Journal of Pharmaceutical Chemistry
http://www.vensel.org/index.php/jphchem
VENSEL

Comparative study of isolated Guggulsterones as marker compounds from Guggulu, *Commiphora mukul* with Ayurvedic Guggulu containing formulations by HPTLC, an in-house quality control method

[1]Selvakumar Duraipandi, [1,*]Vijaya Selvakumar

[1]*School of Pharmacy, Taylor's University Lakeside, Subang Jaya, 47500, Malaysia*

Abstract: The guggulu containing polyherbal formulations in Ayurveda have been used for treating various inflammatory conditions. A simple HPTLC method has been developed to qualitatively analyze the formulations that claimed to have contained guggulu using the Guggulsterones isolated from the guggulu raw material as a marker. The isolation of Guggulsterones from the resinous gum obtained from the plant was used as a marker to determine the Guggulsterone content in the formulations. The study showed that all the preparations taken for analysis that claimed to contain Guggulsterones was originally having the contents but in variable amounts depending on the amount of resin taken for the preparation. Due to the fact that Guggulsterones are very expensive marker compounds if procured separately as Guggulsterone E and Z forms, this method can be used for routine qualitative analysis of presence of Guggulsterones as an in-house quality control method.

Keywords: Guggulsterones; HPTLC; Guggulu(Gulgulu); ayurveda

1. Introduction

Ayurveda, an indigenous system of medicine practiced for more than 3000 years in India, Sri Lanka and some other countries. Guggulu, a resin obtained from *Commiphora mukul,* a flowering plant from burseraceae and it commonly found in all the parts of India. It is an important component of many Ayurvedic preparations, Gulgulu in particular[1]. The Ayurvedic proprietary medicines Gulgulutiktakam Kashayam Tablets, Gulgulutiktakam Ghritam, Triphala Gulgulu Churna DS are gulgulu containing preparations used to treat various inflammatory conditions[1]. These are polyherbal formulations claimed to containing variable concentrations of guggulu in the final products. The disadvantage of these polyherbal formulations is the lack of appropriate method to analyze the contents qualitatively and quantitatively due to either disproportionate availability or very expensive of marker compounds. In the present study, Guggulsterones are isolated from resin obtained from *Commiphora mukul* and purified in the laboratory. Then the isolated Guggulsterone is qualitatively compared with commercial Ayurvedic formulations (Gulgulutiktakam Kashayam, Gulgulutiktakam Ghritam, Triphala Gulgulu Churna) by High Performance Thin Layer Chromatography (HPTLC).

2. Result and Discussion

Gulgulutiktakam Kashayam tablets contains 850mg of dried polyherbal extract per tablet(1g), Gulgulutiktakam Ghritam contains 1.5 g in each 10 mL; Triphala gulgulu tablet contains 425 mg of polyherbal extract per gram of the tablet. The study was related t o a method using laboratory produced marker of guggulsterones for the identification of the guggulsterones present the above preparations. Standard Guggulsterones were obtained from the raw guggulu by ethyl acetate extraction followed by alkalization and then extracted with petroleum ether. The guggulsterones obtained were a mixture of Guggulsterone E and Z rather than individual components.

The developed HPTLC plates visualized under UV cabinet at 254 nm and 366 nm and their corresponding fingerprint displays are shown in **Figure 1**. The spectral display showing the absorption of Rf 0.51 and 0.76 that is thought to be corresponding to guggulsterones (Since these spots fluoresce strongly under UV light) were shown in **Figure 2**. The **Figure 3** and **Figure 4** were showing peak display (densitometeric) of developed spots of test samples under UV light at 254 nm and 366 nm respectively. The calculated R_f values and AU (Area Under the peak), a measure of peak intensity of spots were given in the **Table 1** and **Table 2** for the observations under UV light at 254 nm and 366 nm respectively.

The UV scan and the AU obtained for Rf values of 0.51 & 0.76 for both standard and test sample, which

Submitted on: Mar 08, 2014
Revised on: Apr 04, 2014
Accepted on: Apr 07, 2014

*corresponding author: VS Tel: +60102595347 ; E-mail: vijirx2008@gmail.com

corresponds to the fluorescent spots (Guggulsterones fluoresces brightly under 366nm in very small concentrations). The comparison of the concentration (AU) of all the samples resulted from the densitometric scanning have also showing the corresponding increase/decrease in the concentrations of the expected presence of guggulsterones in the formulations.

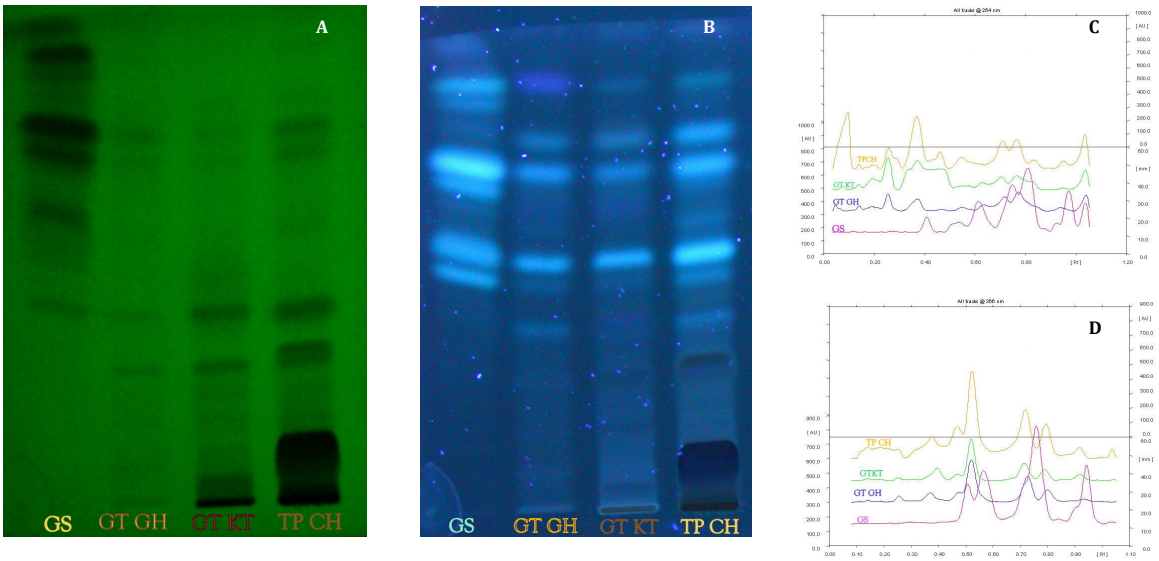


Figure 1. Visualization under UV light (A) @254nm (B) @366nm; 3D-display of Fingerprint (C) @254 nm (D) @366 nm

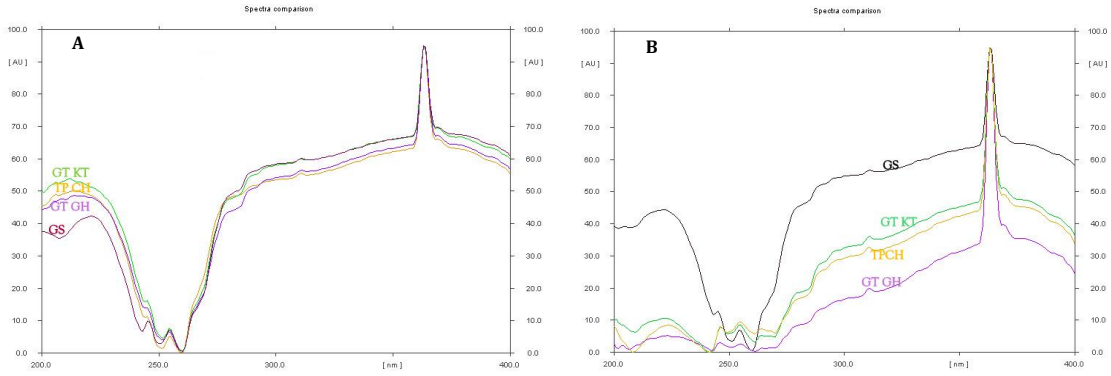


Figure 2. Spectral display @ 366 nm (A) R_f = 0.51(B) R_f = 0.76

Table 1. Showing the peaks of spots and their respective concentration (AU) observed under @254 nm

Peaks	Rf value Tracks				Area (AU) Tracks			
	1	2	3	4	1	2	3	4
1		0.05	0.08	0.10		586.9	296.6	12035.6
2		0.14	0.14	0.14		593.8	635.1	270.1
3		0.19	0.19	0.20		1102.0	2787.7	486.0
4		0.26	0.26	0.26		2458.1	5536.4	4203.6
5		0.37	0.37	0.37		2791.6	10921.5	13205.8
	0.41				2467.1			
6		0.47	0.45	0.46		273.7	5800.6	3676.4
7	[a]0.53	[a] 0.55	[a] 0.57	[a] 0.54	[a] 3146.0	[a] 873.7	[a] 1015.1	[a] 3441.7
8	0.61	0.63	0.63		9706.1	1345.5	1512.3	
9		0.72	0.70	0.71		3681.4	3460.2	7459.3
10	[a] 0.75	[a] 0.77	[a] 0.77	[a] 0.77	[a] 13695.9	[a] 5945.8	[a] 4470.5	[a] 7067.4
11	0.81		0.82	0.83	18370.0		1171.4	1578.3
12	0.87				459.3			
13	[a] 0.92	[a] 0.94	[a] 0.95	[a] 0.95	[a] 1334.2	[a] 692.0	[a] 230.4	[a] 1257.5
	0.97				8533.3			

[a] The values shown in bold letters are the peaks of comparison between isolated markers from the guggulu and the test formulations

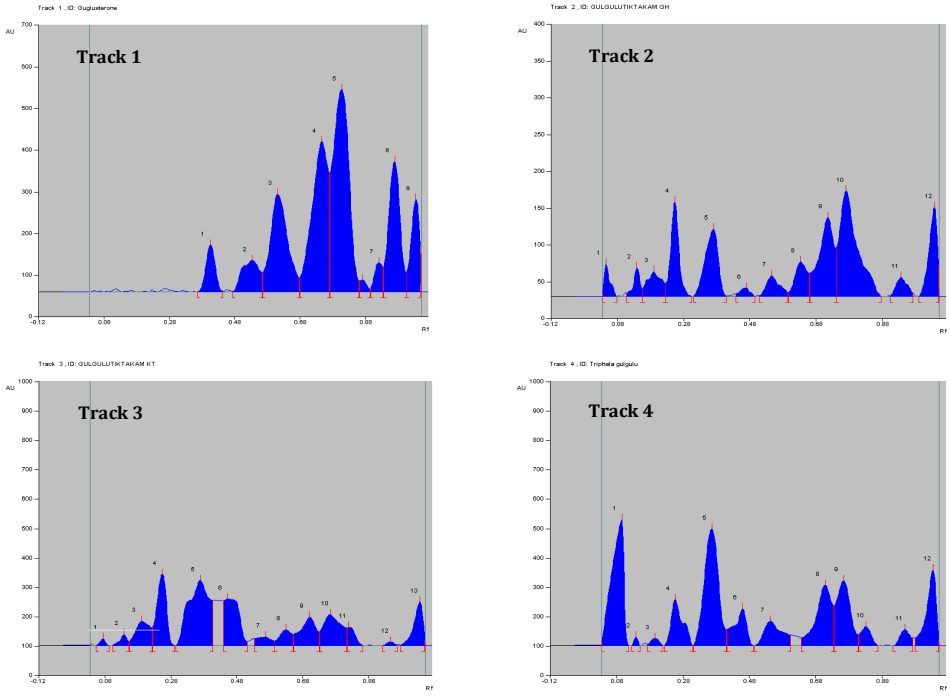


Figure 3. Peak display at 254 nm

It can be concluded that the levels of guggulsterones in the Ayurvedic preparations cannot be controlled but a uniform concentration can be maintained throughout a batch by adapting a method like this to use as an in-house method for the quality control check in Ayurvedic industries.

3. Experimental

Materials and Methods: All the solvents used in the extraction process were analytical grade. The solvents used in the HPTLC analysis were HPLC grade. The test samples are obtained commercially from the market and used for the study.

3.1. Sample Preparation, Test:

Powdered material of the test products of interest has been refluxed in 10 mL of methanol and filtered. The filtered extract was concentrated on a water bath to obtain 1 mL of extract and used for the analysis.

3.2. Extraction and Isolation of Guggulsterones.[2, 3, 4]

The resin taken and loaded in soxhlet extractor and extracted with ethyl acetate about five time the weight of gum. The temp is kept at 65-70 °C. The extracted fluid is taken for solvent recovery. The oleoresin (thick paste) obtained after solvent removal can be purified for enrichment of guggulsterones by solvent frication method. Take 2 g sample of guggulu extract in 250 mL round bottom flask, add 35 mL of 0.5 M alcoholic KOH and reflux for 90 min on a water bath. Transfer the content of flask to a separator rinse the flask with 50 mL lukewarm water. Extract while the liquid is warm by shaking vigorously with three successive quantities of 50 mL petroleum ether (60-80°). Combine the petroleum ether layers and wash with 20 mL water. Evaporate the petroleum ether and weigh the residue.

