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A Selective and Accurate LC-MS/MS Method for Simultaneous Quantification of Valsartan and Hydrochlorothiazide in Human Plasma

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Abstract: The fixed dose combination of valsartan (VAL) and hydrochlorothiazide (HCTZ) is the most commonly prescribed medicine for the effective treatment of hypertension. In this study, a simple sensitive and accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the simultaneous quantitation of VAL and HCTZ in human plasma by using irbesartan (IRB) and hydroflumethiazide (HFMZ) as their specific internal standards (ISs). HLB cartridge-based solid-phase extraction was used for the extraction of analytes and ISs. The chromatographic separation was achieved on Lichrocart RP Select (125 × 4 mm), 5 nm with the mobile phase composition of acetonitrile: 10 mM ammonium acetate buffer: 95:05, *v*/*v*, at flow rate of 0.5 mL/min. The turbo ion electrospray ionization in negative mode was used as ion source for the sample ionization. The precursor to product ion transitions were 434.10 > 179.10 (VAL), 295.70 > 204.90 (HCTZ), 427.10 > 192.90 (IRB), and 329.90 > 302.40 (HFMZ) for detection and quantification of analytes and their ISs. The retention times of VAL and HCTZ were 1.90 min and 2.30 min, respectively. The range for the calibration curves of VAL and HCTZ were 50.2–6018.6 ng/mL and 1.25–507.63 ng/mL, respectively, with good linearity having correlation coefficient values of ≥0.995 for both VAL and HCTZ. All validation parameter results (selectivity, precision and accuracy, matrix effects and stabilities) were within the acceptable range as per USFDA guideline for bioanalytical method validation. The intra-day and inter-day accuracy data for VAL were within the range of 105.68–114.22% and 98.41–108.16%, respectively, whereas for HCTZ they were 87.01–101.18% and 95.16–99.37%, respectively. The ion suppression effects produced for VAL and ion enhancement effects produced for HCTZ were insignificant according to the proposed sample cleanup procedure. The developed LC-MS/MS method was successfully applied to bioequivalence study on healthy volunteers.

Keywords: valsartan; hydrochlorothiazide; LC-MS/MS; human plasma; validation

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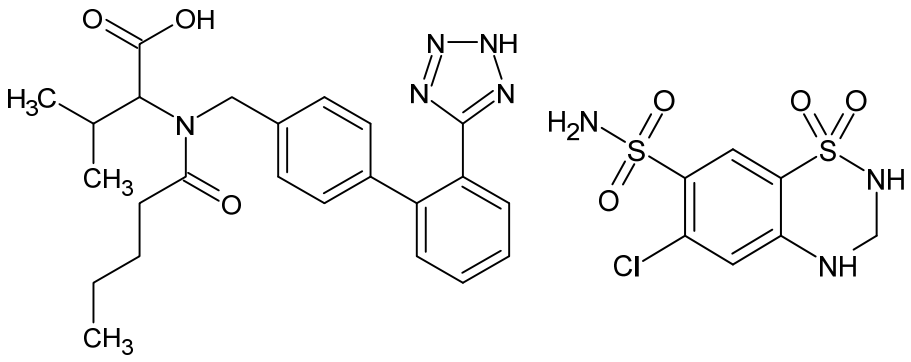
1. Introduction

Hypertension is the most prominent chronic disease, leading to public-health challenges across the world. If not treated properly, it may lead to concomitant risks of cardiovascular and kidney disease [1]. Globally, 26% of the world's population are hypertensive, and according to recent data its prevalence is expected to increase to 29% by 2025 [2]. Fixed dose combination (FDC) of diuretics and an angiotensin II receptor antagonist is the best approach for the treatment of hypertension [1,2]. Valsartan (VAL) is an angiotensin II (AT1) receptor antagonist, which is chemically designated as N-(1-oxopentyl)-N-[[2′-(1H-tetrazol-5-yl) [1,1′-biphenyl]-4-yl] methyl]-L-valine (Figure 1a). It is most commonly prescribed and established as an antihypertensive agent, which works by improving cardiac function,

endothelial function, and lipid profiles [3]. Hydrochlorothiazide (HCTZ) is a thiazide diuretic, which chemically designated as 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide,1,1-dioxide, which control the blood pressure by inhibiting Na-Cl transport system in the kidney which results to reduces plasma volume (Figure 1b). Long-term treatment with an FDC of VAL and HCTZ effectively reduces blood pressure by improvements in cardiac output, reduction in arterial pressure, and heart rate and reduces cardiovascular risk factors in patients [4].



(**a**) (**b**)

Figure 1. Chemical structure of (**a**) VAL and (**b**) HCTZ.

