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In vitro incompatibility study of Valsartan and Hydrochlorothiazide by spectroscopic and RP-HPLC Method

M Manoj Kumar, D Himaja & Sandip Sen*

Srikrupa Institute of Pharmaceutical Sciences, Velikatta, Siddipet, Telangana-502277, India

E-mail: sandipsen2010@gmail.com

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The present investigation is based on an *in vitro* incompatibility study between valsartan and hydrochlorothiazide by spectroscopic and reverse-phase high-pressure liquid chromatography (RP-HPLC) methods. The method has been developed and validated by ultraviolet (UV) spectroscopic method using methanol and water (1:4) as the solvent. The RP-HPLC study has been carried out using acetonitrile, methanol and 50 Mm phosphate buffer (20:50:30 %) adjusted to pH 3 with orthophosphoric acid. The methods show linearity at the concentration range of 1-5 µg/ml with correlation coefficient 0.9994 for valsartan and 0.9978 for hydrochlorothiazide in the concentration range of 1-5 µg/ml. Both the methods have shown a percentage relative standard deviation values less than 2. The *in vitro* incompatibility study has been carried out by UV-spectroscopic and RP-HPLC methods. The results of the study show that there is a change in the concentration level of both drugs in combination. The structure of the interacting compound has been determined by spectral analysis using IR, ^{1}H NMR and LC-MS study. The spectral analysis confirms the formation of the new complex between valsartan and hydrochlorothiazide. The results obtained from the LC-MS study also correlate the reason behind an increase in the concentration of valsartan and decreases the concentration of hydrochlorothiazide during recovery study by spectroscopic and RP-HPLC method.

Keywords: Valsartan, Hydrochlorothiazide, Spectroscopic method, RP-HPLC, *In vitro* drug interaction

In recent years the Indian union health ministry banned most of the combinational drugs due to their adverse effects and drug-drug interactions. These interactions lead to change in the chemical property of the dosage form which is known as chemical incompatibility[1]. Valsartan is chemically *N*-pentanonyl-*N*-[*2'*-(1 *H*-tetrazole-5-yl) biphenyl-4-yl methyl]-L-valine. This is mainly used as anti-hypertensive[2]. Valsartan is an angiotensin II receptor blocker. Activation of angiotensin II includes constricting blood vessels and activating aldosterone to reduce blood pressure[3]. Hydrochlorothiazide is chemically 6-chloro-3, 4-dihydro-2H-1, 2, 4benzothiadiazine-7-sulphonamide 1,1-dioxide. This is mainly used as a diuretic. Hydrochlorothiazide belongs to the thiazide class of diuretics. It reduces blood volume by acting on the kidneys to reduce sodium (Na^+) reabsorption in the distal convoluted tubule[4]. The combination drug mainly used to treat high blood pressure. Lowering high blood pressure helps prevent strokes, heart attacks and kidney problems[5]. The literature review reveals that valsartan and hydrochlorothiazide were previously estimated by spectrophotometry[6-8], RP-HPLC[9, 10], HPTLC[11], UPLC[12] and some hyphenated techniques like HPLC-MS/MS also used for estimation of valsartan in plasma[13]. The present study describes there is a chemical incompatibility between valsartan and hydrochlorothiazide. It leads to the formation of a new chemical molecule by the synthesis of these two drugs. These types of studies were previously explained by using UV spectroscopy, HPLC[14], IR and NMR[15].

Materials and Methods

Original standards of valsartan hydrochlorothiazide were provided by the Caplin point laboratories limited, Pondicherry. Formulations of valsartan (Valzaar-40 mg), hydrochlorothiazide (Cipla-12.5 mg) and valsartan and hydrochlorothiazide combination dosage form (Valembic) were purchased from local markets. HPLC grade methanol, acetonitrile, and water were purchased from Merck, Mumbai, India. Potassium dihydrogen orthophosphate & orthophosphoric acid was purchased from Research-Lab fine chem industries.