Table 2. Showing the peaks of spots and their respective concentration (AU) observed under @366 nm

Peaks	Rf value Tracks				Area (AU) Tracks			
	1	2	3	4	1	2	3	4
1		0.14	0.14	0.14		179.6	262.5	2137.7
2				0.19				2384.4
3				0.21				1555.3
4		0.26	0.25	0.25		903.8	306.6	1208.4
5		0.37	0.39	0.38		2210.9	2573.5	8141.3
6		0.47	0.47	0.47		1518.5	981.4	7290.5
7	[a] 0.51	[a] 0.52	[a] 0.52	[a] 0.52	[a] 7518.8	[a] 8343.0	[a] 6436.8	[a] 18718.1
8	0.57				14079.4			
9	[a] 0.76	[a] 0.73	[a] 0.71	[a] 0.72	[a] 30288.2	[a] 5123.2	[a] 3588.6	[a] 14757.1
10	0.84	0.80	0.79	0.79	808.5	2381.3	1907.6	7282.6
11	[a] 0.94	[a] 0.93	[a] 0.92	[a] 0.92	[a] 12105.4	[a] 843.6	[a] 1138.2	[a] 2249.5
12	0.98			0.98	234.8			189.4
13								943.0

[a]The values shown in bold letters are the peaks of comparison between isolated markers from the guggulu and the test formulations

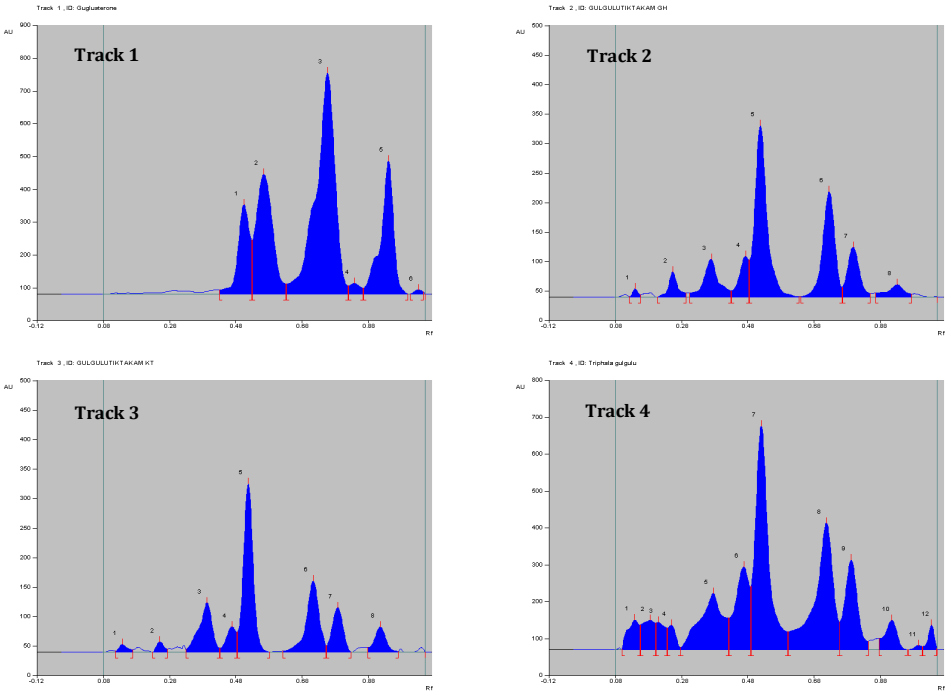


Figure 4. Peak display @ 366 nm

3.3. HPTLC Analysis[5-9]

The HPTLC analysis was carried out using the following conditions and the developed plates were visualized under UV light at 254 and 366 nm and densitometric scanning was performed to obtain the Rf Values and corresponding concentration of the spots (AU). The scanning was also performed by CAMAG densitometry scanner 3 between 200-400 nm to determine any additional existing spots. The spectral data was obtained by scanning the specific spots of Rf values of 0.51 and 0.72 under UV light at 366 nm.

3.4. HPTLC Conditions

Instrument used : CAMAG make HPTLC.
Software : winCATS 1.4.3
Sample Applicator : Linomat 5.
Detection : @254 nm & @366 nm in Densitometry TLC Scanner 3
STD Preparation : 1 mg of STD is dissolved in 0.25 mL water and 0.25 mL Ether
Stationary Phase : HPTLC plates silica gel 60 F 254.
Mobile Phase : Toluene: Ethyl acetate: Methanol (7:2:1)
Spectral Detection : Scanned at 200 nm to 400 nm
Sample Applied : 2µl sample is applied as 8mm band.
Track 1, **GS** : Guggulsterone Standard,
Track 2, **GT GH**: Gulgulutiktakam Ghritam
Track 3, **GT KT**: Gulgulutiktakam KashayamTablets
Track 4, **TP CH**: Triphala Gulgulu Churna

Acknowledgement

The authors are thankful to the Research and Development, AVN Ayurveda Fomulations Pvt Ltd., Madurai, India for providing the commercial samples and facilities for carrying out this research.

References

1. Ayurvedic Pharmacopoeia of India, Part II (Formulations). 1 ed.; Government of India: New Delhi, 2007; Vol. 1.
2. Badmaev, V.; Majeed, M.; Pacchetti, B.; Prakash, L., Standardiation of Commiphora Mukul extract in dislipidemia and cardiovascular disease. *NUTRA Foods* 2003, 2, 45-51.
3. Soni, V., Isolation of E & Z guggulsterones from young aerial stems of Commiphora wightii without destruction of plants. *Planta Medica* 2006, 72, S_046.
4. Patel, S.; Patel, J.; Patel, K., Comparison and Quantification of Marker Compound of Triphala Guggulu by using HPTLC method. *Am J PharmTech Res* 2012, 2, 999-1013.
5. Rajani, M.; Ravishankara, M. N.; Shrivastava, N.; Padh, H., HPTLC-aided phytochemical fingerprinting analysis as a tool for evaluation of herbal drugs. A case study of Ushaq (ammoniacum gum). *J Planar Chromatogr Mod TLC* 2001, 14, 34-41.
6. Agrawal, H.; Kaul, N.; Paradkar, A. R.; Mahadik, K. R., HPTLC method for guggulsterone: II. Stress degradation studies on guggulsterone. *J Pharm Biomed Anal* 2004, 36, 23-31.
7. Musharraf, S. G.; Iqbal, N.; Gulzar, U.; Ali, A.; Choudhary, M. I.; Atta ur, R., Effective separation and analysis of E- and Z-guggulsterones in Commiphora mukul resin, guggulipid and their pharmaceutical product by high performance thin-layer chromatography-densitometric method. *J Pharm Biomed Anal* 2011, 56, 240-5.
8. Shelke, N.; Kuber, V.; Bhope, S.; Jadhav, R., Simultaneous HPTLC analysis of E -guggulsterone, Z -guggulsterone, 11-keto-β-boswellic acid, and 3-acetyl-11-keto-β-boswellic acid in an anti-arthritic formulation. *J Planar Chromatogr Mod TLC* 2011, 24, 242-7.
9. Jariwala, J.; Saluja, A.; Anajwala, C.; Dakhara, S., Simultaneous estimation of Guggulsterone E & Z and Tinosporaside in Jivitprada vati by HPTLC method. *Int J Green Pharm* 2011, 5, 113-7.

Journal of Pharmaceutical Chemistry
http://www.vensel.org/index.php/jphchem
VENSEL

Synthesis Antimicrobial and Anticancer Activity of 1-[(arylalkylidene)amino]-3-(4*H*-1,2,4-triazol-4-yl)thiourea

[1,*]Umasankar Kulandaivelu, [1]Chawada Bhawatha, [1]Boyapati Shireesha, [1]Alavala Rajasekhar Reddy

[1]Medicinal Chemistry Research Division, Vaagdevi College of Pharmacy, Hanamkonda, AP, India

Abstract: Arylalkylidene derivatives of aminotriazoles (**3a-3j**) were synthesized and tested for their antimicrobial and anticancer activity. Four non-pathogenic bacteria [*E. coli* (NCIM 2068), *K. pneumoniae* (NCIM 2957), *S. aureus* (NCIM 2079), *B. subtilis* (NCIM 2921)] two fungi [*C. albicans*, *A. niger*] and two cancer cell lines [HBL-100 and HT-29] were employed in the study. All the compounds were found to have better antibacterial activity against *B. subtilis* than Ciprofloxacin (standard) and compound **3i** was equivalent to Ciprofloxacin in inhibiting *S. aureas*. Similarly all the compounds inhibited the growth of *A. niger* better than Fluconazole and compound **3c** was equivalent to Fluconazole (standard) in inhibiting *C. albicans*. In case of anticancer activity none of the molecule exhibited activity better than the standard used (Methotrexate), though they have inhibitory concentration at submicromolar level.

Keywords: aminotriazoles; thiosemicarbazones; antimicrobial; antifungal; anticancer

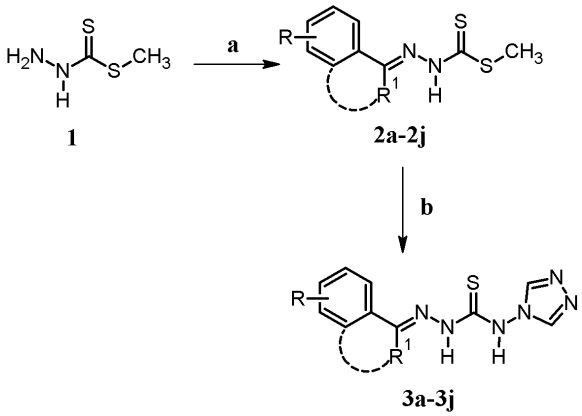
1. Introduction

Many major pathogenic bacteria and parasites have acquired resistance towards currently available antibiotics in the market during the last decade. This has led to the adoption of a resolution on antimicrobial resistance in World Health Assembly during 1998.[1] Development of superbugs has raised fears that infectious diseases may once again become major cause of death in developing/developed countries. Chemotherapeutic agents with novel structure and mode of action should be developed to combat the threat due to the superbugs. Thiosemicarbazones were very well known for their antimicrobial & anticancer property.[2-5] Thiosemicarbazones with free primary N4 amino group were reported for their anticancer property.[6-9] We reported anticancer thiosemicarbazones with secondary and tertiary N4 amino group.[10-13] The presented work elucidated the antimicrobial and anticancer activity of a novel triazol ring containing thiosemicarbazones.

2. Result and Discussion

2.1. Chemistry

The final compounds **3a-3j** was synthesized by following the synthetic route outlined in **Scheme 1**.



Scheme 1. Reagents and conditions: (a) R^1-C_6H_4-CO-R/MeOH, H_2SO_4 [cat], reflux, 6-7 h; (b) 1,2,4-triazol-4-amine/EtOH, reflux.

Methyl hydrazine carbodithioate (**1**) was prepared by the reaction of hydrazine hydrate (85%) with carbon disulfide in the presence of potassium hydroxide.[10, 14] Condensation of **1** with aromatic aldehydes/ketones in the presence of catalytic amount of sulphuric acid in methanol provided **2a–2j**.[10, 14] The final compounds **3a–3j** was synthesized by the reaction of 1,2,4-triazol-4-amine with **2a–2j** in ethanol. The reaction comes to completion when evaluation of methyl mercaptan ceases.[10, 14] The compounds (**3a-3j**) were characterized by their spectral (IR, ^{1}H-NMR & ES-MS) and elemental analysis data. CHNS microanalysis revealed that variation in experimental values compared with calculated values is within ±0.4%. All the thiosemicarbazone derivatives (**3a–3j**) displayed characteristic N-H stretch (between 3028-3266 cm^{-1}), C=N stretch (between 1661-1692 cm^{-1}), C-N stretch (between 1255-1389 cm^{-1}) and C=S stretch (between 1181-1259 cm^{-1}). All the derivatives (**3a–3j**) showed a characteristic peak for the aldehydic proton (=C-H) between δ 7.9– 8.9 ppm as a singlet, ketonic methyl proton (-CH_3) between δ 1.3-2.7 ppm as a singlet and aryl proton between δ 6.0-8.1 ppm as doublets or doublet and singlet, aryl proton of triazole between δ

Submitted on: Mar 13, 2014
Revised on: Apr 19, 2014
Accepted on: Apr 19, 2014

*corresponding author: UK- Tel: +91-9989366742; E-mail: youmasankar@gmail.com

7.8-8.6 ppm as singlet, NH proton between δ 1.9-2.3 ppm as signlet, =N-NH proton between δ 11.0-11.5 ppm as singlet. The EI-MS spectra of all the compounds displayed $(M+1)^+$ or $(M-1)^+$ peak. The structure, physico-chemical characterization of compounds **3a–3j** was presented in **Table 1**.

Table 1. Physico-chemical characterization of **3a-3j**

Code	R	R^1	MF	MW	Y (%)	M.P (°C)
3a	4-Cl	H	$C_{10}H_9ClN_6S$	280	58.2	195-197
3b	4-OH	H	$C_{10}H_{10}N_6OS$	262	73.2	212-214
3c	4-OCH_3	H	$C_{11}H_{12}N_6OS$	276	54.4	201-203
3d	3,4-di-OCH_3	H	$C_{12}H_{14}N_6O_2S$	306	52.0	207-209
3e	4-$N(CH_3)_2$	H	$C_{12}H_{15}N_7S$	289	60.2	185-188
3f	3-NO_2	CH_3	$C_{11}H_{11}N_7O_2S$	305	74.0	190-194
3g	4-Cl	CH_3	$C_{11}H_{11}ClN_6S$	294	65.8	210-214
3h	4-OH	CH_3	$C_{11}H_{12}N_6OS$	276	79.7	222-224
3i	4-OCH_3	CH_3	$C_{12}H_{14}N_6OS$	290	73.3	219-221
3j	---Isatin---		$C_{11}H_9N_7OS$	287	50.0	239-241

2.2. Antimicrobial and anticancer studies

All ten thiosemicarbazone derivatives (**3a-3j**) were evaluated for their antibacterial activity in serial double dilution method against non-pathogenic strains of *Escherichia coli* (NCIM 2068), *Klebsiella pneumoniae* (NCIM 2957), *Staphylococcus aureus* (NCIM 2079), and *Bacillus subtilis* (NCIM 2921) and also they were evaluated for their antifungal activity against *Candida albicans* and *Asprgillus niger*.[15] The results were presented in **Table 2**. All the compounds were found to be 3-4 fold potent than standard (Ciprofloxacin) used in the study against *B. subtilis.* Almost all the compounds was found to have antibacterial activity against all the bacteria at a concentration <2.0 μM. Thiosemicarbazones derived from acetophenones (**3g, 3h** and **3i**) were found to be better than those derived from benzaldehydes (**3a, 3b** and **3c**). Among all acetophenone derivatives (**3f-3i**), compound **3i (**p-OCH_3) exhibited better antibacterial activity against *E. coli, K. pneumonia* and *S. aureus* (except *B. subtilis*). Among all benzaldehyde derivatives (**3a-3e**), compound **3b (**p-OH**)** has shown less potent than other derivatives. In the series 4-OH derivative derived from benzaldehyde as well as acetophenone exhibited less antibacterial activity than other compounds substituted with electron donating groups like 4-OCH_3, 3, 4-di-OCH_3 and 4-$N(CH_3)_2$.The compounds were also screened for antifungal activity in serial double dilution method against *C. albicans* and *A. niger*. The results were presented in **Table 2**. They were found to be 5-8-fold potent than standard (Fluconazole) used in the study against *A. niger*. Compound **3c** exhibited potency equivalent to Fluconazole against *C. albicans.* Almost all the compounds were found to have antifungal activity against both the strains at a concentration <2.0 μM. The acetophenone and corresponding benzaldehyde derivatives have shown almost same potency against *A. niger* and *C. albicans* except **3c.**

The compounds **3a-3j** were also evaluated for their anticancer activity against HBL-100 and HT-29 cell lines using MTT assay by serial double dilution method in 96-well plate.[16-18] The results are presented in **Table 2.** All the compounds displayed cytotoxic activity against HBL-100 cell lines at the concentration between 0.15 and 0.33 μM. Compound **3i** (0.15 μM) has shown the best anticancer activity against HBL-100 cell lines within this series and was nearly 4 fold less potent than standard Methotrexate (0.04 μM) used in the study. Thiosemicarbazones derived from acetophenones (**3g, 3h** and **3i**) were found to be better than their corresponding benzaldehyde derivatives (**3a, 3b** and **3c**). The anticancer activity was in the following order: 4-OCH_3>4-OH>4-Cl. In compound **3c** (4-OCH_3; IC_{50}= 0.16 μM) introduced additional substitution offered **3d** (3,4-diOCH_3; IC_{50} = 0.21 μM) and shown less potent. The presence of electron withdrawing group in acetophenone derivatives was found to reduce the potency.