The treatment of hypertension with only oral administration of VAL (monotherapy) was not adequately effective in patients but the combination of VAL and HCTZ has given a better response [5,6] and therefore is considered as the most common combination of treatments across the world for hypertension.

There is ample evidence that VAL/HCTZ is the best and is an effective FDC for hypertension. Coadministration of VAL/HCTZ does not have a clinically relevant pharmacokinetic effect on either drug. FDC of VAL/HCTZ produced dose-proportionate increase in plasma concentrations of each component after oral administration, with peak concentrations achieved within 2–5 h. The half-life of the FDC ranged between 2.5 and 19 h, and clearance of 2.2 L/h for VAL and 20–22 L/h for HCTZ, mainly because of variability in HCTZ values. VAL is excreted mainly through fecal route while HCTZ is excreted renally. VAL/HCTZ has generally similar antihypertensive efficacy to that of other angiotensin receptor blocker/HCTZ combinations. It is a well-tolerated combination and its tolerability profile was superior to that of lisinopril/ HCTZ having similar efficacy profile [3,4].

Simultaneous estimation of drug combinations was reported by many researchers by using chromatographic and hyphenated techniques such as HPLC, GCMS, LCMS, and others. Many cost-effective, simple, accurate, and precise methods have also been developed for the quantification of drugs in routine analysis of its formulation [7]. In literature, simultaneous estimation of VAL and HCTZ by spectrophotometric technique [8] and estimation of amlodipine, VAL, and HCTZ by HPLC in dosage form and spiked plasma were reported [9]. In addition, VAL and HCT were also quantified by five-derivative ultraviolet spectrophotometry and HPLC in pharmaceutical dosage form [10]. Estimation of amlodipine besylate, HCTZ, and VAL by reversed-phase HPLC (RP-HPLC), high-performance thin-layer chromatography (HPTLC), and ultraviolet (UV) spectrophotometric methods [11] were also reported in bulk and combined tablet dosage form. A method for the simultaneous estimation of olmesartan medoxomile, amlodipine besylate, and HCTZ by RP-HPLC was also reported [12]. Many works of literature have revealed different methods for the estimation of VAL alone and in combination with other antihypertensive drugs such as HPLC [13–15] and LC-MS [16–18]. Simultaneous quantification of VAL and HCTZ in combination and with other drugs was also reported in plasma samples [19–22]. The reported HPLC with U/V detection has poor sensitivities (300 ng/mL for both VAL and HCTZ [18]) and a single internal standard (IS) was used for all analyte quantification with high consumption of solvents (mobile phase) [19–21], which cannot be considered as a reliable and environ-

mentally friendly method. The reported LC-MS/MS method has better sensitivities, but these methods were also based on single IS, large consumption of solvent in mobile phase (flow rate 1.2 mL/min), and high matrix effects (mean value 32.3% for HCTZ) [21]. The method reported by Gadepalli SG [22] uses a mixed mode (both positive and negative) of ionization method with high run time (5 min) and does not reveal the matrix effects data. Therefore, the present research work is designed for the determination of VAL and HCTZ by HPLC-MS/MS analytical technique in human plasma by using irbesartan (IRB) and hydrofluoromethiazide (HFMZ) as separate internal standards for VAL and HCTZ, respectively. This method was successfully applied for bioequivalence studies and can be used for routine therapeutic drug monitoring (TDM) as well as for the pharmacokinetics study of this combination.

2. Materials and Methods

2.1. Chemicals and Reagents

The working standards of VAL and IRB (percentage purity $\geq$ 98.5%) were obtained from SynFine Research, Richmond Hill, Ontario, Canada, whereas the HCTZ and HFMZ (percentage purity $\geq$ 97%) were obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were obtained from Spectrochem Pvt Ltd. (Mumbai, India), whereas the ammonium acetate of analytical grade was obtained from Qualigens Fine Chemicals (Mumbai, India). Milli-Q grade water was used for all aqueous sample preparation. Oasis HLB 30 mg/1 cc cartridges was used for the solid phase extraction procedure.

2.2. Instrumentation and Chromatographic Condition

A Shimadzu LC10 HPLC from Shimadzu coupled with API 3000 tandem mass spectrometer (Applied Biosystems, Waltham, MA, USA) is used in this study. Analyst Software (Version 1.4.1, Applied Biosystems, Waltham, MA, USA) was used for sample acquisition, data integration, and processing.

