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VALIDATED UPLC/Q-TOF-MS METHOD FOR SIMULTANEOUS DETERMINATION OF VALSARTAN AND HYDROCHLOROTHIAZIDE IN HUMAN PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ABSTRACT
In the presented work the ultra-performance liquid chromatographic/quadrupole time-of-flight mass spectrometric (UPLC/Q-TOF-MS) method has been developed for simultaneous determination of valsartan and hydrochlorothiazide in human plasma. For identification of drugs, the Q-TOF mass spectrometer was operated in negative ionization mode and quantification was done using the MS/MS transitions at m/z 435.02 to 157.00 for valsartan and 295.80 to 204.94 for hydrochlorothiazide. The chromatographic separation was achieved on Acquity UPLC^{TM} BEH C_{18} (100.0 × 2.1 mm, 1.7µm) column using isocratic mobile phase consisting of acetonitrile-2mM ammonium acetate (50:50, v/v) at a flow rate of 0.25 mL/min. The calibration curves were linear over the concentration range of 1-1000 ng/mL for all the compounds. The developed method was validated according to ICH guidelines. The method was applied for pharmacokinetic study of valsartan and hydrochlorothiazide in human plasma.

KEYWORDS: UPLC/Q-TOF-MS, Valsartan, Hydrochlorothiazide, Pharmacokinetic Study.

INTRODUCTION
The UHPLC/Q-TOF-MS technique is the latest amongst all the chromatographic technique and has been used worldwide in drug discovery and development. It has been applied in pharmaceutical development particularly in the identification and quantitative analysis of drug products. The metabolite profiling has been investigated in various biological samples by applying UHPLC/Q-TOF coupled with MetaboLynx^{TM} software. The Q-TOF mass spectrometry gives the accurate mass, reliable chemical fragmentation of synthetic compounds.[1, 2] Valsartan (VAL) is an antihypertensive drug, belongs to a group of angiotensin converting enzyme (ACE) inhibitors. It is used for the treatment of hypertension.[3] Hydrochlorothiazide (HCTZ) is a diuretic drug used worldwide for lowering the blood pressure individually and in the combination of antihypertensive drugs.[4] Fixed dose combination tablets containing 80 mg of valsartan and 25 mg of hydrochlorothiazide has been approved for the treatment of mild to moderate hypertension and widely available in the Indian market.

The literature survey revealed that few analytical methods have been reported for determination of valsartan as an individual drug by UV spectrophotometry, Liquid Chromatography and LC-MS.[5-7] Determination of hydrochlorothiazide as an individual drug reported by spectrophotometry, HPLC, and LC-MS.[8-12] Simultaneous determination of valsartan and hydrochlorothiazide has been reported by derivative spectrophotometry, TLC-densitometry and spectrofluorimetry, HPLC and LC-MS. [13-15] However a LC-MS/MS method was developed for simultaneous determination of valsartan and hydrochlorothiazide in human plasma but the developed method was found very complicated.[16] Hence in the presented work an UPLC/Q-TOF-MS method is developed and validated for identification and quantitative determination of valsartan and hydrochlorothiazide in human plasma using isocratic elution without use of internal standard.

EXPERIMENTAL
Chemicals and Reagents
Valsartan ($C_{24}H_{29}N_5O_3$, Molecular weight 435.51, and purity 99.98%) and Hydrochlorothiazide ($C_7H_8ClN_3O_4S_2$, Molecular weight 297.72, and purity 99.99%) were kindly supplied as gift sample by Systopic Pharmaceuticals Ltd. (New Delhi, India). Tablets (Co-Diovan, Novartis, India) were obtained

commercially with labeled amounts of 80 mg of valsartan and 12.5 mg of hydrochlorothiazide. LC-MS grade water, acetonitrile, methanol, and ammonium acetate were purchased from Fluka analytical, Sigma-Aldrich Corporation, St. Louis, MO, USA. All other reagents used were of LC-MS grade.