Also all the compounds displayed cytotoxic activity against HT-29 cell lines at the concentration between 0.11 and 0.29 μM. Compound **3d** (0.11 μM) has exhibited the best anticancer activity against HT-29 cell lines within this series and was nearly 5 fold less potent than standard. Thiosemicarbazones derived from benzaldehydes were found to be better than their corresponding acetophenone derivatives (except **3b)**. Further evaluation of this compound and its derivatives are in progress.

3. Experimental

Materials and methods: Melting points were determined using Thermonik Melting Point Apparatus (Campbell electronics, India) by capillary method and are uncorrected. Infrared (IR) spectra were recoded on a Fourier Transform Infrared Spectrophotometer IR-Prestige 21 (Shimatzu Corporation, Japan) from 4000-400 cm^{-1} using KBr. ^{1}H-NMR spectra were recorded at 400 MHz in DMSO-d_6 using a Bruker Avance 400 instrument (Bruker Instruments Inc., USA). Chemical shifts were measured at δ units (ppm) relative to Tetramethylsilane (TMS). Electron Impact (EI) mass spectra were recorded on a VG 7070 H instrument (Micromass, UK) at 70 eV. Elemental analysis was performed on a Vario EL III Elemental Analyser (Elementar, Germany) using sulfanilamide as standard. All chemicals were purchased from Merck, Spectrochem or CDH, India. Solvents were of reagent grade and were purified and dried by standard procedure. Reactions were monitored by thin-layer chromatography on silica gel plates in either iodine or UV chambers. Intermediates were characterized by IR spectroscopic analysis and elemental analysis for CHNS. In the elemental analysis, the observed values were within ±0.4% of the calculated values. Final compounds were characterized by ^{1}H-NMR and EI-MS.

3.1. Chemistry

3.1.1. General procedure for synthesis of methyl hydrazinecarbodithioate (1)

To a cooled solution of potassium hydroxide (0.1 M, 6.6 g/7 mL) was added 2-propanol (7 mL), hydrazine hydrate (85% solution, 0.1 M, 6 mL) with stirring. Ice-cooled carbondisulfide (0.1 M, 10 mL) was added drop wise to the above stirred solution that was maintained below 10 °C over 1.5 h. The bright yellow mixture obtained was further stirred for 1 h and then ice-cooled iodomethane (0.1 M, 7 mL) was added drop wise over a period of 2 h. Stirring was continued for an additional 1.5 h to obtain a white precipitate of **1**. Filtered, washed with ice-cooled water and recrystallized from dichloromethane. Yield: 40 %; mp: 80-82 °C.[14]

3.1.2. General procedure for synthesis of Schiff's bases methylhydrazine carbodithioate (2a-2j)

Methyl hydrazinecarbodithioate **1** (0.01 M, 1.22 g) and (un)-substituted aromatic aldehydes/ketone (0.012 M) were dissolved in methanol (10 mL). To this mixture

Table 2. Antimicrobial and anticancer activity of **3a-3j**

Code	Antibacterial Activity (IC_{50} in μM)*				Antifungal Activity (IC_{50} in μM)*		MTT Assay (IC_{50} in μM)**	
	B. subtilis	*E. coli*	*K. pneumonia*	*S. aureus*	*C. albicans*	*A. niger*	HBL-100	HT-29
3a	1.82	1.79	1.63	1.38	1.78	1.62	0.27	0.12
3b	1.91	2.09	1.94	1.74	2.38	2.11	0.18	0.20
3c	1.65	1.65	1.18	1.51	0.98	1.82	0.16	0.22
3d	1.63	1.19	1.49	1.33	1.79	1.37	0.21	0.11
3e	1.34	1.34	1.33	1.63	1.58	1.39	0.29	0.23
3f	1.27	1.37	1.71	1.61	1.74	1.37	0.31	0.15
3g	1.32	1.23	1.37	1.42	1.95	1.94	0.21	0.25
3h	1.78	1.65	1.80	1.63	1.82	1.70	0.17	0.14
3i	1.30	0.79	1.16	1.27	1.24	1.23	0.15	0.29
3j	1.83	1.97	2.13	1.87	1.86	1.59	0.33	0.16
CIP	4.70	0.03	0.05	1.20	---	---	---	---
FLU	---	---	---	---	0.98	>10.00	---	---
MTX	---	---	---	---	---	---	0.04	0.02

*Mean value of triplicate; ** Mean value of duplicate; MTX: Methotrexate; CIP: Ciprofloxacin; FLU: Fluconazole.

catalytic amount of concentrated sulphuric acid was added and refluxed for 6-7 h. The reaction mixture turned yellow as the methylhydrazine carbodithioate dissolved and the yellow product began to precipitate. The solid obtained was filtered, dried and recrystallized from suitable solvent.

3.1.3. General procedure for synthesis of 2-Arylidene-N-(4H-1,2,4-triazol-4-yl)hydrazine-1-carbothioamide (3a-3j)

1,2,4-triazol-4-amine (0.0056 M, 0.685 g) was added to appropriate schiff's base (**2a-2j,** 0.005 M) in ethanol (25 mL) and refluxed until the evolution of methyl mercaptane was almost completely ceased. Solvent present in the reaction mixture was evaporated under vacuum. The solid obtained was collected, washed with cold ethanol and further purified by recrystallization from suitable solvent.

*2-(4-Chlorobenzylidene)-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3a**)*

IR (KBr, cm^{-1}): 3264 (N-H), 1692 (C=N), 1278 (C-N), 1191 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 2.0 (s, 1H, NH), 7.2 (d, 2H, Ar-H), 7.4 (d, 2H, Ar-H), 7.9 (s, 1H, CH), 8.6 (s, 2H, Ar-H), 11.0 (s, 1H, =N-NH); EI-MS (m/z): 281$[M+1]^+$; Elemental analysis Found (Calcd.): C, 42.64 (42.78); H, 3.30 (3.23); N, 30.68 (29.94); S, 11.62 (11.42).

*2-(4-Hydroxybenzylidene)-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3b**)*

IR (KBr, cm^{-1}): 3028 (N- H), 1682 (C=N), 1283 (C-N), 1195 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 1.9 (s, 1H, N-H), 6.5 (d, 2H, Ar-H), 7.4 (d, 2H, Ar-H), 8.1 (s, 2H, Ar-H), 8.6 (s, 1H, C-H), 9.7 (s, 1H, OH), 11.5 (s, 1H, =N-NH); EI-MS (m/z): 263 $[M+1]^+$; Elemental analysis Found (Calcd.): C, 45.50 (45.79); H, 3.96 (3.84); N, 32.62 (32.04); S, 12.44 (12.22).

*2-(4-Methoxybenzylidene)-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3c**)*

IR (KBr, cm^{-1}): 3264 (N-H), 1683 (C=N), 1389 (C-N), 1195 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 2.1 (s, 1H, NH), 2.5 (s, 3H, OCH_3), 7.0 (d, 2H, Ar-H), 7.5 (d, 2H, Ar-H), 7.8 (s, 2H, Ar-H), 8.2 (s, 1H, CH), 11.3 (s, 1H, =N-NH); EI-MS (m/z): 275$[M-1]^+$; Elemental analysis Found (Calcd.): C, 48.28 (47.81); H, 4.46 (4.38); N, 30.28 (30.41); S, 11.54 (11.60).

*2-(3,4-Dimethoxybenzylidene)-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3d**)*

IR (KBr, cm^{-1}): 3266 (N-H), 1673, (C=N), 1270 (C-N), 1183 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 2.0 (s, 1H, N-H), 3.6 (s, 6H, OCH_3), 6.0 (d, 1H, Ar-H), 6.6 (d, 1H, Ar-H), 7.0 (s, 1H, Ar-H), 8.0 (s, 2H, Ar-H), 8.9 (s.1H, CH), 11.0 (s,1H, =N-NH); EI-MS (m/z): 305 $[M-1]^+$; Elemental analysis Found (Calcd.): C, 46.90 (47.05); H, 4.72 (4.61); N, 27.08 (27.43); S, 10.74 (10.47).

*2-[4-(Dimethylamino)benzylidene]-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3e**)*

IR (KBr, cm^{-1}): 3256 (N-H), 1688 (C=N), 1360 (C-N), 1259 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 2.1 (s, 1H, N-H), 3.6 (s, 6H, CH_3), 7.3 (d, 2H, Ar-H), 7.6 (d, 2H, Ar-H), 8.2 (s, 2H, Ar-H), 8.5 (s, 1H, C-H), 11.1 (s, 1H, =N-NH); EI-MS (m/z): 290$[M+1]^+$; Elemental analysis Found (Calcd.): C, 51.32 (49.81); H, 5.28 (5.23); N, 34.19 (33.88); S 11.25 (11.08).

*2-[1-(3-Nitrophenyl)ethylidene]-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3f**)*

IR (KBr, cm^{-1}): 3265 (N-H), 1661 (C=N), 1255 (C-N), 1181 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 1.3 (s, 3H, $3CH_3$), 1.9 (s, 1H, N-H), 7.2-7.4 (t, 3H, Ar-H), 7.7 (s, 1H, Ar-H), 8.5 (s, 2H, Ar-H), 11.3 (s, 1H, =N-NH); EI-MS (m/z): 306 $[M+1]^+$; Elemental analysis Found (Calcd.): C, 43.16 (43.27); H, 3.74 (3.63); N, 32.20 (32.11); S, 10.88 (10.50).

*2-[1-(4-Chlorophenyl)ethylidene]-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3g**)*

IR (KBr, cm^{-1}): 3256 (N-H), 1690 (C=N), 1360 (C-N), 1205 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 1.4 (s, 3H, CH_3), 2.3 (s, 1H, N-H), 7.7 (d, 2H, Ar-H), 8.1 (d, 2H, Ar-H), 8.2 (s, 2H, Ar-H), 11.3 (s, 1H, =N-NH); EI-MS (m/z): 295 $[M+1]^+$; Elemental analysis Found (Calcd.): C, 44.76 (44.82); H, 3.88 (3.76); N, 28.94 (28.51); S, 10.98 (10.88).

*2-[1-(4-Hydroxyphenyl)ethylidene]-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3h**)*

IR (KBr, cm^{-1}): 3264 (N-H), 1683, (C=N), 1283 (C-N), 1210 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 2.0 (s, 1H, N-H), 2.7 (s, 3H, CH_3), 6.8 (d, 2H, Ar-H), 7.3 (d, 2H, Ar-H), 8.3 (s, 2H, Ar-H), 9.6 (s, 1H, OH), 11.3 (s, 1H, =N-NH); EI-MS (m/z): 275 $[M-1]^+$; Elemental analysis Found (Calcd.): C, 47.86 (47.81); H, 4.42 (4.38); N, 30.36 (30.41); S, 11.83 (11.60).

*2-[1-(4-Methoxyphenyl)ethylidene]-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3i**)*

IR (KBr, cm^{-1}): 3265 (N-H), 1682 (C=N), 1283 (C-N), 1201 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 1.9 (s,1H, NH), 2.7 (s, 3H, CH_3), 4.3 (s 3H, OCH_3), 7.6 (d, 2H, Ar-H), 7.9 (d, 2H, Ar-H), 8.1 (s, 2H, Ar-H), 11.1 (s, 1H, =N-NH); EI-MS (m/z): 291 $[M+1]^+$; Elemental analysis Found

(Calcd.): C, 49.72 (49.64); H, 5.02 (4.86); N, 29.06 (28.95); S, 11.35 (11.04).

*2-(Isatin-3-ylidene)-N-(4H-1,2,4-triazol-4-yl)hydrazinecarbothioamide (**3j**)*

IR (KBr, cm^{-1}): 3112 (N-H), 1670 (C=N), 1285 (C-N), 1206 (C=S); ^{1}H-NMR (DMSO-d6, δ ppm): 2.0 (s, 1H, NH), 6.7-7.1 (m,4H, Ar-H), 8.0 (s, 2H, Ar-H), 10.0 (s, 1H, NHCO), 11.0 (s, 1H, =N-NH); EI-MS (m/z): 288 $[M+1]^+$; Elemental analysis Found (Calcd.) C, 45.28 (45.99); H, 3.24 (3.16); N, 33.74 (34.13); S, 11.63(11.16).