The mobile phase comprising of acetonitrile and 10 mM ammonium acetate buffer (95:05 v/v) was eluted at flow rate of 0.5 mL/min in isocratic mode. The Lichrocart RP Select (125 $\times$ 4 mm) 5 nm column was used for separation of analytes and ISs. The column oven temperature was set at 35 $\pm$ 2 °C, while the auto-sampler temperature was 5 $\pm$ 1 °C. The optimized triple quadrupole detector (TQD) parameters were: Ion source: Turbo Ion Electro Spray ionization (ESI) in negative ion mode, m/z ion: VAL 434.10 > 179.10; HCTZ 295.70 > 204.90; IRB (IS) 427.10 > 192.90, and HFMZ (IS) 329.90 > 302.40. Run Time: 3.0 min, RT: IRB 2.1 min (approx.), HCTZ 2.30 min (approx.), VAL 1.9 min (approx.), HFMZ 2.00 min (approx.) (Table 1).

Table 1. Optimized LC-MS/MS conditions for VAL, IBR, HCTZ, and HFMZ.

Analyte	RT (min)	ESI Mode	Precursor Ion	Product Ion
VAL	1.9	Negative mode	434.10	179.10
HCTZ	2.3	Negative mode	295.70	204.90
IBR	2.1	Negative mode	427.10	192.90
HFMZ	2.0	Negative mode	329.90	302.40

2.3. Stock and Working Solutions Preparation

Stock solution of VAL, IRB, HCTZ, and HFMZ were prepared in methanol: water-50:50 (v/v) to achieve a final concentration of 1mg/mL solution. The stock solution was further diluted with same diluents to prepare working solution for calibration standard (CS) and quality control (QC) samples.

2.4. Preparation of Calibration Standard (CSs) and Quality Control (QCs) Samples

VAL: Eight dilutions over the concentration range from 50.2 to 6018.6 ng/mL were prepared by spiking the appropriate amount of working solutions. QC samples were also prepared at 50.9 ng/mL (LLOQ), 145.5 ng/mL (LQC), 2597.4 ng/mL (MQC), and

3684.2 ng/mL (HQC) by similar procedure. These samples were stored below −80 °C until used. Generation of stability data below −80 °C was transferred to twelve sets of LQC and HQC.

HCTZ: Eight dilutions over the concentration range from 1.25 to 507.63 ng/mL were prepared by spiking the appropriate amount of working solution of HCTZ. QC samples were prepared of 1.27 ng/mL (LLOQ), 3.35 ng/mL (LQC), 186.24 ng/mL (MQC), and 310.40 ng/mL (HQC). These samples were stored below −80 °C until used. Generation of stability data below −80 °C was transferred to twelve sets of LQC and HQC.

2.5. Sample Preparation

The CSs, QCs, and actual samples were withdrawn from the deep freezer and allowed to thaw at room temperature before the processing procedure. After proper vortexing, an aliquot of 500 µL of plasma was transferred into prelabeled polypropylene tubes. Then, 50 µL IS mixture of HFTZ (2 µg/mL) and IRB standard (1 µg/mL) were added and vortex-mixed.

Solid phase extraction method: First, conditioning of the HLB cartridge (30 mg/1 cc) was performed by using 1.0 mL of methanol followed by 1.0 mL of Milli-Q water/HPLC grade water. Then samples were loaded and the cartridges were washed with 1.0 mL of Milli-Q water/HPLC grade water and after that the sample was eluted with 1.0 mL of methanol. The eluted samples were evaporated to dryness at 40 °C at constant pressure in a nitrogen evaporator and then the samples were reconstituted in 500 µL of mobile phase (10 mM ammonium acetate buffer: acetonitrile: 05:95, *v*/*v*) and transferred into HPLC vials. Once the vials were placed in the autosampler, 10 µL was injected into LC-MS/MS for sample analysis.

2.6. Method Validation

Validation of the developed method was performed as per the USFDA guidance and experimented with system suitability, selectivity, carryover, linearity, accuracy and precision, recovery, matrix effect, stability, dilution integrity, and ruggedness [23,24].

2.6.1. System Suitability

The experiment was performed by injecting six samples using an aqueous standard mixture of analytes and ISs at the beginning of each batch. Samples were then injected with one extracted blank (without analytes and ISs) and one extracted LLOQ sample with ISs to check the system performance.

2.6.2. Selectivity

Six blank plasma samples obtained from six different plasma lots were processed along with plasma spiked with LLOQ concentration with ISs. All samples were injected and the responses of blank samples were compared with responses of LLOQ samples. For method acceptance, the blank samples' responses should be ≤20% of LLOQ and ≤5% for IS.

2.6.3. Sensitivity

The sensitivity of the method was determined by measuring the LLOQ of the assay. The LLOQ was determined by measuring the lowest concertation of the CSs whose response should be five times higher than the blank samples. Consequently, its back calculated concentration must be 80–120% accuracy and precision within a ±20% range.