Instruments

The instrument used for the present study was a double beam UV spectrophotometer (ELICO SL 244) using 1.00 cm quartz cuvette. The weighing process was carried out in SHIMADZU AUX 220, RP-HPLC (Make: Cyber labs; Software: LC-100 with C8

column and UV detector), ultrasonicator (Citizen), FTIR (Perkin Elmer), and NMR (Bruker Avance-II 400 MHz), HRLCMS (Make: Agilent Technologies, USA Model: 1290 Infinity UHPLC System, Nano HPLC with Chipcube, 6550 I Funnel Q-TOFs).

Preparation of standard stock solution

Accurately weighed 10 mg of valsartan and hydrochlorothiazide were transferred separately to 10 ml of volumetric flasks followed by the addition of 2 ml methanol. The mixture was sonicated for 10 min and finally, volume was made up with water (1000 µg /ml). From the above stock solutions, 1-5 µg / ml concentration of valsartan and hydrochlorothiazide were prepared.

Preparation of test stock solution

Individually 10 tablets of valsartan and hydrochlorothiazide weighed, their mean weight was calculated and triturated to make a fine powder. Then weight equivalent to 10 mg of valsartan and hydrochlorothiazide were taken in 2 different 10 ml volumetric flasks, made up the volume up to 10 ml with methanol and water (1:4) which gave 1000 µg/ ml (stock solution). The mixture was sonicated for 30 min, above stock solution was filtered and diluted to prepare 1-5 µg /ml concentration of valsartan and hydrochlorothiazide.

Selection of wavelength

The prepared 10µg /ml concentration of valsartan and hydrochlorothiazide were scanned in a UV visible spectrophotometer in the range of 200-400 nm.

UV- spectroscopic method validation

The proposed method was extensively validated in terms of specificity, linearity, accuracy, precision, robustness, ruggedness, limits of detection (LOD) and quantification (LOQ) as per ICH guidelines[16].

***In-vitro* drug interaction studies by the spectroscopic method**

Percentage recovery study using solvent system (methanol and water 1:4)

10 µg /ml solutions of valsartan and hydrochlorothiazide were prepared individually. From the above solutions 0.5 ml of valsartan and 0.5 ml of hydrochlorothiazide was taken in a 10 ml volumetric flask, 2 ml of methanol was added and made up the volume with water and mixed thoroughly and the contents of the volumetric flask transferred into round bottom flask and heated it for 120 min at 37 ºC then the sample was withdrawn for every 30 min. The samples were scanned in UV at respective λ_{max} against reagent blank.

Percentage recovery study using phosphate buffer (pH: 6.8)

10 µg /ml solutions of valsartan and hydrochlorothiazide individually using phosphate buffer (pH: 6.8) were prepared. From the above solutions 0.5 ml of valsartan and 0.5 ml of hydrochlorothiazide were taken in a 10 ml volumetric flask. 5 ml of phosphate buffer (pH: 6.8) was added to mix the contents and made up the volume with phosphate buffer (pH: 6.8). After transfer, the contents of the volumetric flask into the flask, it was heated for 120 min at 37 ºC then the sample was withdrawn for every 30 min. The samples were scanned in UV at respective λ_{max} against the reagent blank.

RP-HPLC method

For RP-HPLC method[17] the mobile phase was acetonitrile: methanol: 50 mM phosphate buffer adjusted to pH-3 with orthophosphoric acid (20:50:30) and Flowrosil C18 (250 x 4.6 mm, 5 µm) column was used. The flow rate, run time, injection volume and wavelength were set to be 1 ml/min, 5 min, 20 µL and 265 nm, respectively.

Preparation of standard stock solution

Accurately 10 mg of valsartan and hydrochlorothiazide were weighed individually and transferred separately to 10 ml of volumetric flasks. 5 ml of mobile phase was added the solutions and sonicated for 10 min and then made up to 10 ml with mobile phase (1000 µg/ml). Then above stock solutions were diluted to prepare 10 µg/ml solutions of valsartan and hydrochlorothiazide separately. These were used for further analysis.

Preparation of test stock solution

10 tablets of valsartan and hydrochlorothiazide fixed-dose combination tablets were taken and weighed and they were transferred into the motor and triturated to make a fine powder. From this weight equivalent to 10 mg of valsartan and hydrochlorothiazide was taken in a 10 ml volumetric flask. The volume was made up to 10 ml with the mobile phase. This gave 1000 µg/ml (stock solution). The above stock solution was filtered and the filtered solution was diluted to prepare 10 µg/ml solution of valsartan and hydrochlorothiazide.