3.2. Antimicrobial study

3.2.1. Antibacterial studies

The antibacterial activities of the newly synthesized compounds (**3a-3j**) were tested using serial double dilution method[15] against non-pathogenic strains of *E. coli* (NCIM 2068), *K. pneumoniae* (NCIM 2957), *S. aureus* (NCIM 2079) and *B. subtilis* (NCIM 2921) in nutrient agar medium by Cup-plate method. Sterilized media was cooled to 40 °C and 0.5 mL of inoculums for 100 mL of media was added. The flasks were shaken gently to avoid formation of air bubbles. This medium was transferred to Petri dishes of 9 cm diameter in 25 mL portions, so as to obtain 4-5 mm thickness of the media layer. The plates were left at room temperature to allow solidification of the media. In each Petri plate, 4 cups of suitable diameter were made with a sterile borer. All these procedures were conducted aseptically under laminar air flow workstation (Elite; Elite Scientific and Equipments). The test compounds and Ciprofloxacin were dissolved in DMSO (0.5%) and solution ranging between 0.1 and 100 µM were prepared. DMSO control was also maintained. Test compounds (40 µL) and standard (40 µL) were added into each cup with the help of a micropipette. Plates were kept undisturbed for at least 2 h at room temperature to allow for proper diffusion. Petri plates were then incubated at 37±1 °C for 24 h. Zone of inhibitions (in mm) were measured after incubation and IC_{50} values were calculated by plotting a graph between log concentrations and percentage inhibition values. All the studies were performed in triplicate and results were presented in **Table 2**.

3.2.2. Antifungal studies

The antifungal activities of the test compounds were assayed using serial double dilution method against *C. albicans* and *A. niger* in Sabouraud dextrose agar medium by Cup-plate method.[15] The sterile medium was inoculated using 24 h slant cultures of test organisms and transferred into sterile Petri dishes and allowed to solidify. Four cups of suitable diameter were made on the solidified media. The test compounds and Fluconazole were dissolved in DMSO (0.5% v/v) and solution ranging between 0.1 and 100 µM were prepared. DMSO control was also maintained. Test compounds (40 µL) and standard (40 µL) were added into each cup with the help of a micropipette. Zones of inhibition (in mm) were measured after 24 h of incubation and IC_{50} values were calculated by plotting a graph between log concentrations and percentage inhibition value. All the studies were performed in triplicate and results were presented in **Table 2**.

3.2.3. Anticancer studies (MTT assay)

The compounds **3a-3j** were evaluated for their anticancer activities on HBL-100 and HT-29 cell lines using MTT assay by serial double dilution method in 96-well plate.[16-18] Cells seeded in plate at 5000 cells/well. Different dilutions of test and standard (0.1-100 µM) were made with growth medium in such a way that the final DMSO concentration is around 0.5% v/v. 100 µL of cell suspension and 100 µL of test and standard were transferred aseptically to each well. The plate was then incubated at 37 °C for 72 h in CO_2 incubator. After incubation, 20 µL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide give the chemical name of the dye (MTT) was added to each well and plate was wrapped in aluminum foil to prevent the oxidation of the dye. The plate was again incubated for 2 h. 80 µL of lysis buffer was added to each well and the plate was placed on a shaker overnight. The absorbance was recorded on the ELISA reader at 562 nm. The absorbance of the test was compared with that of DMSO control to get the percentage inhibition and IC_{50} values are calculated by plotting a graph between log concentrations and percentage inhibition value. All the studies were performed in duplicate and results were presented in **Table 2**.

Acknowledgement

Authors are thankful to Secretary, Viswabhara Educational Society, Warangal for providing the facilities and Director, IICT, Hyderabad for providing spectral data.

References

1. WHO, *Containing antimicrobial resistance: review of the literature and report of a WHO Workshop on the Development of a Global Strategy for the Containment of Antimicrobial Resistance, Geneva, Switzerland, 4-5 February*; 1999.
2. El-Sharief, M. A.; Abbas, S. Y.; El-Bayouki, K. A.; El-Gammal, E. W., Synthesis of thiosemicarbazones derived from N-(4-hippuric acid)thiosemicarbazide and different carbonyl compounds as antimicrobial agents. *Eur J Med Chem* 2013, 67, 263-8.
3. Mohamed, N. A.; Mohamed, R. R.; Seoudi, R. S., Synthesis and characterization of some novel antimicrobial thiosemicarbazone O-carboxymethyl chitosan derivatives. *Int J Biol Macromol* 2014, 63, 163-9.
4. Rollas, S.; Küçükgüzel, S. G., Biological activities of hydrazone derivatives. *Molecules* 2007, 12, 1910-39.
5. Mashayekhi, V.; Haj Mohammad Ebrahim Tehrani, K.; Azerang, P.; Sardari, S.; Kobarfard, F., Synthesis, antimycobacterial and anticancer activity of novel indole-based thiosemicarbazones. *Arch Pharm Res* 2013.
6. Liu, M. C.; Lin, T. S.; Sartorelli, A. C., 1 Chemical and Biological Properties of Cytotoxic α-(*N*)-Heterocyclic Carboxaldehyde Thiosemicarbazones. *Prog Med Chem* 1995, 32, 1-35.
7. Alvero, A. B.; Chen, W.; Sartorelli, A. C.; Schwartz, P.; Rutherford, T.; Mor, G., Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) induces apoptosis in ovarian cancer cells. *J Soc Gynecol Investig* 2006, 13, 145-52.
8. Finch, R. A.; Liu, M. C.; Cory, A. H.; Cory, J. G.; Sartorelli, A. C., Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-AP): an inhibitor of ribonucleotide reductase with antineoplastic activity. *Adv Enzyme Regul* 1999, 39, 3-12.
9. Finch, R. A.; Liu, M.; Grill, S. P.; Rose, W. C.; Loomis, R.; Vasquez, K. M.; Cheng, Y.; Sartorelli, A. C., Triapine (3-aminopyridine-2-carboxaldehyde- thiosemicarbazone): A potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. *Biochem Pharmacol* 2000, 59, 983-91.
10. Chetan, B.; Bunha, M.; Jagrat, M.; Sinha, B. N.; Saiko, P.; Graser, G.; Szekeres, T.; Raman, G.; Rajendran, P.; Moorthy, D.; Basu, A.; Jayaprakash, V., Design, synthesis and anticancer activity of piperazine hydroxamates and their histone deacetylase (HDAC) inhibitory activity. *Bioorg Med Chem Lett* 2010, 20, 3906-10.

11. Krishnan, K.; Prathiba, K.; Jayaprakash, V.; Basu, A.; Mishra, N.; Zhou, B.; Hu, S.; Yen, Y., Synthesis and Ribonucleotide reductase inhibitory activity of thiosemicarbazones. *Bioorg Med Chem Lett* 2008, 18, 6248-50.
12. Kulandaivelu, U.; Padmini, V. G.; Suneetha, K.; Shireesha, B.; Vidyasagar, J. V.; Rao, T. R.; K N, J.; Basu, A.; Jayaprakash, V., Synthesis, antimicrobial and anticancer activity of new thiosemicarbazone derivatives. *Arch Pharm (Weinheim)* 2011, 344, 84-90.
13. Ali, A. Q.; Teoh, S. G.; Salhin, A.; Eltayeb, N. E.; Khadeer Ahamed, M. B.; Majid, A. M., Synthesis of isatin thiosemicarbazones derivatives: In vitro anti-cancer, DNA binding and cleavage activities. *Spectrochim Acta A Mol Biomol Spectrosc* 2014, 125, 440-8.
14. Klayman, D. L.; Bartosevich, J. F.; Griffin, T. S.; Mason, C. J.; Scovill, J. P., 2-Acetylpyridine thiosemicarbazones. 1. A new class of potential antimalarial agents. *J Med Chem* 1979, 22, 855-62.
15. Barry, A., Procedure for testing antimicrobial agents in agar media: Theoretical considerations. *Antibiotics in Laboratory Medicine. Edition* 1986, 2, 1-26.
16. Grever, M. R.; Schepartz, S.; Chabner, B. In *The National Cancer Institute: Cancer Drug Discovery and Development Program*, 1992; p 622.
17. Boyd, M. R.; Paull, K. D., Some practical considerations and applications of the National Cancer Institute in vitro anticancer drug discovery screen. *Drug Develop Res* 1995, 34, 91-109.
18. Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A., Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991, 83, 757-66.

Journal of Pharmaceutical Chemistry
http://www.vensel.org/index.php/jphchem
VENSEL

Method Development and Validation of Hydrochlorothiazide and Quinapril in bulk and tablet dosage form by RP-HPLC

[1]Harini Kanchukommula, [1,*]Ananda Thangadurai Subramaniam, [1]Jambulingam Munusamy, [1]Kamalakannan Danapal, [1]Saravanakumar Sengodan

[1]Department of Pharmaceutical Analysis, Swamy Vivekanandha College of Pharmacy, Elayampalayam-637205, TN, India

Abstract: A RP–HPLC chromatographic method was developed and validated for the determination of Quinapril and Hydrochlorothiazide in bulk powder and in pharmaceutical formulations. Quinapril and Hydrochlorothiazide can be separated on Zorbax Eclipse XDB, C18 column (150 x 4.6 mm, 5 µm) at 30 °C using Acetonitrile: Phosphate buffer, pH 4.5 was adjusted with o-phosphoric acid in the ratio of 35:65 v/v as a mobile phase at flow rate of 0.9 mL min^{-1} and detected at 210 nm. The retention time of Quinapril and Hydrochlorthiazide was found to be 2.099 min and 5.537 min respectively. The validation of the proposed method was carried out for specificity, linearity, accuracy, precision, LOD, LOQ and robustness. Calibration was linear over a range of 50-300 µg mL^{-1} and 31.25–187.5 µg mL^{-1} with correlation coefficient of 0.999 for Quinapril and Hydrochlorthiazide, respectively. The robustness of the method was evaluated by deliberately altering the chromatographic conditions. The method developed can be applicable for quality control analysis.

Keywords: Quinapril; Hydrochlorothiazide; RP-HPLC; method development; validation

1. Introduction

Quinapril (QUI) **(Figure 1)** is chemically (3S)-2-[(2S)-2-{[(2S)-1-ethoxy-1-oxo-4- phenylbutan-2-yl] amino} propanoyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid. QUI is an angiotensin-converting enzyme inhibitor (ACE inhibitor) used in the treatment of hypertension and congestive heart failure. Hydrochlorothiazide (HCTZ) **(Figure 1)** is chemically 6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7- sulphonamide. It is a diuretic drug of the thiazide class that acts by inhibiting the kidneys' ability to retain water. This reduces the volume of the blood, decreasing blood return to the heart and thus cardiac output and, by other mechanisms, is believed to lower peripheral vascular resistance. The review of literature revealed that various analytical methods involving Spectrophotometry UV-Visible,[1,2] Ion-pair HPLC,[3] UPLC-MS/MS,[4] HPLC,[5,6] HPTLC[7] have been developed for HCTZ in pharmaceutical dosage forms and biological fluids individually or in combination with other drugs.

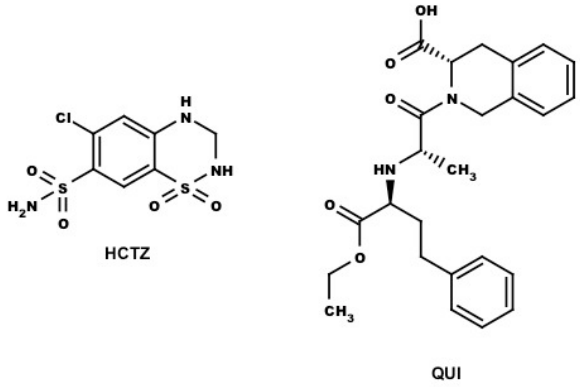


Figure 1. Structure of Hydrochlorthiazide (HCTZ) and Quinapril (QUI)

To the best of our knowledge, there are two methods reported by RP-HPLC for this combination.[8,9] So, the present paper describes a simple, accurate and precise method for simultaneous estimation QUI and HCTZ in combined pharmaceutical formulation by RP-HPLC method. The developed method was validated in accordance with ICH Guidelines.[10,11] The developed method has been successfully employed for the assay of QUI and HCTZ in their combined dosage form.

2. Result and Discussion

2.1. Method Development

2.1.1. Detection of wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Drug solution containing 20 µg mL^{-1} of QUI and 12.5 µg mL^{-1} of HCTZ were prepared separately in 100 mL volumetric flask and made up the volume with methanol. The above solutions were scanned from 400 nm to 190 nm and their spectra were overlaid. The wavelength selected was 210 nm as both the drugs showed significant absorbance at this wavelength (**Figure 2**).

Submitted on: Mar 14, 2014
Revised on: Apr 19, 2014
Accepted on: Apr 19, 2014
*corresponding author: Initial Tel: +91-9443367700; E-mail: anands17@gmail.com

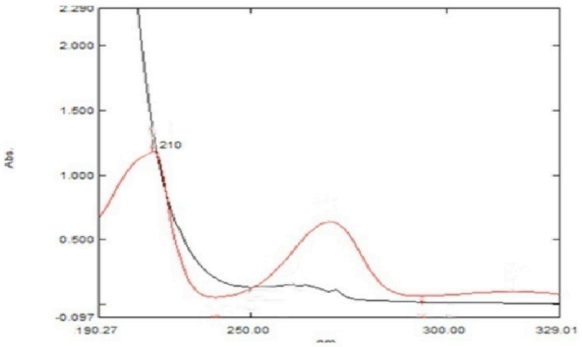


Figure 2. Overlaid spectra of HCTZ (—) and QUI (—)

2.1.2. Preparation of standard stock solution for QUI and HCTZ

Standard stock solution of pure drugs were prepared separately by dissolving 20 mg of QUI with 7 mL diluent into a 10 mL clean dry volumetric flask and make up to 10 mL with mobile phase [(ACN:PB$^{4.5}$)$_{35:65}$]. Pipette out 1 mL and make up to 10 mL to get a final concentration of 200 µg mL^{-1} (stock solution A). Dissolve 12.5 mg of HCTZ with 7 mL diluent into a 10 mL clean dry volumetric flask and made up to 10 mL (1250 µg mL^{-1}) with mobile phase (name). Pipette out 1 mL (1250 µg mL^{-1}) solution and dilute to 10 mL to get a final concentration of 125 µg mL^{-1} (stock solution B). A series of standard solution of HCTZ from stock solution A and QUI from stock solution B were prepared to obtain the concentration of 31.25 - 187.5 µg mL^{-1} and 50-300 µg mL^{-1}, respectively (**Figure 3**).