2.6.4. The Calibration Curves (CCs) and Linearity

The linearity was determined by plotting the CCs between response ratio of analytes/IS versus nominal concentration of analytes. Three different CCs were prepared between the concentration ranges of 50.2 to 6018.6 ng/mL for VAL and 1.25 to 507.63 ng/mL for HCTZ. A regression equation with weighting factors of 1/x, 1/x2, and none was judged to produce the best fit for the concentration-detector response relationship for HCTZ and

VAL in human plasma. To obtain the goodness of fit, the back-calculated concentration of CC standards that meet the acceptance criteria using (1/x and 1/x2 weighing) was measured.

2.6.5. Precision and Accuracy

The precision of the assay was measured by the percentage coefficient of variation (% CV) over the concentration range of LLOQ and all three QC concentrations (LQC, MQC, and HQC) samples during the validation study. Similarly, the accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ and all QCs (LQC, MQC, and HQC) samples to their respective nominal values, expressed in percentage (%). The intra-day precision and accuracy were evaluated by measuring on same day, while inter-day were evaluated by measuring on three consecutive days in six replicates for each sample. The acceptance limit for precision was $\leq$15% of % CV (except LLOQ, $\leq$20 %) while for accuracy, it should be within $\pm$15% (except LLOQ, $\pm$20%) as compared to its nominal values.

2.6.6. Stability Studies

The stability of VAL and HCTZ in plasma samples was evaluated at different anticipated conditions by using LQC and HQC concentration in six replicates. The stability of aqueous solutions (stock and working solution) at refrigerator and room temperature were also evaluated. All stability study samples were quantified against freshly prepared CCs.

Short-term (bench top) stability: Short-term benchtop stability was determined by processing of the spiked plasma samples after maintaining them at ambient temperature for around 7 h.

Auto-sampler stability: To assess the auto-sampler stability, the spiked samples were processed and analyzed after putting them in auto-sampler for 66 h.

Freeze-thaw stability: The stability of analytes in human plasma was determined for three freeze-thaw cycles. Six replicates of LQC and HQC were processed and analyzed after undergoing three freeze-thaw cycles.

Long-term stability: The stability of HCTZ and IBR for long-term storage were evaluated by processing the spiked QC samples after storing at -80 °C in a deep freezer for 60 days.

2.6.7. Anticoagulant Effect

Six sets of LQC and HQC were prepared in EDTA pooled plasma for checking the anticoagulant effect. These quality control samples were quantified against the freshly spiked calibration curve standards.

2.6.8. Dilution Integrity

Twelve sets of dilution integrity samples were prepared by spiking 1.5 times (9005.9 ng/mL) with the highest standard concentration (6018.3 ng/mL) of VAL and 1.6 times (871.75 ng/mL) of the highest standard concentration (507.63 ng/mL) of HCTZ. Six sets of dilution integrity samples were processed by diluting them twice and another six sets by diluting them four times using screened pooled plasma. These QCs samples were analyzed along with processed calibration curve standards.

2.6.9. Recovery and Matrix Effect

The recovery and matrix effects of VAL and HCTZ were determined at LQC and HQC concentrations in six replicates. The percentage recovery was determined by comparing the responses of plasma spiked with QCs before extraction (pre-extracted plasma samples) with responses of plasma spiked with QCs sample after extraction (post-extracted plasma samples).

The matrix effects that are produced due to the presence of endogenous, xenobiotic, and other substances in plasma were also evaluated using the precolumn infusion method. The matrix effects that may produce ion suppression or ion enhancement effects were

determined by comparing the responses of plasma spiked with QCs sample after extraction (post-extracted plasma samples) with samples in mobile phase solution (aqueous solution) [25].

2.6.10. Ruggedness

One complete precision and accuracy batch was processed and analyzed by different analysts using different columns to ensure the ruggedness of the method.

2.7. Application of the Developed Method in Bioequivalence Study

The developed assay was successfully applied to a bioequivalence study to compare the developed formulation with innovator formulation of FDC of VAL and HCTZ in healthy subjects. The study was performed to compare the C_{max} and AUC of the developed formulation with innovator product in order to ensure similar pharmacokinetic profile (unpublished data).

