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Simultaneous Determination of Valsartan and Chlorthalidone in Human Plasma by Using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

A sensitive and reliable LC-MS/MS method was established and validated for the simultaneous quantification of valsartan (VAL) and chlorthalidone (CHL) in human plasma. Sample preparation involved a liquid-liquid extraction approach. Chromatographic separations of the analytes and their isotopically labeled internal standards, valsartan-d9 (VALD9) and chlorthalidone-d4 (CHLD4), were achieved using a Waters XBridge C18 column (100 × 4.6 mm, 3.5 µm). Detection was carried out via electrospray ionization in negative mode, employing multiple reaction monitoring (MRM). The ion transitions monitored were m/z 434.2 → 179.1 for VAL and m/z 337.1 → 146.05 for CHL. Calibration curves exhibited good linearity over concentration ranges of 25 - 20,000 ng/mL for valsartan and 2 - 1000 ng/mL for chlorthalidone. The validated method demonstrates suitability for use in bioequivalence assessments involving human plasma samples.

Keywords

Valsartan, Clorthalidone, Valsartan-d9, Clorthalidone-d4, Human Plasma, LC-MS/MS

1. Introduction

Valsartan is chemically described as N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-L-valine [1]. Valsartan is an orally active specific angiotensin II type 1 receptor blocker effective in lowering blood pressure in hypertensive patients [2]. And Chlorthalidone is an oral antihypertensive/diuretic. It is a

monosulfamyl diuretic that differs chemically from thiazide diuretics in that a double-ring system is incorporated into its structure. Chlorthalidone is chemically described as 2-chloro-5(1-hydroxy-3-oxo-1-isoindolinyl) benzenesulfonamide [3].

Various analytical techniques have been established for the quantification of valsartan (VAL) in human plasma, either alone or in combination with other pharmaceutical agents [4]-[10]. Similarly, multiple chromatographic approaches have been documented for the analysis of chlorthalidone (CHL) [9]-[15]. A literature was reported about VAL and CHL for application to commercially available drug products in tablet dosage form [16]. Iriarte *et al.* have developed an UPLC-UV method for simultaneous determination of valsartan and chlorthalidone using solid phase extraction [17]. While their method demonstrated acceptable performance, UV detection typically lacks the sensitivity and selectivity required for pharmacokinetic or bioequivalence studies, especially at low plasma concentrations. However, to date, no LC-MS/MS method utilizing liquid-liquid extraction for the simultaneous quantification of VAL and CHL in plasma which was suitable for pharmacokinetic or bioequivalence studies, has been reported in the literature. Given the need for more accessible and cost-effective sample preparation techniques in bioanalysis, liquid-liquid extraction was selected over solid-phase extraction due to its simplicity and reduced cost. Therefore, the objective of this study was to develop a straightforward, rapid, economical, and highly sensitive LC-MS/MS method for the simultaneous determination of VAL and CHL in human plasma samples, with potential application in clinical trial settings.

2. Experimental

2.1. Chemicals and Materials

Valsartan (purity 99.9%) was kindly supplied by Jubilant Life Sciences (Mysore, India). Chlorthalidone (purity 100.7%) was purchased from IPCA Laboratories (Mumbai, India). Valsartan-d9 (purity 99%) and Clorthalidone-d4 (purity 99%) was obtained from TLC pharmaceutical standards (Vaughan, Canada). Methanol, acetonitrile, formic acid, diethyl ether, hydrochloric acid HCl) 37% and ammonia solution 25% were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Lithium heparin blank human plasma was obtained from Equitech Enterprises Inc (Texas, USA). The water was purified in a Millipore MilliQ water purification system (USA).

2.2. Stock Solutions, Calibration Standards and QCs

Stock solutions of VAL and CHL were prepared by accurately dissolving appropriate quantities of each compound in methanol. Subsequent standard solutions were obtained through serial dilution of the stock solutions with methanol. A working internal standard (IS) solution containing VALD9 and CHLD4 was also prepared in methanol at a final concentration of 2.5 μg/mL. All stock solutions, including those of VAL, CHL, VALD9 and CHLD4 were stored at −20˚C to ensure

stability.

Calibration standards were prepared by spiking blank human plasma with defined volumes of the standard solutions to yield final concentrations of 25, 50, 500, 3000, 6000, 10,000, 18,000, and 20,000 ng/mL for VAL and 2, 4, 20, 50, 250, 500, 900 and 1000 ng/mL for CHL. Quality control (QC) samples were similarly prepared at concentration levels of 25 ng/mL (lower limit of quantification, LLOQ), 75 ng/mL (low QC), 8000 ng/mL (medium QC), 15,000 ng/mL (high QC), and 20,000 ng/mL (upper limit of quantification, ULOQ) for VAL, and 2 ng/mL (LLOQ), 6 ng/mL (low QC), 400 ng/mL (medium QC), 750 ng/mL (high QC), and 1000 ng/mL (ULOQ) for CHL. All calibration and QC plasma samples were stored at −70˚C until analysis.