Q-TOF-MS and UPLC Conditions

Mass spectrometry was performed on a Waters Synapt Q-TOF Premier (Micromass MS Technologies, Manchester, UK) mass spectrometer. Quantification was done by using MS/MS transitions, m/z 435.02 to 157.00 for valsartan and 295.80 to 204.94 for hydrochlorothiazide. UPLC was performed with Waters Acquity UPLC system (Waters Corporation, MA, USA) equipped with a binary solvent manager, an auto-sampler, column manager and a tunable MS detector. The various parameters for Q-TOF-MS and UPLC conditions are presented in **Table 1.**

Table 1: Various Parameters for Q-TOF-MS and UPLC Conditions.

Q-TOF-MS Conditions		**UPLC Conditions**	
Capillary voltage	3.0 kV	Chromatography	Waters Acquity UPLC system
Sampling cone voltage	40 V	Column	Acquity UPLC BEH C_{18}
Extraction cone voltage	4 V	Column dimension	100.0 × 2.1 mm, 1.7µm
Source temperature	80ºC	Mobile phase	Acetonitrile–2 mM ammonium acetate (50:50, v/v)
Cone gas flow	50 L/h	Mobile phase flow rate	0.25 mL/min
Source gas flow	0.50 mL/min	Elution mode	Isocratic
Collision gas (Argon)	2.5×10^{-4} mbar	Total run time	3 min
Collision energy	12 V	System pressure	2450 to 2500 psi

Preparation of Standard Solutions

Each of valsartan and hydrochlorothiazide were weighed accurately and transfer to 50 mL volumetric flasks separately. The powders were then dissolved with approximately 25 mL of methanol and ultrasonicated for 5 min. The final volume was made up with methanol. The solutions were further diluted with methanol: water (50:50, v/v) to give a series of standard solutions containing required concentrations for each compound.

Preparation of sample solutions

500 µL of plasma sample was transferred to 10 mL glass tube. To this 5 mL of extraction solvent (diethyl ether: dichloromethane 70:30, v/v) was added. The sample was mixed by vortexer for 5 min. The organic layer was transferred to another glass tube. The solid residue was evaporated to dryness using evaporator at 40 ºC under a stream of nitrogen. The dried extract was reconstituted in 200 µL of diluent (methanol: water, 50:50, v/v). This solution was filtered through 0.45 µm nylon membrane filter to remove all the particulate materials. 20 µL aliquot was injected in to UPLC system.

Validation of the Method

The developed method was validated according to ICH validation guidelines.[17] Different standard concentrations each of the compound in the range of 1-1000 ng/mL (1, 10, 50, 100, 200, 500, and 1000 ng/mL) was spiked to 100 µL of blank human plasma separately in methanol: water (50:50, v/v). Similarly the low, medium and high concentration QC samples containing different concentrations of both the drugs were prepared independently using the same procedure. The solutions were filtered through 0.20 µm nylon syringe filter and injected in to the UPLC/QTOF-MS system for analysis. Linearity graph was prepared by average peak area of each concentration. Intraday and interday precision and accuracy and was also evaluated. The specificity of the method was examined by analyzing blank plasma extract.

Pharmacokinetic Study

The method was applied to determine the plasma concentrations of valsartan and hydrochlorothiazide from a clinical trial in which 3 healthy male volunteers received a FDC tablet containing 80 mg valsartan and 12.5 mg hydrochlorothiazide. Blood samples were collected before and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h post-dosing. Plasma was separated by centrifugation of the heparinized samples at 2000 × g for 10 min and was stored at -20 ºC until analysis.

RESULTS AND DISCUSSION

All the compounds have strong responses in the negative ionization mode. Therefore, the negative ions, $[M-H]^-$ at m/z 435.02 valsartan and m/z 295.86 for hydrochlorothiazide were selected as the precursor ions. Under the selected MS/MS conditions the precursor ions were fragmented to major product ions at m/z 435.02 to 157.00 for valsartan, 295.91 to 204.94 for hydrochlorothiazide as shown in **Figure 1 and Figure 2**, respectively.