3. Results and Discussion

3.1. Method Development

Initially, for mass spectrometry parameter optimization, the 500 µg/mL standard solutions of VAL, HCTZ, IRB, and HFMZ were infused directly into the system for ESI-based ionization in both positive and negative ion modes. The observed parent mass spectra in negative mode showed prominent deprotonated molecular ions [M-H] of m/z 434.10, 295.70, 427.10, and 329.90 for VAL, HCTZ, IRB, and HFMZ, respectively. These prominent molecular ions of respective analytes were subjected to collision-induced dissociation (CID), which produced the most intense fragment ions of *m/z* 179.10, 204.90, 192.90, and 302.40 for VAL, HCTZ, IRB, and HFMZ, respectively. The MS/MS spectra of analytes are presented in Figure S1. For better chromatographic separations, several combinations of organic modifiers (acetonitrile, methanol) with aqueous buffers (formic acid, ammonium acetate) were attempted with different percentages, ratios, and flow rates. Overall, acetonitrile in combination with 10 mM ammonium acetate buffer (95:05 *v*/*v*), at flow rate of 0.5 mL/min, produced better peak shape and appropriate retention time for all analytes and IS and was considered as the mobile phase of this method. Attempts have also been made to optimize the sample preparation method for better recovery and low matrix effects. Among the available sample extraction methods, SPE by using HLB cartridge produced high recovery and insignificant matrix effects.

3.2. System Suitability

The precision (% CV) for system suitability was in the range of 0.32% to 0.85% for the retention time and 1.2% to 1.6% for the area response of all the analytes and ISs during study. Moreover, the signal-to-noise ratio for system performance was $\geq$16 for all the analytes and ISs.

3.3. Selectivity

No significant interference was shown at the retention time and *m/z* of HCTZ, VAL (analyte), HFMZ, and IRB (IS) in all the batches screened. These results confirmed that the developed method is selective for simultaneous determination of VAL and HCTZ in plasma samples. The representative chromatogram of blank plasma samples is presented in Figure 2.

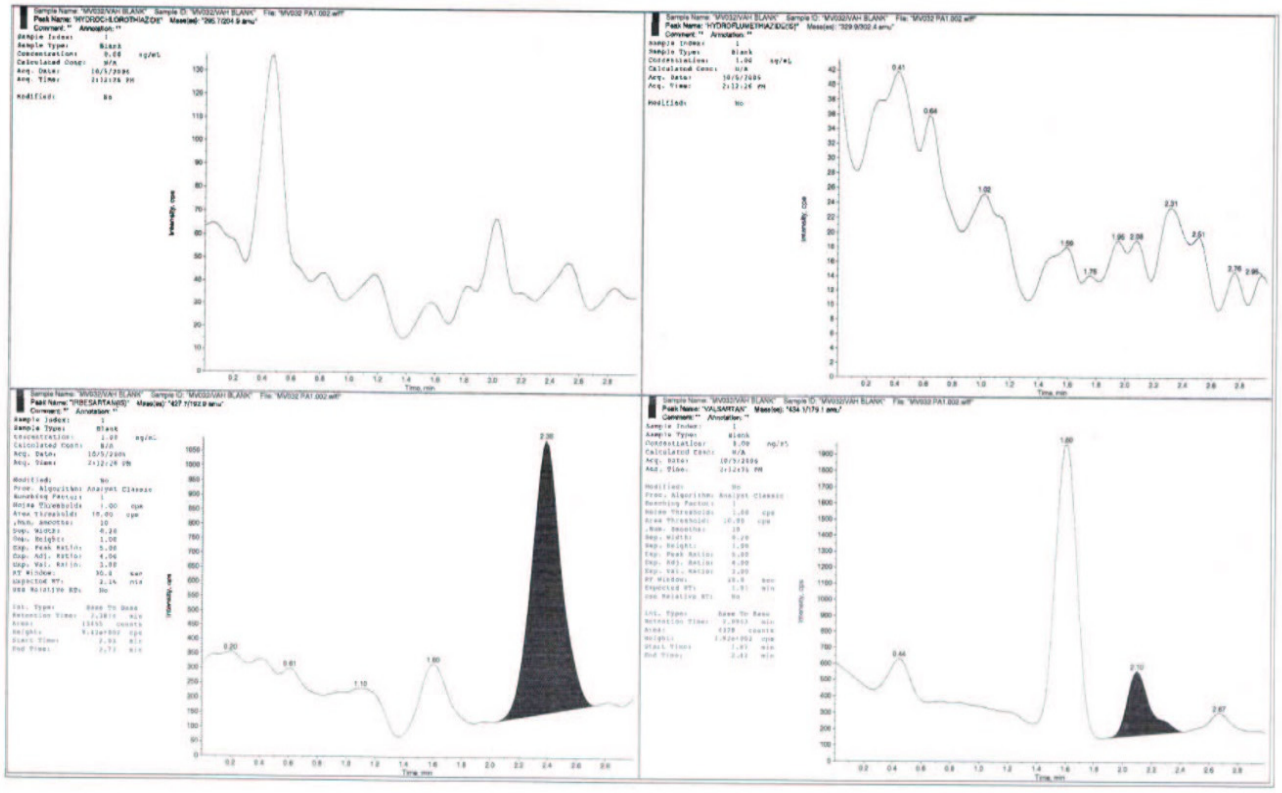


Figure 2. The representative chromatograms of HCTZ (**upper left**), HFMZ (**upper right**), IRB (**lower left**) and VAL (**lower right**) in human blank plasma samples.