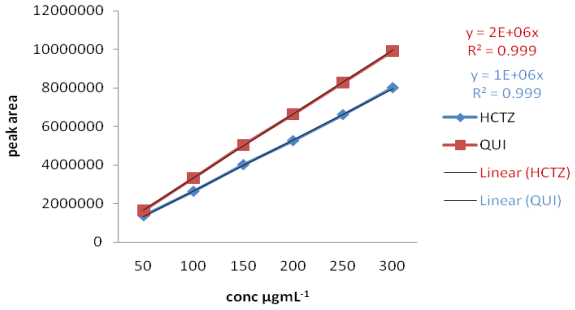


Figure 3. Calibration curve of HCTZ and QUI

2.1.3. Preparation of standard solution containing mixture of QUI and HCTZ

Pipette out 1 mL each from stock solution A of QUI and stock solution B of HCTZ in to a 10 mL volumetric flask and made up to volume with mobile phase [(ACN:PB$^{4.5}$)$_{35:65}$] to get a mixed standard solution (QH$_s$) containing 20 µg mL^{-1} of QUI and 12.5 µg mL^{-1} of HCTZ as final concentration.

2.1.4. Preparation of test solution containing mixture of QUI and HCTZ

Twenty tablets were weighed and crushed. An accurately weighed quantity of powder equivalent to 50 mg of QUI was transferred to 10 mL volumetric flask and dissolved in 7 mL of diluent. It was then sonicated for 25 min, further the volume was made up with mobile phase [(ACN:PB$^{4.5}$)$_{35:65}$] and filtered. From the filtered solution 2 mL was pipetted out into a 10 mL volumetric flask and was made up to 10 mL with mobile phase [(ACN:PB$^{4.5}$)$_{35:65}$] to get test solution (QH$_t$).

2.1.5. Chromatographic method for the determination of QUI and HCTZ

With optimized chromatographic condition a steady base line was recorded with mobile phase [(ACN:PB$^{4.5}$)$_{35:65}$] that was followed by the analysis of the sample solutions (QH$_s$ and QH$_t$). A 10 µL quantity of sample solution was injected and the chromatogram was recorded in triplicate. The retention times of HCTZ and QUI in QH$_s$ and QH$_t$ were found to be 2.097, 5.541 and 2.099, 5.537 min, respectively (**Figure 4**). Content of HCTZ and QUI in tablet was calculated by comparing mean peak area of sample with that of the standard. Concentrations of both drugs were calculated. Representative chromatogram of the test was shown in **Table 1**.

Table 1. Analysis of marketed formulations

Commercial formulation	Ingredients	Label amount (mg)	Amount present (mg)	% purity
Accupril -H	QUI	20	19.8	98%
	HCTZ	12.5	12.25	97%
D 19	QUI	20	19.5	97.5%
	HCTZ	12.5	12.05	96.4%

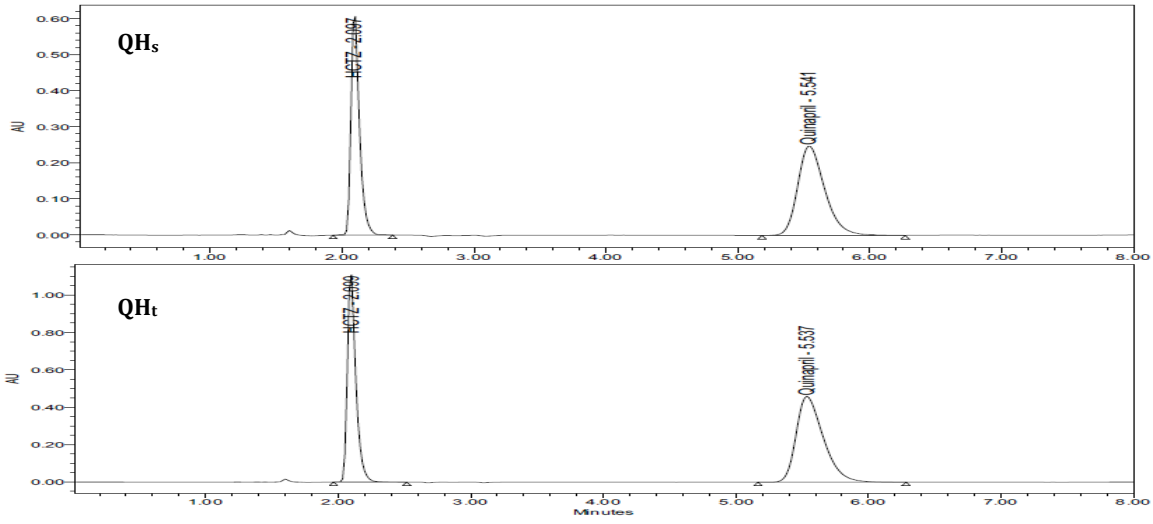


Figure 4. Chromatogram of samples containing mixture of HCTZ and QUI in QH$_s$ and QH$_t$

2.2. Method Validation

2.2.1. Linearity and Range

The linearity of the method was determined at five concentration levels ranging from 31.25-187.50 µg mL^{-1} for HCTZ and 50-300 µg mL^{-1} for QUI. The calibration curve was constructed by plotting response factor against concentration of drugs.

2.2.2. Accuracy

To ascertain the accuracy of the proposed method recovery studies were carried out by standard addition method, adding known amount of each drug to the pre analyzed tablet at three levels 50%, 100% and 150% of the label claim. Recovery studies were carried out in triplicate and the percentage recovery and standard deviations, which are within acceptance limits as shown in **Table 2**.

Table 2. Accuracy studies

Amount Spiked	Amount added[a] (Amount recovered)[a]		% Estimated[b] (%RSD)	
	HCTZ	QUI	HCTZ	QUI
50%	6.25 (6.22)	10 (10.35)	99.72±0.73 (0.73)	100.92±0.6 (0.65)
100%	12.5 (12.6)	20 (20.17)	100.82±0.42 (0.42)	100.82±0.47 (0.47)
150%	18.75 (18.89)	30 (30.33)	100.81±0.61 (0.61)	101.11±0.12 (0.12)

[a]Values are in mg; [b]Mean±SD of triplicate

2.2.3. Precision

Precision was the measure of the degree of repeatability of an analytical method under normal operation and it was normally expressed as the relative standard deviation for a statistically number of samples. Precision should be performed at three different levels: repeatability, intermediate precision and reproducibility, six repeated injections of standard and sample solutions were made and the response factor of drug peaks and %RSD. This shows that the percentage RSD is not more than 2% the chromatogram as shown in **Table 3 & 4**.

Table 3. Method of precision

S.NO	HCTZ		QUI	
	Rt (min)	Peak area (n=3)	Rt (min)	Peak area (n=3)
1	2.099	4939962	5.463	6461393
2	2.101	4939495	5.487	6565316
3	2.105	4979099	5.490	6442481
Mean		4952852		6489730
SD		22731.77		66138.86
%RSD		0.05		0.21

Table 4. Intermediate precision

Parameter	Inj	HCTZ		QUI	
		Rt (min)	AUC (n=3)	Rt (min)	AUC (n=3)
Intraday Precision Analyst-1	1	2.079	4947356	5.481	6349954
	2	2.083	4936963	5.484	6364801
	3	2.084	4962116	5.501	6353063
	Mean		4948812		6355939
	%R.S.D		0.26		0.12
Interday Precision Analyst-2	1	2.084	4942883	5.504	6363923
	2	2.090	4933657	5.521	6344797
	3	2.093	4953878	5.577	6356320
	Mean		4943473		6355013
	%R.S.D		0.20		0.15

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

LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. LOD and LOQ for both the drugs were calculated by using the values of slopes and intercepts of the calibration curves as shown in **Table 5**.

Table 5. Limit of detection and quantification

Contents	HCTZ	QUI
LOD (S/N)	3	3.1
LOQ (S/N)	9.8	9.1

2.2.5. Robustness

Robustness of the proposed method was ascertained by deliberately changing the chromatographic conditions such as change in flow rate of the mobile phase (±0.1 mL min^{-1}), change in composition of the mobile phase. Effect of change in chromatographic parameters on resolution and tailing factor of peak was studied. The condition with variation and their result were shown in **Table 6**.

Table 6. Robustness studies

	Flow rate (mL^{-1}) Peak Area			
	HCTZ		QUI	
	1.2 (mL)	1.4 (mL)	1.2 (mL)	1.4 (mL)
	4951359	5004839	5681577	6457829
	4985132	4995256	5736681	6451940
	4968245	5000048	5709129	6454885
Mean	4968245	5000048	5709129	6454885
SD	16886.5	4791.5	27552	2944.5
%RSD	0.34	0.10	0.48	0.05
	Mobile phase Peak Area			
	HCTZ		QUI	
	30 : 70	30 : 70	35 : 65	35 : 65
	5004839	4997275	6457829	6480049
	4995256	4998272	6451940	6480896
	5000048	4997773	6454885	6480472
Mean	5000048	4997773	6454885	6480472
SD	4791.5	498.5	2944.5	423.5
%RSD	0.10	0.01	0.05	0.01

2.2.6. System suitability studies

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall within ±2% standard deviation range during routine performance of the method the chromatogram as shown in **Table 7** & **Figure 5**.

Table 7. System suitability parameters

S. No.	Parameters	HCTZ	QUI
1	Theoretical plates	5136	3516
2	Symmetric factor	1.5	1.7
3	Resolution	6.9	6.3
4	Tailing factor	1.02	1.29

3. Experimental

Materials and methods: Standard samples of QUI and HCTZ were provided as a gift samples from Pfizer Limited, Mumbai. The marketed formulation ACCUPRIL-H & D-19 tablets containing 20 mg and 12.5 mg of QUI and HCTZ, respectively were procured from local market. HPLC grade water and acetonitrile were

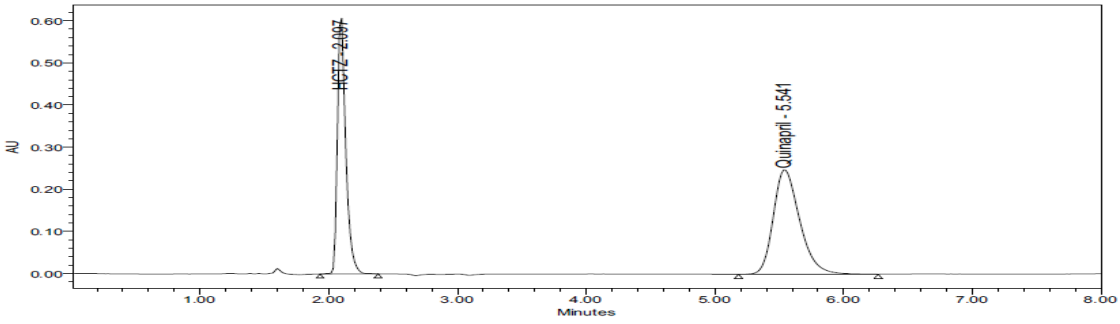


Figure 5. System suitability study for HCTZ and QUI

purchased from Merck, India. Weighing of samples was performed on Secura 224-1S Analytical balance (Sartorius, USA). Degassing of mobile phase using Branson 1510MTH Ultrasonic Cleaner (Balkowitsch Enterprises Inc., USA). Mobile phase pH was checked and adjusted using pH-meter. All chromatographic analyses were performed on HP Agilent 1100 (Agilent Technologies, USA) machine with Zorbax Eclipse XBD column (Agilent Tehnologies, USA). Empower 3 CDS (Waters, USA) has been used to plot chromatographic data. Excel 2007 (Mirosoft Office 2007, Microsoft) was used for plotting calibration curves.

3.1. Method development

3.1.1. Chromatographic conditions

Flow rate: 0.9 mL min^{-1}
Column : Zorbax Eclipse XDB, C18, 150 x 4.6 mm, 5µm.
Detector wave length: 210 nm
Column temperature: 30 °C
Injection volume: 10 µL
Run time: 10 min
Mobile phase (diluent): $[(ACN:PB^{4.5})_{35:65}]$

3.1.2. Preparation of buffer ($PB^{4.5}$)

Weigh accurately about 2.72 gm of Potassium dihydrogen orthophosphate (KH_2PO_4) and dissolved in 900 mL of milli-Q water in a 1000 mL beaker, sonicated and pH 4.5 was adjusted with orthophosporic acid and finally make up to the volume.

3.1.3. Preparation of mobile phase [$(ACN:PB^{4.5})_{35:65}$]

A 65 mL of potassium dihydrogen orthophosphate buffer of pH 4.5 and 35 mL of acetonitrile was taken into 100 mL volumetric flask, sonicated for 25 min and filtered.

3.2. Method validation

3.2.1. Specificity

It is evaluated by injecting the blank, placebo and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of QUI and HCTZ.

3.2.2. Linearity and Range

QUI and HCTZ standard stock solution was transferred to volumetric flask of 10 mL capacity. The volume was adjusted to the mark with methanol to give solutions containing 31.25 to 187.5 µg mL^{-1} HCTZ and 50 to 300 µg mL^{-1} QUI. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

3.2.3. Method precision

The repeatability was evaluated by assaying the sample solution for 6 times. Different concentration of QUI (31.25, 62.5, 93.75 µg mL^{-1}) and HCTZ (50, 100, 150 µg mL^{-1}) were used for the estimation of intraday and interday precision.

3.2.4. Accuracy

Accuracy was assessed by determination of the recovery of the method by addition of standard drug to the pre-quantified placebo preparation at 3 different concentration levels 50%, 100% and 150%, taking into consideration percentage purity of added bulk drug samples. Each concentration was analyzed 3 times and average recoveries were measured.

3.2.5. Intermediate precision (Ruggedness)

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e, different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (*i.e.* from laboratory to laboratory, from analyst to analyst). Acceptance criteria for ruggedness, the %RSD for the area of five standard injections should not be more than 2%.

3.2.6. Robustness

As part of robustness, deliberate change in the flow rate and mobile phase composition was made to evaluate the impact on the method. The mixed standard solution is injected in two replicates and %RSD was calculated.

3.2.7. LOD and LOQ

LOD is the smallest concentration of the analyte that gives measurable response (signal to noise ratio of 3). The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10).