2.3. Instrumentation

The LC-MS/MS analysis was performed using a Waters ACQUITY liquid chromatography system coupled with a tandem quadrupole (TQ) mass spectrometer equipped with an electrospray ionization (ESI) source (Waters Corp., Milford, MA, USA). Data acquisition and processing were carried out using MassLynx version 4.1 software in conjunction with the QuanLynx application (Waters Corp., USA).

Chromatographic separation was achieved on a Waters XBridge C18 column (100 × 4.6 mm, 3.5 µm particle size). The mobile phase consisted of acetonitrile and water (80:20, v/v) containing 0.0075% ammonia solution (25%), delivered isocratically at a flow rate of 0.5 mL/min. The column oven was maintained at 30˚C, and the autosampler was kept at 10˚C. The total run time for each analysis was 3.5 minutes.

Mass spectrometric detection was carried out in negative electrospray ionization (ESI) mode using multiple reaction monitoring (MRM). The monitored MRM transitions were m/z 434.2 → 179.1 for VAL, m/z 337.1 → 146.05 for CHL, m/z 443.2 → 179.1 for VALD9, and m/z 341.1 → 150.05 for CHLD4. The cone voltage was set at 40 V for VAL and VALD9, and at 35 V for CHL and CHLD4.

The ion source temperature and desolvation temperature were maintained at 150˚C and 400˚C, respectively. High-purity nitrogen (generated using a Peak Scientific NL-60 system) was employed as both the cone and desolvation gas. The desolvation gas and cone gas flow rates were adjusted to 600 L/h and 50 L/h, respectively. The capillary voltage was set to 3.2 kV. Data acquisition and processing were performed using MassLynx™ Version 4.1 software (Waters Corp., USA).

2.4. Sample Preparation

A volume of 125 µL of 0.2 M hydrochloric acid (HCl) solution and 50 µL of the internal standard (IS) solution were added to 100 µL of plasma sample. The mixture was vortexed for 10 seconds to ensure thorough mixing. Subsequently, 3 mL of diethyl ether was added, followed by vortexing for an additional 30 seconds to facilitate extraction. Samples were then centrifuged at 4600 rpm for 10 minutes.

The resulting organic (upper) layer was carefully transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen at 40˚C. The dried residue was reconstituted with 200 µL of the mobile phase. Finally, the reconstituted samples were transferred to autosampler vials, and a 20 µL aliquot was injected into the LC-MS/MS system for analysis.

3. Results and Discussion

3.1. Method Validation

3.1.1. Selectivity

Figure 1 shows the chromatograms (a) blank plasma spiked with IS and analytes at LLOQ and (b) drug-free human plasma. The method selectivity was determined by analyzing ten different sources of human plasma to demonstrate the lack of chromatographic interference at the retention time of VAL, CHL, VALD9 and CHLD4.