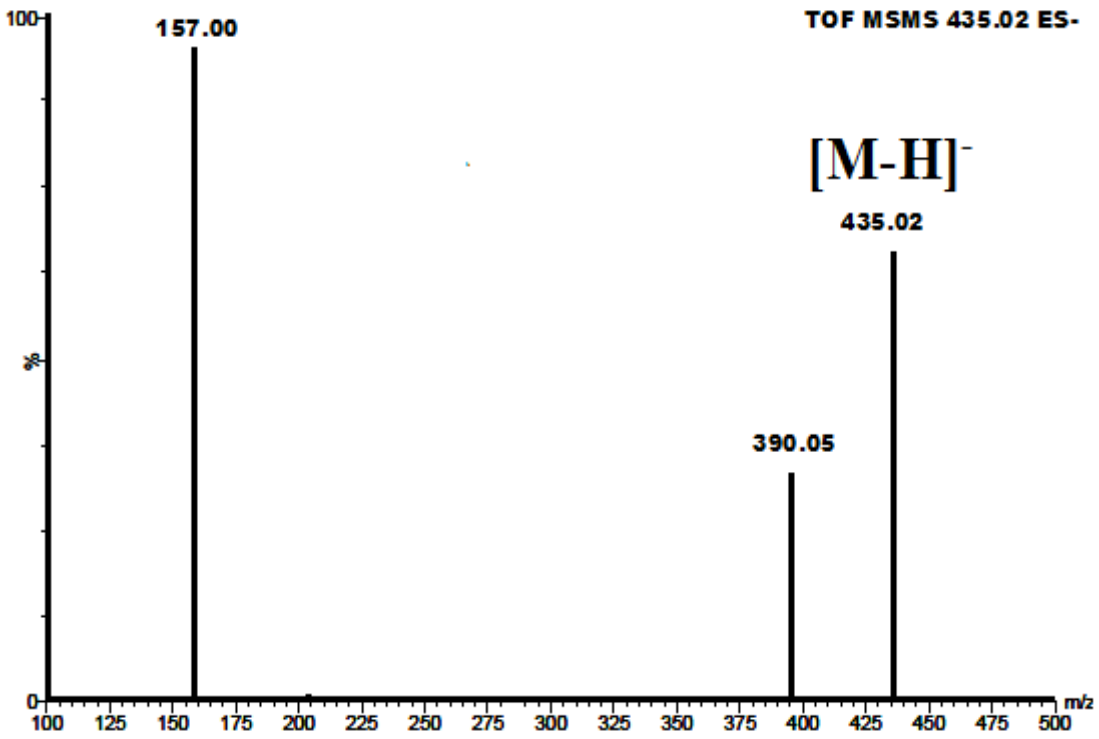

Figure 1: TOF-MS/MS Spectra of Valsartan.