***In vitro* drug interaction studies by RP-HPLC method**

Percentage recovery study using RP-HPLC solvent system

100 µg/ml solutions of valsartan and hydrochlorothiazide were prepared individually using

the mobile phase. From the above solutions, 0.75 ml of valsartan and 0.75 ml of hydrochlorothiazide were taken in a 10 ml volumetric flask, 5 ml of mobile phase was added and the contents were mixed and made up to volume with the mobile phase. The content of the volumetric flask was transfer into the RBF flask and heated for 120 min at 37 ºC then the sample was withdrawn after every 30 min. Then the solution was filtered and injected into the HPLC system. The % recovery of each drug was calculated.

Synthesis of the mixture of valsartan and hydrochlorothiazide

The equimolar ratio of valsartan and hydrochlorothiazide was taken in 50 ml of round bottom flask then add 10 ml of methanol and refluxed for 3 h. After that the mixture was cooled, filtered and dried by a solvent evaporation method. The dried synthetic product was delivered for spectral analysis (IR, NMR, and LC-MS) to determine the structure of the formed compound.

Results and Discussion

The absorption spectra for valsartan and hydrochlorothiazide are shown in Fig. 1. The maximum wavelength (λ_{max}) for valsartan and hydrochlorothiazide was found to be 231 and 271 nm, respectively. This new cheapest UV method was developed using methanol and water (1:4) and validated according to ICH guidelines. It is evident from Table 1 and 2 that both the drugs show linearity at the concentration range of 1-5 µg/ml. Fig. 2 shows the calibration curves of valsartan and hydrochlorothiazide in UV-spectroscopic method. The correlation coefficient is found to be 0.9994 for valsartan and 0.9978 for hydrochlorothiazide.

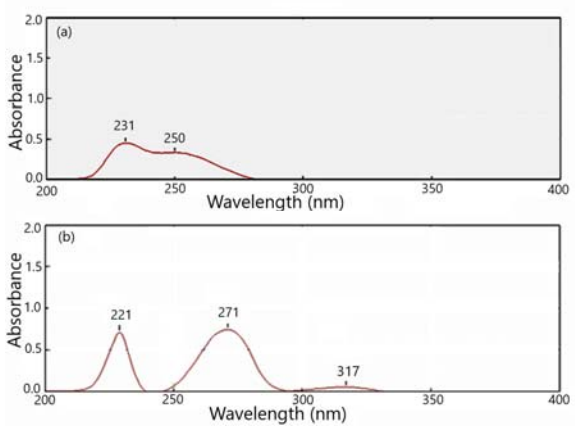


Fig. 1 — Absorption spectra of (a) valsartan and (b) hydrochlorothiazide solution (Solvent- methanol: water (1:4)).

The assay results of valsartan in tablet dosage form at 2 µg/ml and hydrochlorothiazide at 1 µg/ml are given in Table 3 and 4, respectively. Both the methods were shown %RSD values less than 2. The percentage recovery study was also carried out individually and it was found to be 98% for valsartan and 90% for hydrochlorothiazide as shown in Table 5.

The RP-HPLC study was carried out using acetonitrile, methanol, and 50 Mm phosphate buffer (20:50:30 %) adjusted to pH-3 with orthophosphoric acid by the previously reported method developed by Jothieswari D (2011). The plots are given in Fig. 3. The proposed method also exhibited a correlation coefficient of 0.999 and the method is well accepted. The optimized chromatogram of valsartan and hydrochlorothiazide are given in Fig. 4.

The *in vitro* drug interaction study was carried out using the developed method for UV-spectroscopy and

Table 1 — Linearity study of valsartan at 1-5 µg /ml

S. N.	Concentration (µg/ml)	Absorbance (mean, n=6)	Standard deviation	%RSD
1	1	0.0887	0.0016	1.75472
2	2	0.1857	0.00081	0.54475
3	3	0.2753	0.0009	0.34679
4	4	0.3696	0.00305	0.97452
5	5	0.4525	0.00668	1.60284