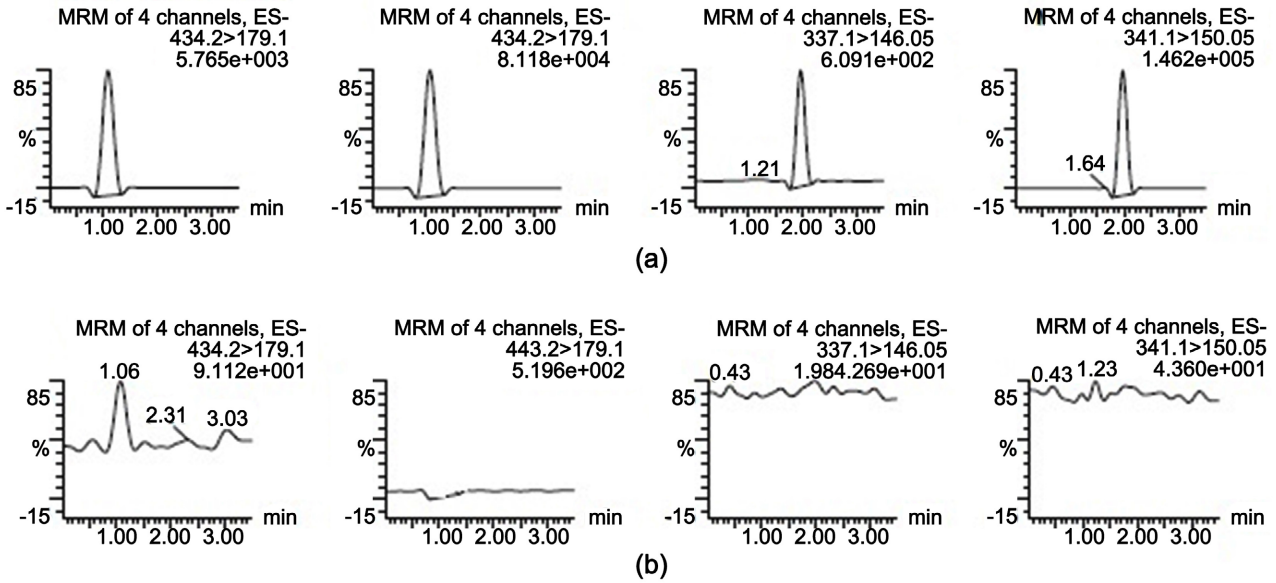

Figure 1. MRM chromatograms of 25 ng/mL (LLOQ) for valsartan and 2 ng/mL (LLOQ) for chlorthalidone spiked with internal standards (a) and blank human plasma (b).

3.1.2. Linearity

The calibration curves were created by plotting the peak area ratios of the various analytes to internal standard versus nominal concentration of the analytes standards. Both calibration curves were regressed using a linear equation with weighting factor of $1/X^2$. Calibration curves were linear with coefficient of correlation (r^2) values more than 0.9971. Range was 25 - 20,000 ng/mL and 2 - 1000 ng/mL for VAL and CHL, including the LLOQ. Calibration curves were constructed from the blank sample (processed matrix sample without analyte and without IS), a zero sample (processed matrix with IS) and eight non-zero samples. The standard concentration must be within 15% deviation from the nominal value except at LLOQ, for which the maximum acceptable deviation was set as 20%. At least 75% of eight

non-zero samples must be met exception criteria including acceptable LLOQ and ULOQ [18] [19].

3.1.3. Accuracy and Precision

The intra- and inter-day precisions and accuracies of LLOQ, QC Low, QC Medium and QC High and ULOQ of the analytes were summarized in **Tables 1(a)-(d)**. Six replicates of QC samples were analyzed at five concentration levels respectively to evaluate the intra-day precisions and accuracies. This process was repeated on three consecutive days in order to evaluate inter-day precisions and accuracies.

Table 1. (a) Intra-day precision and accuracy of the method for determining valsartan in plasma samples; (b) Inter-day precision and accuracy of the method for determining valsartan in plasma samples; (c) Intra-day precision and accuracy of the method for determining chlorthalidone in plasma samples; (d) Inter-day precision and accuracy of the method for determining chlorthalidone in plasma samples.

(a)

	Batch No: 1 (n = 6)			Batch No: 2 (n = 6)			Batch No: 3 (n = 6)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
25	24.63 ± 0.71	−1.45	2.88	25.65 ± 0.56	2.62	2.19	24.66 ± 0.79	−1.36	3.22
75	70.39 ± 2.59	−6.14	3.67	76.12 ± 1.77	1.50	2.32	75.68 ± 1.35	0.91	1.78
8000	7462.17 ± 164.82	−6.72	2.21	8059.89 ± 262.41	0.75	3.25	8226.45 ± 72.85	2.83	0.89
15,000	14234.37 ± 625.69	−5.10	4.39	15939.20 ± 691.33	6.26	4.34	16071.10 ± 430.34	7.14	2.68
20,000	17893.35 ± 722.55	−10.53	4.04	20115.05 ± 671.17	0.58	3.34	20715.77 ± 474.25	3.58	2.29

(b)

	Batch No: 1 - 3 (n = 18)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
25	24.98 ± 0.81	−0.06	3.26
75	74.07 ± 3.26	−1.24	4.40
8000	7916.17 ± 379.23	−1.05	4.79
15,000	15414.89 ± 1025.21	2.77	6.65
20,000	19574.72 ± 1382.94	−2.13	7.06

Conc: Concentration, n: Replicates at each concentration, RE: Relative error, CV: Coefficient of Variation, SD: Standard Deviation.