Abbreviations

ACN-Acetonitrile; **HCTZ**-Hydrochlorthiazide; **PB**-Phosphate buffer; **QUI**-Quinapril

Acknowledgement

The authors are very much thankful to the management and faculty of Swamy Vivekanandha College of Pharmacy, Elayampalayam for providing support to carry out this work.

References

1. Khan, S.; Kulkarni, S.; Biyani, K.; Khan, B., Development and Validation of A UV Spectrophotometric Method for the Simultaneous Determination of Quinapril Hydrochloride and Hydrochlorothiazide in Tablet Dosage Form. *Int J Res Pharm Biomed Sci* 2013, 4, 795-801.
2. Patel, R.; Patel, J., Simultaneous Equation Spectrophotometric Method for the Estimation of Nebivolol HCL and Hydrochlorthiazide in their Combined Dosage Form. *Int J Pharm App Sci* 2011, 2, 6-10.

3. Gandhimathi, M.; Ravi, T., Ion pair-HPLC method for the simultaneous estimation of quinapril and hydrochlorothiazide in tablets. *Indian J Pharm Sci* 2009, 71, 311-3.
4. Dasandi, B.; Shah, S.; Shivprakash, Determination of quinapril and quinaprilat in human plasma by ultraperformance liquid chromatography–electrospray ionization mass spectrometry. *Biomed Chromatogr* 2009, 23, 492-8.
5. Abbara, C.; Aymard, G.; Hinh, S.; Diquet, B., Simultaneous determination of quinapril and its active metabolite quinaprilat in human plasma using high-performance liquid chromatography with ultraviolet detection. *J Chromatogr B* 2002, 766, 199-207.
6. Kugler, A. R.; Olson, S. C.; Smith, D. E., Determination of quinapril and quinaprilat by high-performance liquid chromatography with radiochemical detection, coupled to liquid scintillation counting spectrometry. *J Chromatogr B* 1995, 666, 360-7.
7. Bhavar, G.; Chatpalliwar, V.; Patil, D.; Surana, S., Validated HPTLC method for simultaneous determination of quinapril hydrochloride and hydrochlorothiazide in a tablet dosage form. *Indian J Pharm Sci* 2008, 70, 529-31.
8. Jaiswal, R.; Patel, P., Development and Validation of RP-HPLC method for simultaneous estimation of Quinapril HCl and Hydrochlorthiazide in combined pharmaceutical formulation. *Int J Pharm Res Dev* 2013, 5, 45-56.
9. Sujita Rani, G.; Mohan Gandhi, B.; G, M. K.; Rekha, M.; Pullam Raju, K.; Manjusha, N., A New RP-HPLC method for simultaneous estimation of Quinapril and Hydrochlorthiazide in pharmaceutical dosage forms. *World J Pharm Pharm Sci* 2013, 2, 6220-34.
10. ICH guidelines Q2R1, Validation of Analytical procedures: Text and Methodology. In *International Conference on Harmonisation*, London, UK, 2005.
11. ICH Guidelines Q2A, Validation of Analytical Procedures: Definition and Terminology (CPMP III/5626/94). In *International Conference on Harmonisation*, Geneva, Switzerland, 1995.

Journal of Pharmaceutical Chemistry
http://www.vensel.org/index.php/jphchem
VENSEL

Effect of viscosity of hydrophilic coating polymer on lag time of atenolol pulsatile press coated tablets

[1,*]Rajesh A. Keraliya; [2]Madhabhai M. Patel

[1]Kalol Institute of Pharmacy, Department of Pharmaceutics, Kalol–382721, Gujarat, India
[2]Shankarsingh Vaghela Bapu College of Pharmacy, Department of Pharmaceutics, Vasan-382650, Gujarat, India

Abstract
A method for the development of press coated tablet of atenolol for pulsatile delivery was investigated for chronotherapy of hypertension. Effect of viscosity of Hydroxypropylcellulose (HPC) on pulsatile release of atenlol was studied by press coating atenolol core tablet using different viscosity grade HPC and varying coat weight. L-HPC, M-HPC and H-HPC viscosity garde with 75, 100 and 150 mg coat weight were press coated over atenolol core tablets to delay release of atenolol. The batches, HP1-HP9, exhibited an increase in lag time in response to increase in viscosity and coat weight. Two of the batches HP5 and HP7 have shown a burst release of atenolol after 6.5 and 6.0 h lag time respectively, which is suitable for pulsatile drug delivery of atenolol for chronotherapy of hypertension.

Keywords: Atenolol; chronotherapy; pulsatile release; press coating; hydroxypropylcellulose; hypertension

1. Introduction

Chronobiology is the study of biological rhythms and their mechanisms.[1] Every biological rhythm has a periodicity of about 24 h. It is well known that circadian rhythms influence disease processes and physiological events. In case of cardiovascular diseases, several functions (e.g. blood pressure, heart rate, stroke volume, cardiac output, blood flow) of the cardiovascular system (CVS) are subject to circadian rhythms. It has been reported that more shocks and heart attacks occur during morning hours.[2] The level of cortisol is higher in the morning hours, and its release is reported to decline gradually during the day.[3] Capillary resistance and vascular reactivity are higher in the morning and decreases latter in the day. Thus, Adjusting the administration of drug at an appropriate timing as predicted by circadian rhythm of disease could be advantageous in CVS disease control.[4] An emerging discipline, Chronotherapeutics is concerned with delivery of the drugs to acheive maximum concentration at the time of onset of symptoms that is predicted based on inherent activity of disease over a period of time.[5] In order to achieve the chronopharmaceutical design for the time controlled pulsatile type of colon targeted preparations, a formulation design should consider controlling the lag time is prior to the immediate release of drug.

Atenolol, a β-blocker, is prescribed widely in diverse cardiovascular diseases like hypertension, angina pectoris, arrhythmias and myocardial infarction. An oral administration of colon targeted pulsatile delivery of atenolol at bed time releasesing the drug after a desired lag time of about 6–6.5 h could be effective in controlling hypertension due to peak levels of cortisol, capillary resistance, platelet agreeability and vascular reactivity in the morning hours. The presented study investigates the effect of hydroxypropyl cellulose (HPC) on lag time of press coated pulsatile formulation of atenolol.

It was postulated that when the barrier layer was exposed to dissolution media, the HPC particles swell and erode, a process which was depends upon the viscosity grades and quantity of HPC, demonstrating that manipulation of both controls the lag time.[5] HPC has an ability to swell upon gellification once in contact with water. The gel becomes a viscous layer around a core, acting as a protective barrier to both the influx of water and the efflux of the drug in solution.[6] The HPC gel layer delays the atenolol release because of lengthening of the drug diffusion pathway. The rupturing or breaking time of the outer coat greatly depend upon the viscosity and coat weight of HPC.[7] Thus, in the present investigation, an attempt has been made to study the effect of viscosity and coat weight of HPC on lag time of time controlled pulsatile release tablets of atenolol.

2. Results and discussion

2.1. Spectrophotometric estimation of atenolol

Atenolol exhibited its maximum absorption at 225 nm and obeyed Beer's law with linear response in the range of 2.5-35 µg/mL. Calibration curves for atenolol in different solvents (methanol, 0.1 *N* HCl, phosphate buffer pH 6.8 and pH 7.4) were shown in **Figure 1**. All the curves have shown a linear regression of absorbance with regression coefficient near to 1.

Submitted on: Mar 06, 2014
Revised on: Apr 12, 2014
Accepted on: Apr 13, 2014

*corresponding author: Initial Tel: +91-9067036717; E-mail: pharmarx19@gmail.com

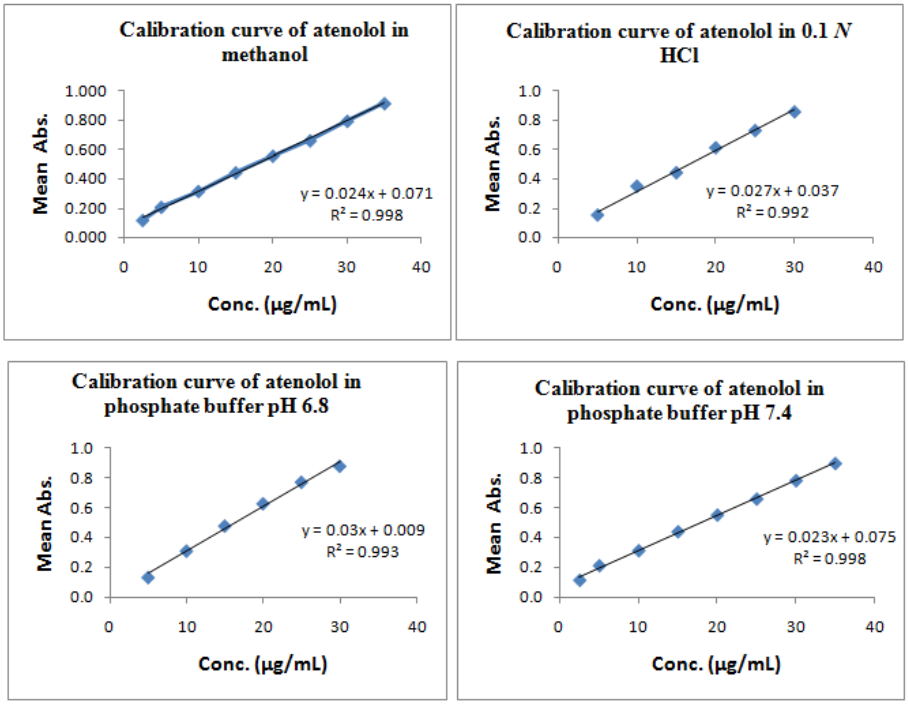


Figure 1: Calibration curves of atenolol in different solvent

2.2. Drug-Excipient compatibility study

2.2.1. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy was used to analyze the pure sample powder of atenolol and powder of atenolol core tablet. FTIR spectra of these samples were shown in **Figure 2**. They were identical and the absorption bands due to functional groups of atenolol were present in all the spectra. Absorption band due to N-H stretch of $CO\text{-}NH_2$ group appeared at 3340 cm^{-1} and 3160 cm^{-1} in the both the spectra. The absorption band due to -C=O (amide I) and –N-C=O (amide II) stretch were located at 1625 cm^{-1} and 1500 cm^{-1} in spectra of both the samples and there was no shift in its position, a clear indication of drug-excipient compatibility. Other peaks due to =C-H stretching (2940 cm^{-1}), $-CH(CH_3)_2$ (1383 cm^{-1} & 1170 cm^{-1}) and Ar-O-C (1390 cm^{-1} & 1235 cm^{-1}) were also present in both the spectra.[8] The FTIR spectra of the tested samples have shown the prominent characterizing peaks of pure atenolol confirming no chemical modification due to any drug-excipient interaction at the molecular level.

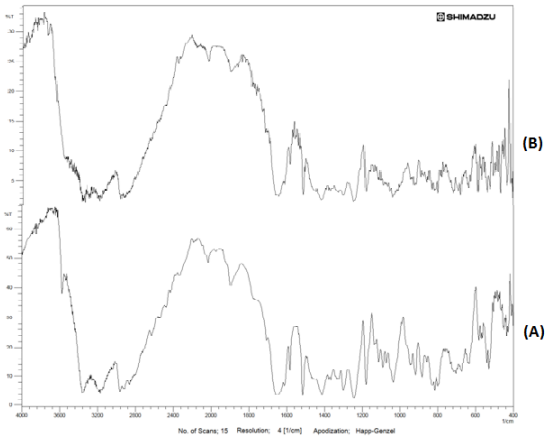


Figure 2. FTIR spectra of: pure sample powder of atenolol (A) and powder of atenolol core tablet (B)

2.2.2. Differential Scanning Calorimetry (DSC)

DSC thermographs of pure sample powder of atenolol and powder of atenolol core tablet has shown a sharp exothermic peak (Tm) at 156.11 °C and 156.21 °C respectively, which corresponding to melting point of atenolol (154 °C to 156 °C)**.8, 9** Melting exotherm not appreciably change in powder of atenolol core tablet as compared to atenolol pure sample. This observation confirmed the absence of any chemical interaction of drug with excipients of core tablet, further supporting the results of IR spectroscopy. The DSC results of pure sample powder of atenolol and powder of atenolol core tablet were shown in **Figure 3**.

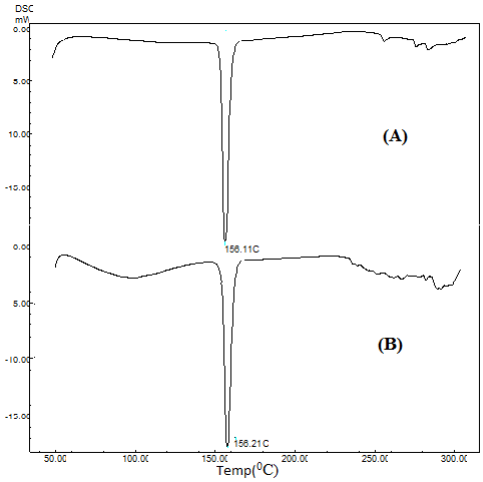


Figure 3. DSC thermographs of: pure sample powder of atenolol (A) and powder of atenolol core tablet (B)

2.3. Flow property study of powder blend

The bulk density, tapped density, angle of repose, hausner's ratio and carr's index of powder blend for atenolol core tablets are 0.280±0.03, 0.33±0.04, 29.74°±0.40, 1.178±0.12 and 15.15±0.65 respectively (**Table 1**). The results indicated that the powder blend

has good flow property and compressibility compared with pure sample powder of atenolol and suitable for direct compression method.

The angle of repose, hausner's ratio and carr's index of powder blend used for coating of core tablets were ranged from 28.72°±0.54 to 29.74°±0.25, 1.15±0.15 to 1.171±0.12 and 13.04±0.43 to 14.63±0.35 respectively as shown in **Table 1**. The values of pre-compression parameters indicated a good free flowing property and suitable for direct compression method.