Table 2 — Linearity data of hydrochlorothiazide at 1-5 µg /ml

S. N.	Concentration (µg/ml)	Absorbance (Mean, N=6)	Standard Deviation	%RSD
1	1	0.13032	0.000162	1.2443
2	2	0.21053	0.00237	1.1246
3	3	0.34073	0.05714	1.6770
4	4	0.45087	0.00557	1.2348
5	5	0.54818	0.008716	1.5900

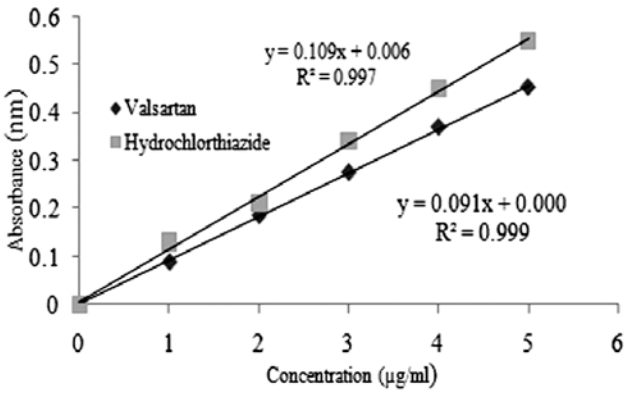


Fig. 2 — Calibration curves of valsartan and hydrochlorothiazide in UV-spectroscopic method.

RP-HPLC. The study was carried out by a percentage recovery study using a combination of both drugs. In UV-spectroscopic method methanol and water (1:4) (Table 6) and phosphate buffer (pH 6.8) (Table 7) were used. For both, the study equal ratio of each drug was taken with the designed concentration of 1 µg/ml. The mixture was warmed at body temperature at 37 ºC in a thermostat water bath and the absorbance was recorded at 30 min interval for 2 h. Fig. 5 shows the calibration curves of valsartan and hydrochlorothiazide in RP-HPLC method. During 2 h of recovery study, it was found that there is a change in the concentration level of both the drugs. The concentration of valsartan was increased whereas that of hydrochlorothiazide was decreased.

Table 3 — Assay result of valsartan in tablet dosage form at 2 µg/ml

Mean of assay values (µg/ml) (N=6)	Standard Deviation	1 % RSD
0.144	0.00077	0.541%

Table 4 — Results of the assay for hydrochlorothiazide at 1 µg/ml

Mean of assay values (µg/ml) (N=6)	Standard Deviation	% RSD
0.07861	0.00012	0.14769%

Table 5 — Summary of validation parameters for the proposed methods

S. N.	Parameters	Results	
		Valsartan	Hydrochlorothiazide
1	Absorption maximum (λ_{max})	231 nm	271 nm
2	Beer's Law limit (µg/ml)	1-5 µg/ml	1-5 µg/ml
3	Slope	0.009463	0.100141
4	Intercept	0.001	0.0117
5	Coefficient of correlation	0.9994	0.9978
6	Accuracy (%recovery)	98%	90%
7	Precision (%RSD)	0.54%	0.98%
8	Robustness (%RSD)	1.50%	0.93%
9	Ruggedness (%RSD)	1.71%	0.91%
10	Limit of detection (LOD)	0.054 µg/ml	0.053 µg/ml
11	Limit of quantification (LOD)	0.163 µg/ml	0.161 µg/ml

Similarly, the *in vitro* interaction study was carried out using the RP-HPLC method using solvent system acetonitrile, methanol and 50 mM phosphate buffer (20:50:30%) adjusted to pH 3 with orthophosphoric acid (Table 8). It was found that the concentration of valsartan was increased and hydrochlorothiazide was decreased continuously during 2 h of study. Fig. 6

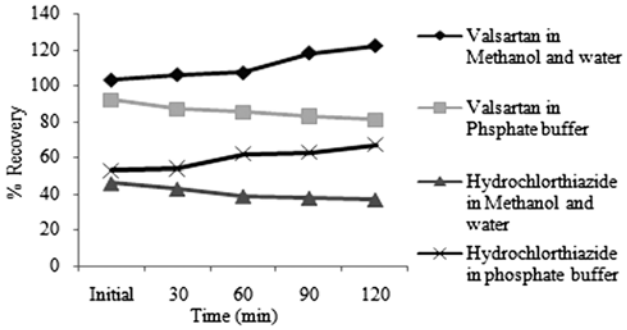


Fig. 3 — *In-vitro* interaction studies by percentage recovery of valsartan and hydrochlorothiazide in methanol and water (1:4) and Phosphate buffer.