3.4. Sensitivity

For the six replicate injections of VAL and HCTZ into LLOQ, the mean value ± % CV was 51.52 ng/mL ± 1.57 for VAL and 1.206 ng/mL ± 1.91 for HCTZ, respectively. Therefore, the LLOQ of the analytes, VAL and HCTZ, were fixed at 50.9 and 1.27 ng/mL, respectively. The representative chromatogram of plasma spiked at LLOQ level samples are presented in Figure 3.

3.5. Linearity and Goodness of Fit

The developed assay was found to be linear between the concentration ranges of 50.2 and 6018.6 ng/mL and 1.25 and 507.63 ng/mL, for VAL and HCTZ, respectively. The back-calculated concentration of VAL and HCTZ were found to be within the acceptable range of ±15% accuracy. The correlation coefficient (R2) was ≤0.995. The results of linearity parameters are presented in Table 2. Experiment results indicated that 1/x2 was found to be the best fit for regression as shown in Supplementary Figure S2.

Table 2. Concentration-response linearity of VAL and HCTZ in spiked plasma.

Drugs	Valsartan	Hydrochlorothiazide
Concentrations range (ng/mL)	50.2–6018.6	1.25–507.63
SD	0.807–524.011	0.023–16.438
%CV	1.57–8.67	1.91–4.73
Linearity equation	Y = 9.74005x + 0.0027	Y = 0.00754x + 0.00378
R^2	0.9967	0.9956
LLOQ (ng/mL)	50.9	1.27
LOQ (ng/mL)	145.5	3.35

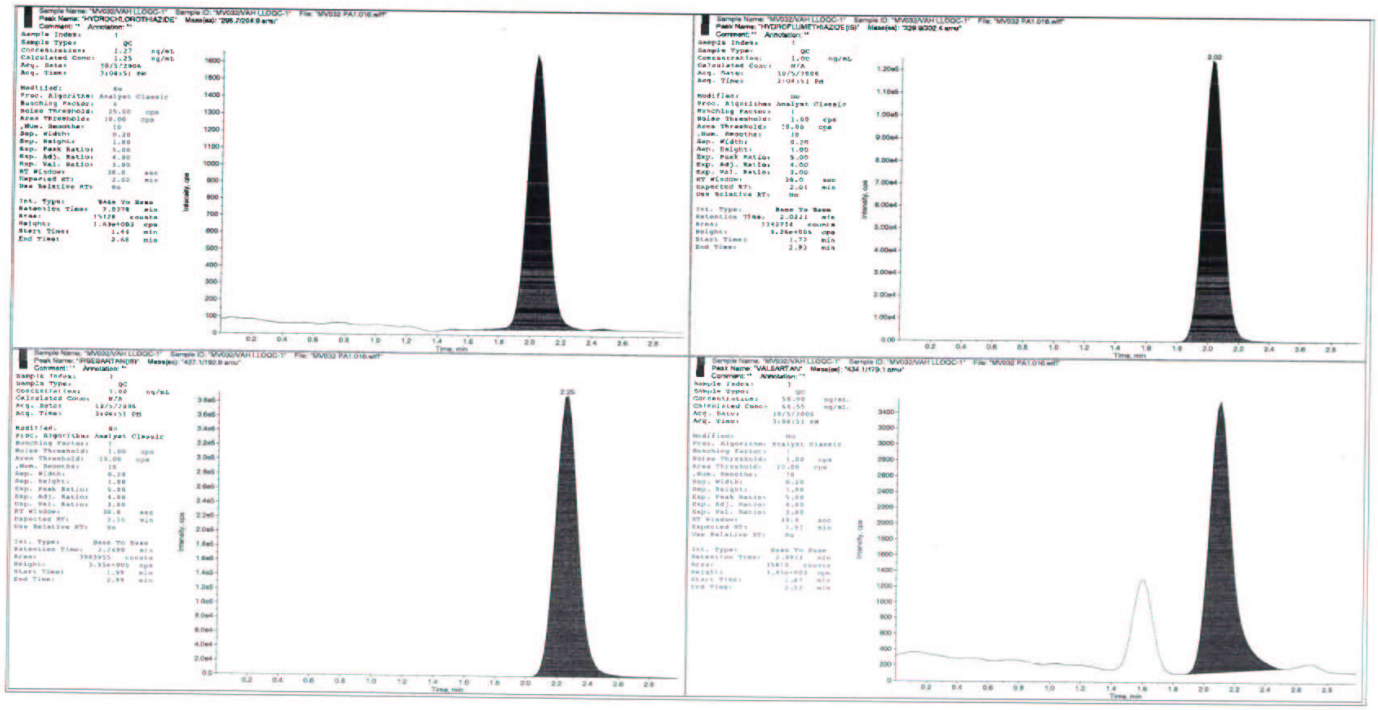


Figure 3. The representative chromatograms of HCTZ (**upper left**), HFMZ (**upper right**), IRB (**lower left**) and VAL (**lower right**) in human blank plasma spiked at LLOQ concentration level.