Table 1. Flow property study for powder blends of various grades of HPC

Formulation Code	BD[a] (g/cm^3)	TD[a] (g/cm^3)	CI (%)[a]	HR (H_R) [a]	AoR (Θ) [a]
AT2	0.28 ±0.03	0.33 ±0.04	15.15 ±0.65	1.18 ±0.12	29.74° ±0.40
L - HPC	0.35 ±0.05	0.41 ±0.04	14.63 ±0.35	1.17 ±0.12	29.74° ±0.25
M –HPC	0.40 ±0.04	0.46 ±0.03	13.04 ±0.43	1.15 ±0.15	28.72° ±0.54
H - HPC	0.39 ±0.03	0.45 ±0.05	14.44 ±0.37	1.17 ±0.16	29.39° ±0.47

[a](mean± SD); BD-Bulk Density; TD-Tapped Density; CI-Carr's Index; HR-Hausner's Index; AoR-Angle of Repose

2.4. Post compression study of core tablets and press coated tablets

The data obtained from post-compression study of core and press coated tablets such as weight variation, hardness, friability, and drug content are shown in **Table 2**. The hardness of core tablets of atenolol was 3.25±0.15, indicated that core tablets had good crushing strength. The friability, drug content, weight variation and thickness of atenolol core tablets were 0.12±0.05%, 99.97±1.3%, 79.57±2.57 and 3.2±0.04 respectively, which indicated that atenolol core tablets passed the post compression study. Atenolol core tablets have shown disintegration time of 28±1.52 sec, which is important parameter for burst release after a 6 h lag time.

Table 2. Post-compression parameters of rupturable and erodible type press coated tablets

Formulation Code	H[a] (kg/cm^2)	F[a] (%)	DC[a] (%)	WV[a] (mg)	TT[a] (mm)
AT2	3.25 ±0.15	0.12 ±0.05	99.97 ±1.3	79.57 ±2.57	3.2 ±0.04
HP1	4.0 ±0.35	0.17 ±0.06	99.80 ±1.3	155.67 ±1.45	4.0 ±0.04
HP2	5.5 ±0.5	0.12 ±0.06	99.57 ±1.5	188.40 ±2.95	5.0 ±0.05
HP3	6.5 ±0.3	0.08 ±0.05	100.95 ±1.2	228.35 ±2.25	5.9 ±0.04
HP4	4.0 ±0.4	0.15 ±0.08	99.15 ±1.8	154.25 ±2.57	4.0 ±0.02
HP5	6.0 ±0.6	0.11 ±0.05	100.65 ±1.6	189.50 ±2.50	5.0 ±0.05
HP6	7.0 ±0.4	0.06 ±0.07	99.84 ±1.4	229.26 ±2.76	5.9 ±0.04
HP7	4.5 ±0.5	0.12 ±0.04	100.54 ±1.2	153.8 ±3.15	4.0 ±0.03
HP8	6.0 ±0.3	0.09 ±0.08	99.75 ±1.5	187.75 ±1.55	5.0 ±0.04
HP9	7.5 ±0.45	0.06 ±0.05	99.45 ±1.3	230.67 ±2.20	6.0 ±0.03

[a](mean± SD); H-Hardness; F-Friability; DC-Drug Content; Weight Variation; Tablet Thickness

In all press coated formulations, the hardness test indicated good mechanical strength. Hardness has ranged from 4.0-7.5 kg/cm^2. Friability was ranged from 0.06±0.05 to 0.17±0.06. Friability is less than 1% which indicated that tablets had good mechanical resistance. Drug content was found to be high (>99.15%). It was ranged from 99.15±1.8 to 100.95±1.2% and uniform in all tablet formulations. In weight variation test, twenty tablets were selected randomly and average weight was calculated. Then individual tablet was weighed and was compared with average weight. None of tablets showed more than 7.5% weight variation from average weight. So, all formulations pass the weight variation test as per Indian Pharmacopoeia, 2007. Tablet thickness varied from 4.0 to 6.0 mm.

2.4.1. Swelling study

Table 3 describes the percentage swelling ratio of different batches of press coated tablets (HP1-HP9). The study indicated that as the amount of HPC increases, the swelling of tablet increases. The batches from HP1-HP3 have shown complete disintegration within 0-1.5 h due to low viscosity of L-HPC.[5] But, as viscosity of HPC increases to medium level, it did not show any disintegration but started to show swelling. The percentage swelling of M-HPC coated tablets (HP4-HP6 batches) increases as the coat weight of M-HPC increases from 91.86±5.85 to 168.39±4.87. The higher viscosity grade of HPC (H-HPC) has shown high percentage of swelling (227.45±4.34%) with high coat weight.

Table 3. Lag time and % swelling index of atenolol press coated tablets

Formulation Code	Lag Time (h)	% Swelling Index
HP1	0	Disintegrated
HP2	1	Disintegrated
HP3	1.5	Disintegrated
HP4	5	91.86±5.85
HP5	6.5	135.64±6.57
HP6	8.5	168.39±4.87
HP7	6	127.69±3.96
HP8	8.5	179.37±5.53
HP9	11	227.45±4.34

2.4.2. *In vitro* dissolution study of atenolol press coated tablets

In time controlled press coated tablets, different batches (HP1-HP9) have shown a variable lag time depending on the concentration and viscosity grade of HPC in the outer coating layer. The press coated tablets showed a lag time before the drug release because the atenolol core tablets were completely surrounded by the polymer layer that prevented the release of drug from the core tablets. Burst release after a specific lag time occurred due to swelling and erosion of the outer hydrophilic HPC layer. When the polymer layer swelled adequately, it allowed sufficient dissolution medium to enter into it and reach the core tablet. The superdisintegrant in the core swelled extensively which exerts a pressure on the outer layer resulting in burst release of the drug.[10]

Figure 4 shows the dissolution profile of various press coated tablet batches (HP1-HP9). Press coated tablets (HP1-HP9) exhibited distinct lag time as given **Table 3**. It is possible to obtain lag time of 1-11 h using different HPC viscosity grades and coating weight (**Table 4**). The swelling and subsequent erosion of outer coating layer of HPC determines the lag time of atenolol press coated tablets. Formulations HP1-HP9 have shown an increase in lag time with increase in weight ratio of HPC because upon contact with dissolution medium HPC forms a gel like structure that delays the release of drug depending

on quantity of gel layer. The lag time should be longer with increase in HPC viscosity, because the dissolution rate or erosion rate of the HPC polymer would be delayed as molecular weight of HPC polymer increases (Shinde and Mayee, 2012).[11]

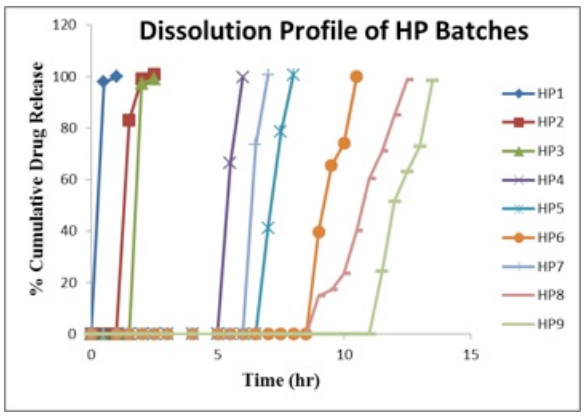


Figure 4. Dissolution profile of atenolol press coated tablets

Table 4. Formulations of press coated tablets of atenolol

Formulation code	Core tablet	Grade of HPC	Coat Weight (mg)
HP1	A2	L-HPC	75
HP2	A2	L-HPC	100
HP3	A2	L-HPC	150
HP4	A2	M-HPC	75
HP5	A2	M-HPC	100
HP6	A2	M-HPC	150
HP7	A2	H-HPC	75
HP8	A2	H-HPC	100
HP9	A2	H-HPC	150

The dissolution profile of batche HP1 has shown the rapid drug release within 30 min as 75 mg coat weight of L-HPC is insufficient to retard the release of atenolol. The dissolution study of batches H2 and H3 as shown in **Figure 4** revealed that even at higher concentrations of L-HPC, the drug release was retarded only for about 1-1.5 h respectively, as low viscosity grade of HPC was not found suitable. Moreover L-HPC is a disintegrant and had been used to cause rapid disintegration of tablets.[5] Thus, a higher viscosity grade of HPC was used in further trials.

The lag time of drug release was delayed with the increase of the viscosity grade and coat weight of HPC and the release rate was also decreased. The dissolution profile of batches H4-H6 have shown the lag time of 5, 6.5 and 8.5 h respectively. As shown in **Figure 4**, the higher viscosity grade of HPC (M-HPC) was found to be sufficient to design the desired lag time to release the drug at the scheduled time and then release most of the drug within a short time period in the colon. Formulation HP5 exhibited the lag time of 6.5 h, after that it released drug by bursting effect as pressure generated in core tablet is enough to break the outer coating layer of M-HPC after some erosion of outer gel layer of M-HPC.[11] It has also shown that lag time decreases with coat weight of M-HPC. M-HPC swells due to absorption of water, which delayed the release of drug, thus as the coat weight of M-HPC was increased, lag time increases. It showed that Formulation HP6 having coat weight of M-HPC at 150 mg, showed lag time of 8.5 h.

If a higher viscosity grade of HPC (H-HPC) was used to prepare press coated atenolol tablet, than formulation HP7 having lower coat weight of H-HPC (75 mg), is sufficient to achieve desired lag time of 6 h. But, formulations HP8 and HP9 containing higher coat weight of H-HPC was not suitable for this purpose, as it showed the delayed lag time of 8.5 and 11 h respectively due to formation of stable gel layer of H-HPC around atenolol core tablets for a long period of time. As shown in **Figure 4**, dissolution profile of formulation HP8 and HP9 showed the initial slow drug release prior breakage of gel layer of H-HPC, because as dissolution medium penetrate into the coating layer and it hydrates the core, but due to tight gelled structure of H-HPC around core tablet, some drug is eject out through gel layer by diffusion mechanism. When internal pressure was build inside the core is enough to break the gel layer of H-HPC, rapid drug release was observed at later stage.[12]

Results of *in vitro* dissolution study of atenolol press coated tablets were shown in **Table 5**. The two batches HP5 and HP7 have achieved a burst release after 6.5 and 6 h lag time respectively. Thus, the dosage forms can be taken at bedtime, so, that the content will be released in the morning hours, i.e., at the time of symptoms. The release of drug was rapid and complete after the lag time. Lag time was greatly affected by viscosity and coat weight of HPC.

3. Experimental

Materials and methods: Atenolol was obtained as a gift sample from Zydus Cadila Healthcare Ltd., Ahmedabad, India. Sodium starch glycolate IP was obtained as a gift sample from Maruti Chemicals, Ahmedabad, India. L-HPC, M-HPC, H-HPC were received as a gift sample from Nippon Soda Co. Ltd., Tokyo, Japan. Polyvinyl pyrollidone K 30 (PVP) (S. D. Fine Chemicals Ltd., Mumbai, India); microcrystalline cellulose, talcum powder, magnesium stearate and sodium hydroxide (Chemdyes Corporation, Rajkot, India); potassium dihydrogen phosphate (Merck Specialities Pvt. Ltd., Mumbai, India) and methanol (Ranbaxy Fine Chemicals Ltd., New Delhi, India) were purchased from respective vendors.

3.1. Evaluation of flow property of powder blends

Powder blends used for preparation of atenolol core tablets and press coated tablets were evaluated for flow property by measuring bulk density, tapped density, carr's index, hausner's ratio and angle of repose.

3.2. Preparation of atenolol core tablets

The core tablets of atenolol (A2) were prepared by direct compression method. An optimized core tablet was formulated using various concentrations of dry binder and super disintegrant as describe in **Table 6**. An accurately weighed quantity of atenolol, microcrystalline cellulose, polyvinyl pyrroloide (PVP K30) and sodium starch glycolate were passed through 40# sieve and mixed in a double cone blender for 15 min. Talc (2% w/w, 40#) was added into the blend and mixed for 10 min. that was followed by magnesium stearate (1% w/w, 40#) and continued for another 5 min. The resultant powder mixture was compressed into tablets (average tablet weight=80 mg) by 6 mm standard concave plain punches using rotary tableting machine (Hardik Engineering Works, Ahmedabad, India) and compression force was controlled to produce more than 3±0.5 kg/cm^2 tablet hardness. The prepared

Table 5. % cumulative drug release study of atenolol press coated tablets with HPC

Time (h)	HP1	HP2	HP3	HP4	HP5	HP6	HP7	HP8	HP9
0	0	0	0	0	0	0	0	0	0
0.5	97.75	0	0	0			0	0	0
1	99.87	0	0	0	0	0	0	0	0
1.5		83.14	0	0	0	0	0	0	0
2		98.96	97.25	0	0	0	0	0	0
2.5		100.84	99.12	0	0	0	0	0	0
3				0	0	0	0	0	0
4				0	0	0	0	0	0
5				0	0	0	0	0	0
5.5				66.43	0	0	0	0	0
6				99.64	0	0	0	0	0
6.5					0	0	73.41	0	0
7					41.25	0	100.42	0	0
7.5					78.46	0		0	0
8					100.48	0		0	0
8.5						0		0	0
9						39.67		14.88	0
9.5						65.35		17.21	0
10						74.00		23.47	0
10.5						99.99		40.37	0
11								60.33	0
11.5								71.08	24.4
12								85.01	51.55
12.5								98.83	63.20
13									72.80
13.5									98.51

atenolol core tablets were tested for weight variation, hardness, thickness, drug content, disintegration time, friability and *in vitro* dissolution study by standard methods.[12-14]

Table 6. Composition of atenolol core tablet

Ingredients	Quantity/tablet (mg)
Atenolol (API)	45 mg
Sodium Starch Glycolate (SSG) (5%)	4 mg
Polyvinyl pyrollidone K30 (PVP K30) (5%)	4 mg
Microcrystalline Cellulose (MCC)	24.6 mg
Talc (2%)	1.6 mg
Mg Stearate (1%)	0.8 mg
Total Weight	80 mg

3.3. Preliminary Trial Batches

As shown in **Table 7** preliminary trials batches of atenolol press coated tablets were prepared randomly using different viscosity grade and coat weight of HPC on trial and error basis to select the desired coat weight.