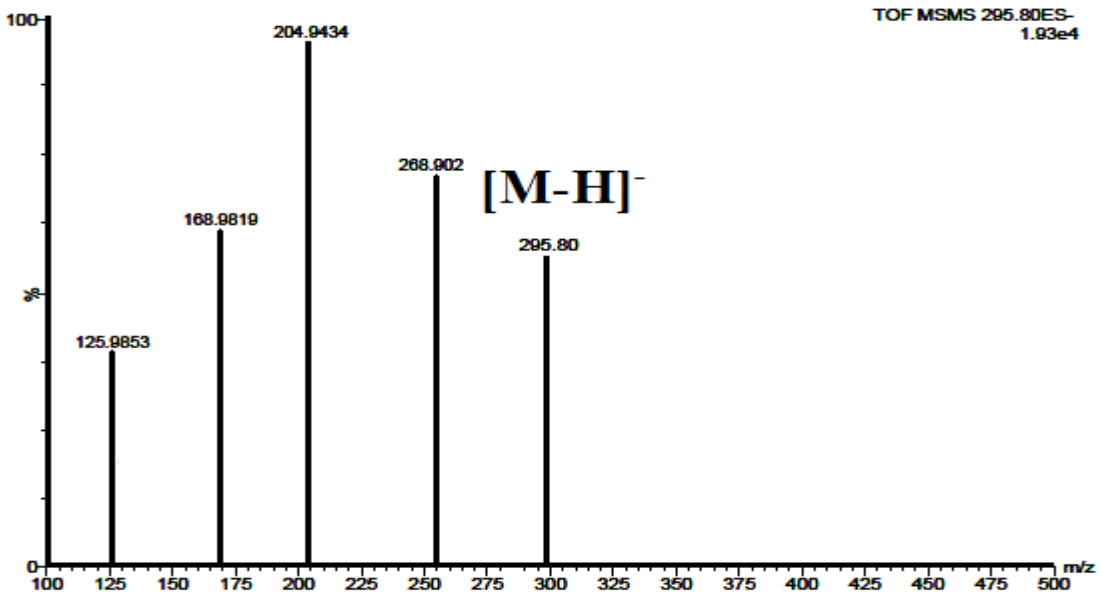

Figure 2: TOF-MS/MS Spectra of Hydrochlorothiazide.

The proposed MS/MS fragmentation mechanism of valsartan is shown in **Figure 3.** The product ion spectra of hydrochlorothiazide was occurred due to the fragmentation of compound via loss of neutral molecule namely HCN and degraded by hydrolysis, resulted in the formation of one intermediate product ion, at m/z 283.95 which was identified as 4-amino-6-chloro-1,3-benzenedisulfonamide, a major degradation product of hydrochlorothiazide. This is further fragmented in to another product ion with higher intensity at m/z 268.90 by the loss of NH_3 molecule. The product ion at intensity m/z 268.90 was identified as chlorothiazide. This product ion is further converted into major product ion with highest intensity at m/z 204.93 by the loss of SO_2. This in turn fragmented into smallest intensity ion at m/z 169.00. The proposed MS/MS fragmentation mechanism of hydrochlorothiazide and chlorothiazide is shown in **Figure 4**.

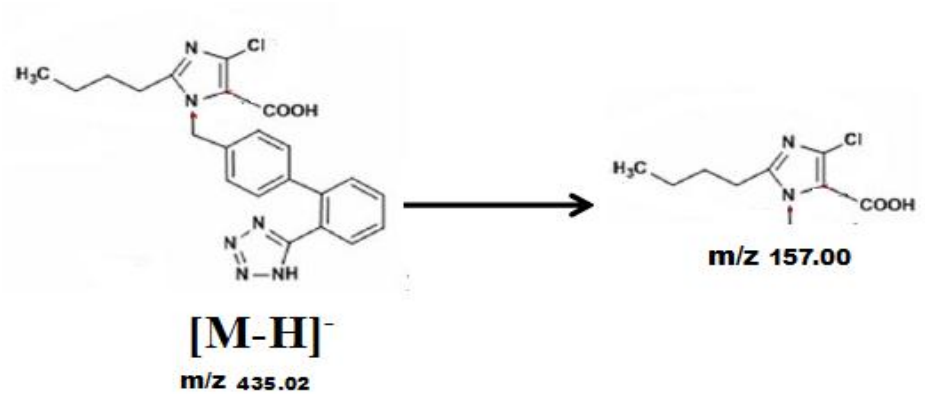

Figure 3: Proposed MS/MS Fragmentation Mechanism of Valsartan.