3.6. Precision and Accuracy

Within-batch (intra-day) and between-batch (inter-day) precision and accuracy data for HCTZ and VAL were determined for each QC sample value including LLOQ and are presented in Table 3. The intra-day and inter-day precision values for VAL were ≤10.22 and 12.45% (%, RSD), respectively, whereas for HCTZ they were ≤3.71 and 10.78% (%, RSD), respectively. The intra-day and inter-day accuracy data for VAL were within the range of 105.68–114.22% and 98.41–108.16%, respectively, whereas for HCTZ they were 87.01–101.18% and 95.16–99.37%, respectively. These results were found to be within the acceptable limits of FDA guidelines that confirmed that the developed method in human plasma is reliable and accurate for simultaneous quantification of VAL and HCTZ.

Table 3. Intra and inter-day precision and accuracy data of VAL and HCTZ in human plasma.

Nominal QC (ng/mL)	Precision (RSD, %)		Accuracy (%)	
	Intra-Day (n = 5)	Inter-Day (n = 15)	Intra-Day (n = 5)	Inter-Day (n = 15)
VAL				
50.9	10.22	12.40	114.22	108.16
145.5	4.76	12.0	108.06	99.13
2597.4	6.76	10.90	105.68	101.02
3684.2	8.68	11.15	106.60	98.41
HCTZ				
1.27	3.71	7.41	101.18	99.37
3.35	2.82	10.78	87.01	95.16
186.24	1.57	5.19	93.32	96.79
310.40	2.18	2.52	95.83	96.03

3.7. Extraction Recovery and Matrix Effects

The ER % and MF % data for both VAL and HCTZ are presented in Table 4. The overall mean ER % of VAL and HCTZ were 78.57 and 86.70%, respectively, with % RSD of ≤15% by using the proposed SPE method. These results confirmed that the recovery is consistent and

concentration independent for both analytes of interest. Similarly, the % MF for VAL and HCTZ, which was evaluated by using the precolumn infusion (quantitative) method, were also within the range of 85–115% with % RSD $\leq$ 15%, and was insignificant as mentioned in the guideline for bioanalytical method validation [23,24]. Ion suppression effects were observed for VAL, while ion enhancement effects were observed for HCTZ and were produced due to the endogenous plasma sample substances. Overall, the proposed SPE method for sample preparation is suitable for better cleanup procedure with high recoveries and suitable for accurate quantification of VAL and HCTZ in human plasma samples.

Table 4. Extraction recovery and matrix effects data of VAL and HCTZ in human plasma (n = 5).

Compound	QC Level	ER %		MF %	
		% Mean	% RSD	% Mean	% RSD
VAL	LQC	73.51	6.95	88.34	4.29
	MQC	71.54	5.80		
	HQC	90.65	3.69	94.43	1.96
	Mean	78.57	13.38		
HCTZ	LQC	86.65	6.00	109.02	9.44
	MQC	78.61	6.67		
	HQC	94.84	3.01	89.93	9.70
	Mean	86.70	9.36		

3.8. Anticoagulant Effect

The mean overall accuracy of VAL is 101.512% (LQC) to 102.537% (HQC) and precision ranged from 6.97% (LQC) to 15.32% (HQC), and for HCTZ, mean overall accuracy ranged from 96.915% (LQC) to 98.035% (HQC) and precision ranged from 5.48% (HQC) to 11.87% (LQC). The result showed the absence of any anticoagulant effect.

3.9. Dilution Integrity

The quality control sample concentrations were calculated using the appropriate dilution factor. The within-batch precision and accuracy, for a dilution factor of 4 of VAL, were 3.25% and 90.931%, respectively. The dilution integrity with dilution factor-2 of VAL was 7.94% and 96.801%, respectively. The within-batch precision and accuracy, for a dilution factor of 4 of HCTZ were 3.52% and 100.426%, respectively. The dilution integrity with dilution factor-2 of HCTZ was 2.43% and 106.082%, respectively. Results demonstrated acceptable dilution integrity for both VAL and HCTZ in human plasma samples.