(c)

	Batch No: 1 (n = 6)			Batch No: 2 (n = 6)			Batch No: 3 (n = 6)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
2	1.91 ± 0.09	−4.69	4.67	1.94 ± 0.33	−2.97	16.8	1.98 ± 0.13	−0.92	6.62
6	5.39 ± 0.18	−10.15	3.33	5.53 ± 0.35	−7.87	6.39	5.84 ± 0.36	−2.63	6.09
400	375.21 ± 5.75	−6.20	1.53	420.30 ± 11.16	5.07	2.66	419.51 ± 8.01	4.88	1.91
750	680.48 ± 27.91	−9.27	4.10	776.45 ± 20.20	3.53	4.34	761.79 ± 13.12	1.57	1.72
1000	890.49 ± 23.31	−10.95	2.62	1022.86 ± 23.66	2.29	2.31	1001.12 ± 9.68	0.11	0.97

Continued

(d)			
	Batch No: 1 - 3 (n = 18)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
2	1.94 ± 0.20	−2.86	10.25
6	5.59 ± 0.35	−6.88	6.23
400	405.01 ± 23.14	1.25	5.71
750	739.57 ± 47.82	−1.39	6.47
1000	971.49 ± 62.62	−2.85	6.44

3.1.4. Matrix Effect

The matrix effect for VAL and CHL was assessed at two concentration levels, QC Low and QC High, using six distinct human plasma lots, including one hemolytic and one lipemic sample. All selected plasma samples were confirmed to be free from significant interference at the retention times of the analytes and internal standards.

To evaluate the matrix effect, the mean peak areas of post-extraction spiked samples were compared with those of analyte solutions prepared in the mobile phase, following the procedure outlined in Section 2.4. This approach aligns with established practices for quantifying matrix effects in LC-MS/MS analyses.

The precision, expressed as the coefficient of variation (%CV), for VAL at QC Low and QC High levels was 2.06% and 1.32%, respectively. For CHL, the %CV values were 10.40% at QC Low and 3.39% at QC High. These results indicate acceptable variability, adhering to the commonly accepted threshold of 15% for matrix effect evaluations. Detailed results of the matrix effect assessments are presented in Table 2(a) and Table 2(b).

Table 2. (a) Results of matrix effects for valsartan (n = 6); (b) Results of matrix effects for chlorthalidone (n = 6).

(a)											
	Plasma QC Low						Plasma QC High				
	Mean Peak Area	Matrix Factor	Mean Peak Area IS (n = 6)	IS Matrix Factor	IS Normalized Matrix Factor		Mean Peak Area	Matrix Factor	Mean Peak Area IS (n = 6)	IS Matrix Factor	IS Normalized Matrix Factor
Pure Solution	7450.81	-	126828.02	-	-	Pure Solution	732897.52	-	126828.02	-	-
Matrix 1	7171.37	0.96	132514.40	1.04	0.92	Matrix 1	727744.68	0.99	132514.40	1.05	0.95
Matrix 2	7240.64	0.97	135755.04	1.07	0.91	Matrix 2	735008.62	1.00	135755.04	1.07	0.94
Matrix 3	7385.65	0.99	135025.08	1.06	0.93	Matrix 3	724758.82	0.99	135025.08	1.06	0.93
Matrix 4	7127.02	0.96	135201.73	1.07	0.90	Matrix 4	722838.34	0.99	135201.73	1.07	0.92
Matrix 5	7550.31	1.01	135115.73	1.06	0.95	Matrix 5	718677.85	0.98	135115.73	1.06	0.92
Matrix 6	7393.78	0.99	137724.71	1.09	0.91	Matrix 6	729791.65	1.00	137724.71	1.09	0.92
				Mean IS Normalized Matrix Factor	0.92					Mean IS Normalized Matrix Factor	0.93
				CV (%)	2.06					CV (%)	1.32

Continued

(b)

	Plasma QC Low						Plasma QC High				
	Mean Peak Area	Matrix Factor	Mean Peak Area IS (n = 6)	IS Matrix Factor	IS Normalized Matrix Factor		Mean Peak Area	Matrix Factor	Mean Peak Area IS (n = 6)	IS Matrix Factor	IS Normalized Matrix Factor
Pure Solution	351.15	-	126863.44	-	-	Pure Solution	38037.95	-	126863.44	-	-
Matrix 1	443.17	1.26	133389.83	1.05	1.20	Matrix 1	43304.13	1.14	133389.83	1.05	1.08
Matrix 2	346.69	0.99	128504.80	1.01	0.97	Matrix 2	42070.67	1.11	128504.80	1.01	1.09
Matrix 3	349.47	0.99	135102.41	1.06	0.93	Matrix 3	44168.84	1.16	135102.41	1.06	1.09
Matrix 4	351.06	1.00	129761.06	1.02	0.98	Matrix 4	42330.82	1.11	129761.06	1.02	1.09
Matrix 5	338.87	0.96	132824.70	1.05	0.92	Matrix 5	39745.45	1.04	132824.70	1.05	1.00
Matrix 6	336.63	0.96	127244.44	1.00	0.96	Matrix 6	40624.35	1.07	127244.44	1.00	1.06
				Mean IS Normalized Matrix Factor	0.99					Mean IS Normalized Matrix Factor	1.07
				CV (%)	10.40					CV (%)	3.39