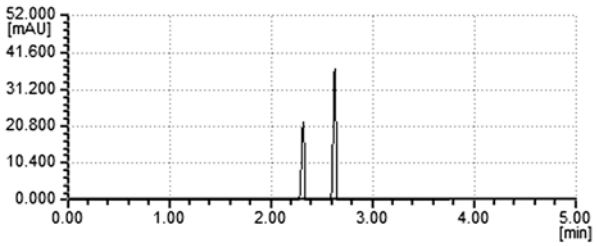


Fig. 4 — Optimized chromatogram of valsartan and hydrochlorothiazide.

Table 6 — *In vitro* incompatibility study by percentage recovery in methanol and water (1:4) using UV spectroscopy

Time	Valsartan		Hydrochlorothiazide	
	At 231 nm	%Recovery	At 271 nm	%Recovery
Initial	0.1899	103	0.0908	46
30 min	0.1956	106	0.0835	43
60 min	0.1963	107	0.0739	39
90 min	0.2171	118	0.0721	38
120 min	0.2241	122	0.071	37

Table 7 — *In vitro* incompatibility study by percentage recovery in phosphate buffer using UV spectroscopy

Time	Valsartan		Hydrochlorothiazide	
	At 231 nm	% Recovery	At 271 nm	% Recovery
Initial	0.1647	92	0.1038	53
30 min	0.1561	87	0.1062	54
60 min	0.1521	85	0.1041	62
90 min	0.1490	83	0.1250	63
120 min	0.1450	81	0.1354	67

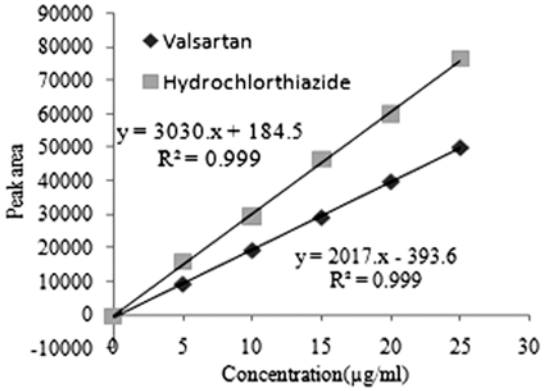


Fig. 5 — Calibration curves of valsartan and hydrochlorothiazide in RP-HPLC method.

Table 8 — *In vitro* incompatibility study by percentage recovery using RP-HPLC method

S. N.	Time	Retention time		Area		% of the drug present	
		VAL	HTCZ	VAL	HTCZ	VAL	HTCZ
1	Initial	2.32	2.72	29407	46444	100	100
2	30	2.32	2.72	30212	45212	102.7	97.3
3	60	2.32	2.72	31123	44201	105.8	95.1
4	90	2.32	2.72	31840	43123	108.2	92.8
5	120	2.32	2.72	32214	42101	109.5	90.6

shows the plots for *in vitro* interaction studies by percentage recovery of valsartan and hydro–chlorothiazide in RP-HPLC using solvent system.

From the recovery study by UV-Spectroscopy and RP-HPLC, it was found that there was a change in the concentration of both valsartan and hydrochlorothiazide. For further verification of result complex of valsartan and hydrochlorothiazide was synthesized using methanol as a solvent. The structure of the complex was characterized by IR, ^{1}H NMR and LC-MS study.

The IR study was carried out by using the KBr pellet technique. Fig. 7 shows the FTIR spectra of hydrochlorothiazide, valsartan and complex of both drugs. The assignment of IR bands was made by comparing the spectra of 2 drugs and the complex formed due to interacting drugs. The valsartan gives a characteristic peak at 1730.35 cm^{-1} indicates the presence of the carbonyl group which is carboxylic in nature. The presence of a characteristic peak at 1598 cm^{-1} (N-H secondary amine) and 1163.43 cm^{-1} (SO_2NH_2) indicates the presence of hydro–chlorothiazide. The missing of the characteristic peak at 1730 cm^{-1} for carboxylic acid and 1163 cm^{-1} for SO_2NH_2 indicates formation of the new complex from participating drug.

