. J Chromatogr B 2004; 813: 347.
7. Gupta SC, Kapoor VK Fundamentals of Mathematical Statistics. 9th ed., New Delhi: Sultan Chand and sons; 1994: 128.
8. Choudhury SK. An LC/MS/MS method for the quantitation of MTIC (5-(3-N-methyltriazene-1-yle imidazole-4-carboxamide), a bioconversion product of temozolamide, in rat and dog plasma. J Pharma Biomedical Analysis 1999: 659-668.
9. The United States of Pharmacopeia.23/NF, 18, United States of Pharmacopoeial Convention, inc., Rock Ville, MD., 1995: 1063, 1961, 1988, 1990.
10. The Merck Index. An Encyclopaedia of chemicals, Drugs & Biological 14th ed., White house station, NJ, USA: Published by Merck Research Laboratories; 2006: 9139.
11. Vasanth kumar Kunithala, Anusha Reddy Byri, Sandeep Karaganchi, Kiran Kumar. New, Simple, Precise, Rapid and Accurate Temozolomide RP-HPLC method has been developed and validated by using low cost materials to estimation of these Temozolomide in dosage forms, Int J Pharma Sci 2012; 4:1781.