3.10. Ruggedness

The mean accuracy for VAL means accuracy ranged from 95.725% (LQC) to 113.883% (LLOQ QC) and the precision ranged from 9.80% (LQC) to 20.00% (LLOQ QC). The mean accuracy for HCTZ ranged from 91.178% (HQC) to 98.294% (LQC) and the precision ranged from 0.87% (MQC) to 10.00% (LLOQ QC).

3.11. Stability Studies

The stabilities data are presented in Table 5. Short-term (bench top) stability: HCTZ and VAL were found to be stable for around 7 h as per the acceptance criteria. The nominal percentage for VAL ranged from 96.770% (HQC) to 107.010% (LQC) and the precision ranged from 7.90% (LQC) to 10.57% (HQC). The nominal percentage for HCTZ ranged from 98.955% (LQC) to 101.416% (HQC), respectively, and the precision ranged from 4.09% (HQC) to 7.54% (LQC), respectively.

Autosampler stability: The nominal percentage at around 66 h ranged from 86.925% (LQC) to 97.992% (HQC) and precision ranged from 6.03% (HQC) to 7.05% (LQC), respectively, for HCTZ, and nominal VAL percentage at around 66 h ranged from 98.281% (HQC) to 101.677% (LQC) and precision ranged from 4.24% (LQC) to 13.44% (HQC), respectively.

Table 5. Stability and dilution integrity data of VAL and HCTZ in spiked plasma at different storage conditions and processes samples (n = 5).

	VAL			HCTZ		
	Nominal Concentration (ng mL^{-1})	**Precision (CV, %)**	**Accuracy (%)**	**Nominal Concentration (ng/mL)**	**Precision (CV, %)**	**Accuracy (%)**
Stability						
Short term (7 h)	145.5	7.90	107.01	3.35	7.54	98.95
	3684.2	10.57	96.77	310.40	4.09	314.79
Freeze thaw (3 cycle)	145.5	4.47	111.16	3.35	7.13	99.15
	3684.2	10.15	98.13	310.40	6.32	97.73
Auto-sampler (66 h)	145.5	4.24	101.67	3.35	7.05	86.92
	3684.2	13.44	98.28	310.40	6.03	90.93
Long term (60 days)	145.5	11.94	87.55	3.35	5.81	112.58
	3684.2	7.68	102.17	310.40	5.00	105.85
Dilution integrity	2-times	7.94	96.80		2.43	106.08
	4-times	3.25	90.93		3.52	100.42

Freeze-thaw stability—three cycles: The nominal percentage ranged from 97.736% (HQC) to 99.154% (LQC) and precision ranged from 6.32% (HQC) to 7.13% (LQC), respectively, for HCTZ, and for VAL the nominal percentage ranged from 98.138% (HQC) to 111.168% (LQC) and precision ranged from 4.47% (LQC) to 10.15% (HQC), respectively.

Long term stability at −80 °C: The stability for HCTZ and VAL was compared against the nominal mean % of LQC and HQC of precision and accuracy batch-1. The mean stability of HCTZ ranged from 105.850% (HQC) to 112.579% (LQC) and the precision ranged from 5.00% (HQC) to 5.81% (LQC), and for VAL, the mean stability ranged from 87.554% (LQC) to 102.168% (HQC) and the precision ranged from 7.68% (HQC) to 11.94% (LQC).

4. Conclusions

In this study a new simple, accurate, and precise method for the simultaneous determination of VAL and HCTZ in human plasma was developed using separate internal standards for both analytes. The SPE method was used for sample extraction, which produced high recoveries for VAL (78.57%) and HCTZ (86.70%) from plasma samples. The ion-suppression effects produced for VAL and ion-enhancement effects produced for HCTZ were insignificant for the proposed sample cleanup procedure. The results of the validation parameters met the acceptance criteria as described in USFDA and EMEA guidelines for bioanalytical method validation. The ranges for the calibration curves of VAL and HCTZ were 50.2–6018.6 ng/mL and 1.25–507.63 ng/mL, respectively, with good linearity, having correlation coefficient values of $\geq$0.995 for both VAL and HCTZ. The intra-day and inter-day accuracy data for VAL were within the ranges of 105.68–114.22% and 98.41–108.16%, respectively, whereas for HCTZ they were 87.01–101.18% and 95.16–99.37%, respectively. The developed method was successfully applied in bioequivalence study to compare the rate and extent of absorption of the newly developed formulation against the innovator formulation in healthy human subjects. The proposed method can be used for the routine analysis of plasma samples obtained in the conducting of pharmacokinetic studies and therapeutic drug monitoring in clinical therapy.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations10020119/s1, Precursor to product ion spectra (Figure S1). Best fit for regression (Figure S2) and aqueous stability data (Table S1).

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