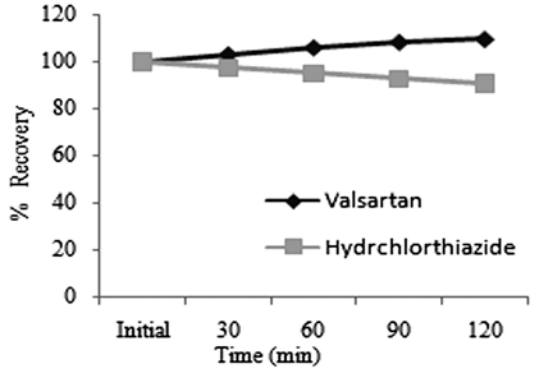


Fig. 6 — *In-vitro* Interaction Studies by percentage recovery of valsartan and hydrochlorothiazide in RP-HPLC using solvent system.

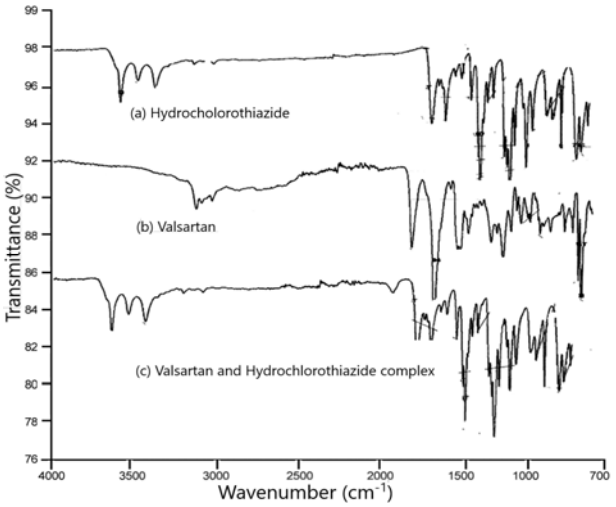


Fig. 7 — FTIR spectra of hydrochlorothiazide, valsartan and complex of both drugs.

Further ^{1}H NMR study was carried out using DMSO d_6 and the plots are shown in Fig. 8. The carboxylic acid of valsartan gives a characteristic singlet peak at δ value 12.01 and hydrochlorothiazide has given a singlet peak at 2.5 for SO_2NH_2 group. But in the case of the complex interacting drug a new singlet peak appeared at δ value 2.01 indicate the formation of the new complex of interacting drug.

The result obtained from the IR and ^{1}HNMR study was further confirmed by the LC-MS study of synthesized compounds. The results indicated the presence of these components in a mixture with retention time value 4.64, 11.32 and 12.56 min (Fig. 9) having molecular mass (m/z) value 295.95 for hydrochlorothiazide, 434.22 for valsartan and 448.23 for the complex compound (Fig. 10).

From FTIR and ^{1}H NMR study it was found that due to chemical incompatibility between valsartan and hydrochlorothiazide the formation of unstable