Table 7. Preliminary trail batches of various grades of HPC

Type of grade	Coat Weight (mg)	Lag time (h)
L-HPC	50	0
L-HPC	200	2
M-HPC	50	0
M-HPC	100	6.5
M-HPC	200	10
H-HPC	50	3
H-HPC	75	6
H-HPC	200	14

3.4. Preparation of atenolol press coated tablets

To study the effect of viscosity on the lag time of press coated colon targeted time release atenolol tablet, the core tablets were press coated with coating material containing various viscosity grades of HPC. The coat weight of various viscosity grades of HPC as decided from the preliminary trial batches (**Table 7**) were used for the compression coating shown in **Table 4**. An accurately weighed quantity of coating material, magnesium stearate (1% w/w) and talc (2% w/w) were passed through sieve no. 22 separately and blended using a mortar and pestle for 10 min. Coating material (43% w/w) was first placed into die cavity (internal diameter 9 mm). Then, the core tablet was carefully placed on it manually at the centre of the die. The remaining coating material (57% w/w) was added into the die and compressed around the core tablet by 9 mm standard concave plain punches using rotary tableting machine (Cadmach Machinery, Ahmedabad, India). Compression force was controlled to produce 5±0.5 kg/cm^2 tablet hardness. The prepared compression coated atenolol tablets were tested for weight variation, hardness, thickness, drug content, friability and *in vitro* dissolution study by standard methods.[15-18]

3.5. Spectrophotometric evaluation of atenolol core and press coated tablets

Atenolol was estimated by UV visible spectroscopy. Calibration curve of atenolol was prepared in different solvents (methanol, 0.1 *N* HCl, phosphate buffer pH 6.8 and phosphate buffer pH 7.4). Accurately weighed 100 mg of atenolol was placed in 100 mL volumetric flask and dissolved in 100 mL of solvent. From this solution, 10 mL solution was withdrawn and further diluted to 100 mL to yield the standard stock solution of atenolol (100 µg/mL). From the stock solution; 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 mL were withdrawn and diluted to 100 mL to yield concentration of 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 µg/mL respectively. Absorbance of each dilution was measured at 225 nm using UV-Visible spectrophotometer (Thermo Scientific Evolution 201). Samples were analyzed in triplicate and the average values were used for plotting the graph of absorbance versus concentration (µg/mL). Regression analysis was done on each beer's plot using Microsoft excel.[8]

3.6. Drug-Excipient compatibility study

3.6.1. Fourier transform infrared spectroscopy

The FTIR spectra of pure sample powder of atenolol and powder of atenolol core tablet were recorded on a FTIR

spectrophotometer (Shimadzu, FTIR-8400S), in the wavelength region of 4000-400 cm^{-1} using KBr with sample concentration of about 1% w/w.

3.6.2. Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (DSC-60, shimadzu corporation, Japan) was used to monitor the thermal events during heating. The DSC was calibrated using indium (156.6±0.2 °C) and zinc (419.5±0.3 °C) standards. Samples of pure sample powder of atenolol and powder of atenolol core tablet weighing 2–3 mg were placed in an open aluminium pans and heated from 50 to 300 °C at a rate of 20 °C per min. Nitrogen was used as a purge gas at a flux rate of 50 mL/min. The onsets of the melting points were recorded using the software, Pyris from Perkin-Elmer.

3.7. Flow property study of powder blend

Flowability of powder blend used for preparation of atenolol core and press coated tablets was evaluated by determining angle of response, carr's consolidation index and hausner's ratio.

3.8. Post compression study of core tablets and press coated tablets

3.8.1. Weight Variation

Twenty tablets from each batch were individually weighed using electronic digital balance (Shimadzu BL-220H) and average weight was calculated. Individual weights of the tablets were compared with the average weight according to the official method in Indian Pharmacopoeia, 2007.[19, 20]

3.8.2. Hardness

Six tablets from each batch were selected and tested for tablet hardness using Monsanto hardness tester. The tablet was placed in contact between the plungers and the handle was pressed, the force of the fracture that causes the tablet to break was recorded.[21]

3.8.3. Thickness

The thickness of ten tablets from each batch was determined using vernier calipers as per Indian Pharmacopoeia, 2007.[22]

3.8.4. Friability (F)

The friability of twenty tablets from each batch was determined using Roche friabilator (Indosati Scientific Lab. Equipments).[23, 24] This device subjects the tablets to the combined effect of abrasions and shock in a plastic chamber revolving at 25 rpm and dropping the tablets at a height of 6 inches in each revolution. A pre-weighed sample (20 tablets) was placed in the friabilator and is subjected to 100 revolutions. Tablets were dedusted and reweighed. The % friability (F) was calculated using the formula given below:

$$F = \left(\frac{W_1 - W_2}{W_1} \right) x100$$

Where, W_1 is the initial weight of twenty tablets before the test and W_2 is the final weight of twenty tablets after the test

3.8.5. Drug content

For determination of drug content, ten tablets were crushed into powder and powder equivalent to 45 mg of atenolol was weighed and dissolved in methanol. It was then filtered through syringe filter (Axiva SFCA25X, 0.45 µm) and the filtrate was analyzed for atenolol content spectrophotometrically using UV-Visible spectrophotometer (Thermo Scientific Evolution 201) at 225 nm with methanol as blank.[25]

3.8.6. Swelling studies

One tablet from each batch of press coated formulation was randomly selected, weighed individually (W_1) and placed separately in petridishes having 8.5 cm diameter containing 20 mL of phosphate buffer (pH 7.4). After 6 h, the tablets were carefully removed from petridishes and excess water was removed using filter paper. The swollen tablets were reweighed (W_2) and swelling index (SI) expressed in percentage for each tablet was calculated using the formula given below.[26, 27]

$$SI = \frac{(W_2 - W_1)}{W_1} x100$$

Where, W_1 is the initial weight of each tablet before test, W2 is the final weight of each tablet after test

3.8.7. Disintegration test

Six tablets from each batch were placed in the glass tube of disintegration test apparatus (Indosati Scientific Lab. Equipments) containing 900 mL of water maintained at a temperature of 37±1 °C and disintegration time of core tablets was determined according to official method in Indian Pharmacopoeia, 2007.[2, 28]

3.8.8. *In vitro* drug release study of atenolol press-coated tablets

In vitro drug release studies were carried out using USP Type II dissolution apparatus (Electrolab, TDT-08L) in a 900 mL of dissolution media at a temperature of 37±1 °C at 100 rpm. In order to simulate the pH changes along the GI tract, multimedia dissolution studies were performed. Three dissolution media with pH 1.2, 6.8 and 7.4 were sequentially used. Initially dissolution study was performed using 0.1 *N* HCl (pH 1.2) as dissolution medium for 2 h (since the average gastric emptying time is 2 h). Then the dissolution medium was replaced with phosphate buffer pH 6.8 and the study was continued for another 3 h (average small intestinal transit time is 3 h). After 3 h, the dissolution medium was again replaced with phosphate buffer pH 7.4 and the study continued for subsequent hours. At regular time intervals, a 10 mL of portion of sample was withdrawn for content analysis and same amount of fresh medium has been replaced. Samples withdrawn were suitably diluted and filtered through syringe filter (Axiva SFCA25X, 0.45µm). The amount of drug released was estimated spectrophotometrically using UV-Visible spectrophotometer (Thermo Scientific Evolution 201) at 225 nm. All studies were carried out in triplicates.[25, 27, 29] The time for which the tablet does not show any release of the drug is known as its lag time. The lag time can be determined from the dissolution profile of the tablet.

Abbreviations

HPC-hydroxy propylcellulose; **L-HPC**-low molecular weight HPC; **M-HPC**-Medium molecular weight HPC; **H-HPC**-high molecular weight HPC; **SI**-swelling index

Acknowledgement

The authors are thankful to Zydus Cadila Healthcare Ltd., Ahmedabad, India and Nippon Soda Co. Ltd., Tokyo, Japan for providing the gift sample for research work.

References

1. Belgamwar, V. S.; Gaikwad, M. V.; Patil, G. B.; Surana, S., Pulsatile drug delivery system. *Asian J Pharm* 2008, 2, 141.

2. Arora, S.; Ali, J.; Ahuja, A.; Baboota, S.; Qureshi, J., Pulsatile Drug Delivery Systems: An Approach for Controlled Drug Delivery. *Indian J Pharm Sci* 2006, 68, 295-300.
3. Lemmer, B., Chronopharmacokinetics: implications for drug treatment. *J Pharm Pharmacol* 1999, 51, 887-90.
4. Youan, B.-B. C., Chronopharmaceutics: gimmick or clinically relevant approach to drug delivery? *J Control Release* 2004, 98, 337-53.
5. Janugade, B.; Patil, S.; Patil, S.; Lade, P., Formulation and evaluation of press-coated Montelukast sodium tablets for pulsatile drug delivery system. *Int J ChemTech Res* 2009, 1, 690-95.
6. Kiil, S.; Dam-Johansen, K., Controlled drug delivery from swellable hydroxypropylmethylcellulose matrices: model-based analysis of observed radial front movements. *J Control Release* 2003, 90, 1-21.
7. Rubinstein, A.; Tirosh, B.; Baluom, M.; Nassar, T.; David, A.; Radai, R.; Gliko-Kabir, I.; Friedman, M., The rationale for peptide drug delivery to the colon and the potential of polymeric carriers as effective tools. *J Control Release* 1997, 46, 59-73.
8. Čaplar, V.; Mikotić-Mihun, Z.; Hofman, H.; Kuftinec, J.; Kajfež, F.; Nagl, A.; Blažević, N., Atenolol. In *Analytical Profiles of Drug Substances*, Klaus, F., Ed. Academic Press: 1984; Vol. Volume 13, pp 1-25.
9. Wander, G. S.; Chhabra, S. T.; Kaur, K., Atenolol Drug Profile. *J Assoc Physicians India* 2009, 57, 13-16.
10. Khadabadi, D.; Chishti, N. H.; Khan, F. M.; Tadvee, A. A., Formulation and evaluation of press coated tablet of Ketoprofen-A chronotherapeutic approach. *Int J Pharm Pharm Sci* 2013, 5, 733-40.
11. Shinde, P.; Mayee, R., Development and Evaluation of Press Coated Tablet of Aceclofenac. *Int J Pharm Innovations* 2012, 2, 6-15.
12. Patel, M. M.; Patel, S. L.; Bhadani, M. N.; Shah, T. J.; Amin, A. F., A synchronous colon-specific drug delivery system for orally administered mesalamine. *Acta Pharma Sci* 2009, 51, 251-60.
13. Sawada, T.; Sako, K.; Fukui, M.; Yokohama, S.; Hayashi, M., A new index, the core erosion ratio, of compression-coated timed-release tablets predicts the bioavailability of acetaminophen. *Int J Pharm* 2003, 265, 55-63.
14. Cheng, G.; An, F.; Zou, M.-J.; Sun, J.; Hao, X.-H.; He, Y.-X., Time-and pH-dependent colon-specific drug delivery for orally administered diclofenac sodium and 5-aminosalicylic acid. *World J Gastroenterol* 2004, 10, 1769-74.
15. Chickpetty, S.; Baswaraj, R., Studies on Design and In Vitro Evaluation of Compression-Coated Delivery Systems for Colon Targeting of Diclofenac Sodium. *Int J PharmTech Res* 2010, 2, 1714-22.
16. Yehia, S. A.; Elshafeey, A. H.; Sayed, I.; Shehata, A. H., Optimization of budesonide compression-coated tablets for colonic delivery. *AAPS PharmSciTech* 2009, 10, 147-57.
17. Sinha, V.; Mittal, B.; Bhutani, K.; Kumria, R., Colonic drug delivery of 5-fluorouracil: an in vitro evaluation. *Int J Pharm* 2004, 269, 101-8.
18. Krishnaiah, Y.; Veer Raju, P.; Dinesh Kumar, B.; Satyanarayana, V.; Karthikeyan, R.; Bhaskar, P., Pharmacokinetic evaluation of guar gum-based colon-targeted drug delivery systems of mebendazole in healthy volunteers. *J Control Release* 2003, 88, 95-103.
19. Fukui, E.; Uemura, K.; Kobayashi, M., Studies on applicability of press-coated tablets using hydroxypropylcellulose (HPC) in the outer shell for timed-release preparations. *J Control Release* 2000, 68, 215-23.
20. Gazzaniga, A.; Iamartino, P.; Maffione, G.; Sangalli, M., Oral delayed-release system for colonic specific delivery. *Int J Pharm* 1994, 108, 77-83.
21. Krishnaiah, Y.; Satyanarayana, S.; Rama Prasad, Y.; Narasimha Rao, S., Evaluation of guar gum as a compression coat for drug targeting to colon. *Int J Pharm* 1998, 171, 137-46.
22. Hariharan, M.; Gupta, V. K., A novel compression-coated tablet dosage form. *Pharmac. Technol* 2001, 14-19.
23. Mastiholimath, V.; Dandagi, P.; Jain, S. S.; Gadad, A.; Kulkarni, A., Time and pH dependent colon specific, pulsatile delivery of theophylline for nocturnal asthma. *Int J Pharm* 2007, 328, 49-56.
24. Matsuo, M.; Arimori, K.; Nakamura, C.; Nakano, M., Delayed-release tablets using hydroxyethylcellulose as a gel-forming matrix. *Int J Pharm* 1996, 138, 225-35.
25. Jagdale, S. C.; Sali, M. S.; Barhate, A. L.; Loharkar, J. N.; Chabukswar, A. R., Design and evaluation of enteric press-coated tablet for pulsatile delivery of atenolol. *Int J Pharma World Res* 2010, 1, 1-15.
26. Yeole, P.; Galgatte, U.; Babla, I.; Nakhat, P., Design and evaluation of xanthan gum-based sustained release matrix tablets of diclofenac sodium. *Indian J Pharm Sci* 2006, 68, 185-89.
27. Veerareddy, P. R.; Manthri, R. P., Formulation and evaluation of compression coated piroxicam tablets for colon specific drug delivery. *Acta Pharma Sci* 2010, 52, 281-94.
28. Gazzaniga, A.; Maroni, A.; Sangalli, M. E.; Zema, L., Time-controlled oral delivery systems for colon targeting. *Expert Opin Drug Deliv* 2006, 3, 583-97.
29. Sungthongjeen, S.; Puttipipatkhachorn, S.; Paeratakul, O.; Dashevsky, A.; Bodmeier, R., Development of pulsatile release tablets with swelling and rupturable layers. *J Control Release* 2004, 95, 147-59.