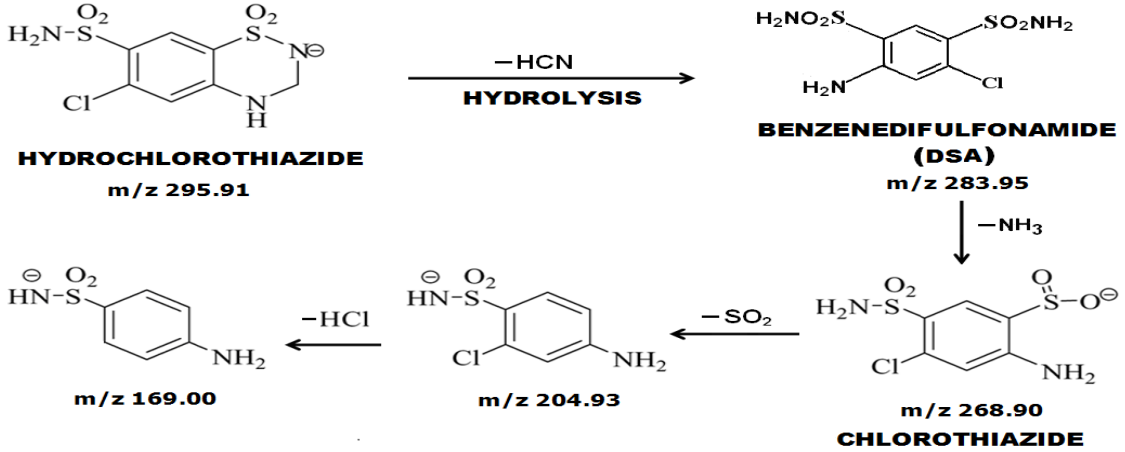

Figure 4: Proposed MS/MS Fragmentation Mechanism of Hydrochlorothiazide.

Optimization of UPLC Conditions

The isocratic mobile phase containing acetonitrile-2mM ammonium acetate (50:50, v/v) at a flow rate of 0.25 mL/min provide peaks with short retention times. The retention time was found to be 2.50 min for valsartan, and 1.25 min for hydrochlorothiazide with the total chromatographic run time of 3.0 min for each compound.

Validation of the method

The results of linearity, LOD and LOQ are presented in **Table 2.** The obtained results indicated that higher sensitivity of the method. The RSD less than 2% were obtained for all the compounds by evaluation of intraday, interday, and different analysts precision suggested an acceptable precision and accuracy of the method. No significant interference in the blank plasma traces was seen from endogenous substances at the retention time of both drugs, suggested that method was specific.

Table 2: Results Obtained from Linearity, LOD, and LOQ.

Parameters	Valsartan	Hydrochlorothiazide
Linear range (ng/mL)	1-1000	1-1000
Correlation coefficient	0.9997	0.9998
LOD (ng/mL)	0.1	0.1
LOQ (ng/mL)	1	1

Pharmacokinetic Study

The method was applied to pharmacokinetic study in human plasma. The results of pharmacokinetic parameters obtained from mean plasma concentration time curve after administration of single FDC tablet (Co-Diovan) containing 80 mg valsartan and 12.5 mg hydrochlorothiazide are presented in Table 5. The results obtained from pharmacokinetic parameters are presented in **Table 3.**

Table 3: Results Obtained from Pharmacokinetic Studies.

Pharmacokinetic Parameter	Valsartan	Hydrochlorothiazide
Tmax (h)	2.5 ± 0.25	2.0 ± 0.15
Cmax (ng/mL)	3525 ± 554	350 ± 225
AUC (ngh/mL)	850 ± 1.12	350 ± 2.20
$T_{1/2}$	7.0 ± 0.15	8.15 ± 1.10

CONCLUSION

The UPLC/Q-TOF-MS method was developed, validated and applied for identification and quantification of valsartan and hydrochlorothiazide. The developed method has shown acceptable precision, accuracy and sensitivity of the drugs in human plasma samples obtained for pharmacokinetic studies. In addition to this, use of isocratic chromatographic separation without any internal standard makes it an advantageous method for

simultaneous determination of valsartan and hydrochlorothiazide in human plasma.

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