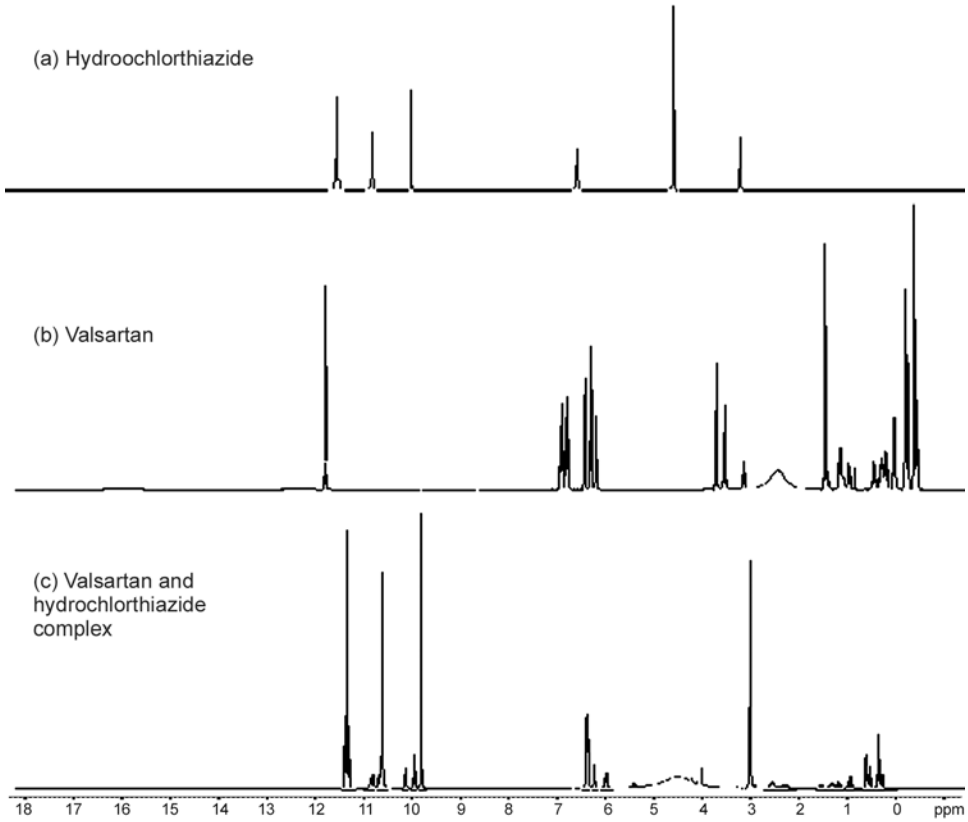


Fig. 8 — ^{1}H NMR spectra of hydrochlorothiazide, valsartan and complex of both drugs.

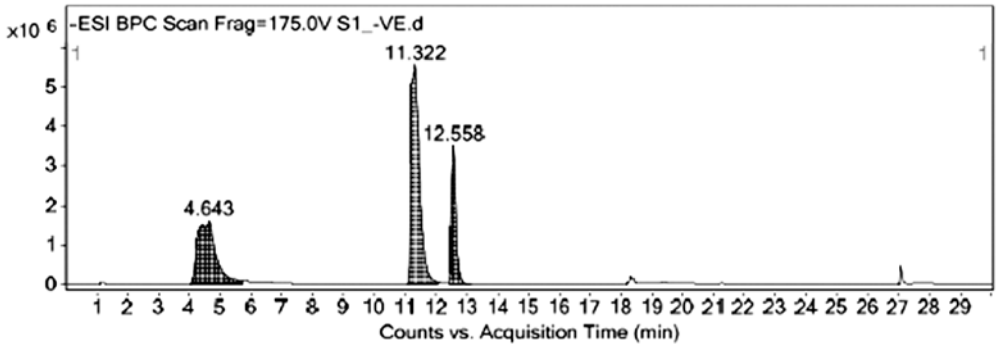


Fig. 9 — LC-MS chromatogram of Hydrochlorothiazide, valsartan and complex of both drugs.

compound named as (S)-N-((2'-(2*H*-tetrazole-5-yl)-[1,1'-biphenyl] -4 -yl) methyl) -N- (1-((6-chloro-1,1-dioxido-3,4-dihydro-2*H*-benzo[e][1,2,4]thiadiazine-7-sulfonamido)oxy)-3-methyl-1-oxobutan-2-yl) pentanamide having molecular formula $C_{30}H_{31}ClN_8O_7S_{22}^{\bullet}$ (Exact Mass: 714.14) was taken place. During LC-MS study it was found there was the presence of a new compound with molecular mass 448.23 at 12.56 min. The compound can be named as (S)-((2-(N-((2'-(2H-tetrazole-5-yl)-[1,1'-biphenyl]-4-yl) methyl) pentanamido)-3-methylbutanoyl) oxy) nitride in which valsartan moiety is prominent. Due to which the concentration of valsartan was increased during the *in vitro* recovery study. The result obtained from the LC-MS study (Table 9) also correlates with increased concentration of valsartan and decreased the concentration of hydrochlorothiazide during recovery study by spectroscopic and RP-HPLC method.

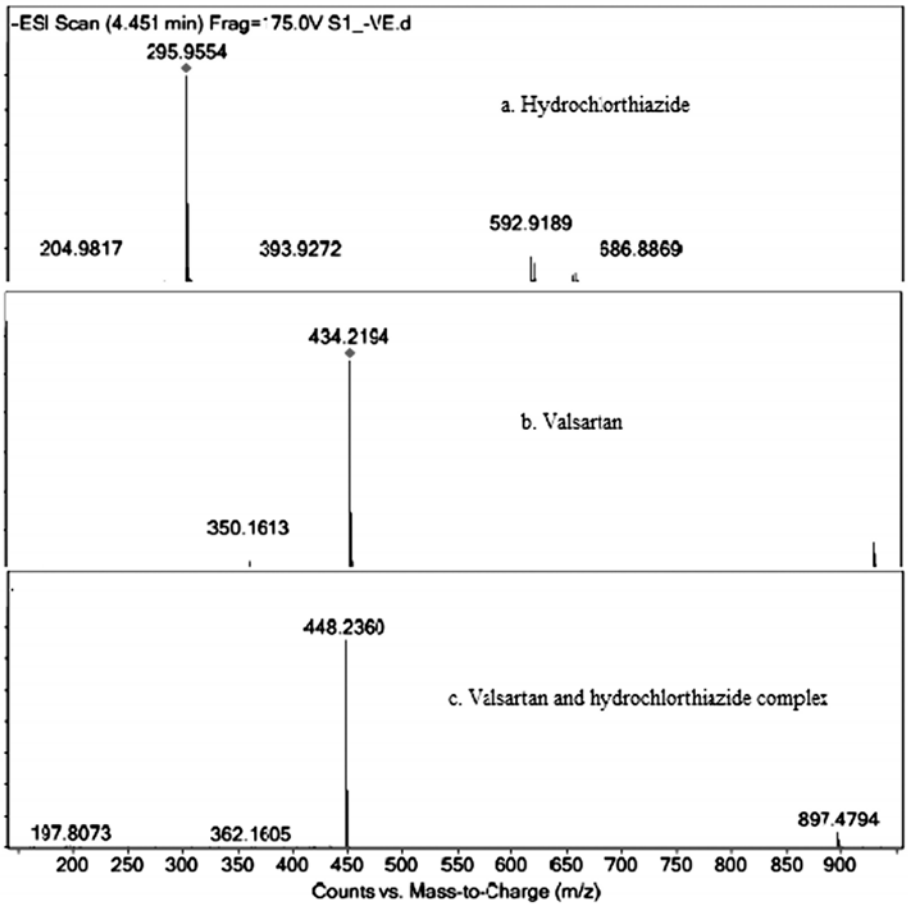
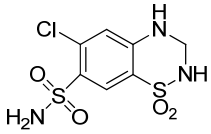
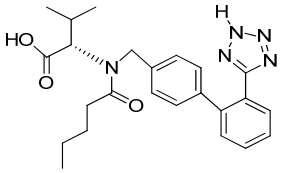
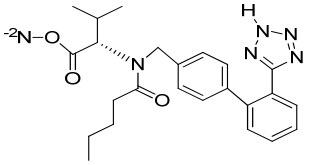


Fig. 10 — ESI mass spectra by LC-MS for hydrochlorothiazide, valsartan and complex of both drugs in negative mode.

Table 9 — Correlation and mass data for reported compounds from HR-LCMS study

S. N.	Retention Time (min)	Peak Height	Peak Area	Molecular Mass Found (m/z)	Type of Peak	Structure of compounds
1	4.64	159313	66549560	295.95		 Hydrochlorothiazide
2	11.32	5557616	109643441	434.22	$(M-H)^-$ Negative ion Base peak	 Valsartan
3	12.56	3480291	34878286	448.23		 Unknown fragmented compound

Conclusions

Based on spectral and RP-HPLC analysis, it is confirmed that there is the formation of an interacting complex between valsartan and hydrochlorothiazide. This indicates there is *in vitro* chemical incompatibility take place between valsartan and hydrochlorothiazide which may produce synergistic or antagonist effect on concurrent administration or in the combined dosage form.

Supplementary Data

Supplementary data associated with this article are available in the electronic form at http://nopr.niscair.res.in/jinfo/ijca/IJCA_59A(08)1120-1127_SupplData.pdf.

Acknowledgment

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