3.1.5. Recovery

Recovery was estimated at three concentration levels (low, medium and high QC) by comparing the mean analytes responses of six extracted samples with those of appropriately diluted standard solutions. The mean overall recovery of valsartan was 109.18% ± 2.66% and the mean overall recovery of CHL was 98.97% ± 3.75%. The recoveries of internal standards were measured in a similar manner using their corresponding medium QC samples as reference. The mean recovery of internal standard was 82.61% for VAL-D9 and 86.85% for CHL-D4.

3.1.6. Stability

The bench-top stability was examined by keeping replicates of spiked plasma with low, medium and high quality control samples at room temperature for approximately 4.5 hours for VAL and CHL. Freeze-thaw stability of samples was obtained over 4 cycles by thawing at room temperature for 1 hour and refreezing for 12 - 24 h. The processed samples were stable up to 48 hours in an autosampler at 10˚C. Long term plasma stability was evaluated at −70˚C over a period of 143 days. The stability results were summarized in Table 3.

Table 3. Results of stability of VAL and CHL in human plasma under different storage conditions.

	VALSARTAN				CHLORTHALIDONE			
Storage Condition	Nominal Conc. (ng/mL)	Conc. Found mean ± SD (ng/mL)	CV (%)	RE (%)	Nominal Conc. (ng/mL)	Conc. Found (mean ± SD (ng/mL)	CV (%)	RE (%)
	75	74.64 ± 2.63	3.52	−0.47	6	5.67 ± 0.22	3.95	−5.40
Autosampler stability[a]	8000	8059.89 ± 147.70	1.83	0.75	400	426.68 ± 12.84	3.01	6.67
	15,000	15889.84 ± 607.37	3.82	5.93	750	788.56 ± 16.58	2.10	5.14

Continued

Short-term plasma stability[b]	75	76.13 ± 2.28	3.00	1.51	6	5.57 ± 0.16	2.83	−7.10
	8000	8036.63 ± 201.33	2.50	0.46	400	407.24 ± 8.90	2.18	1.81
	15,000	15314.47 ± 368.99	2.41	2.10	750	724.31 ± 13.74	1.89	−3.42
Long-term stability[c]	75	77.25 ± 1.75	2.26	3.01	6	5.63 ± 0.44	7.81	−6.2
	8000	7995.42 ± 214.70	2.69	−0.06	400	373.17 ± 10.17	2.73	−6.71
	15,000	15766.94 ± 459.80	2.92	5.11	750	685.86 ± 13.87	2.02	−8.55
Freeze-thaw stability[c]	75	73.32 ± 1.23	1.68	−2.24	6	5.64 ± 0.35	6.28	−5.92
	8000	8145.10 ± 181.85	2.23	−1.81	400	417.50 ± 16.03	3.84	4.37
	15,000	15831.23 ± 569.68	3.60	5.54	750	761.14 ± 22.79	2.99	1.48

RE: Relative error (Accuracy), CV: Coefficient of Variation (Precision), SD: Standard Deviation. [a]Kept at autosampler temperature, 10˚C. [b]Stored at room temperature. [c]Stored at −70˚C.

4. Conclusion

We developed and validated an LC-MS/MS method with ESI interface using negative ion mode for simultaneous determination of VAL and CHL in human plasma. While liquid-liquid extraction (LLE) offers several advantages, such as simplicity, low cost, and effective removal of endogenous interferences, it is not without limitations. One potential drawback is variability in extraction efficiency, particularly when dealing with lipemic or hemolyzed plasma samples, which can influence recovery and reproducibility. Additionally, LLE may require optimization of solvent polarity and pH to ensure consistent analyte partitioning, and emulsion formation during extraction can occasionally complicate phase separation. In this study, careful selection of extraction solvents and pH conditions resulted in consistent recoveries for both valsartan and chlorthalidone. Use of deuterated internal standards guaranteed the success of the assay by eliminating the impact of matrix effects. The developed method was validated to have sufficient sensitivity, satisfactory selectivity and good reproducibility. The liquid-liquid extraction method was established, and diethyl ether was used as extraction solvent to obtain the good extraction recovery and no obvious matrix effect. The validated method could be applied to evaluate the pharmacokinetics and to determine bioequivalence of combination and co-administered VAL and CHL tablets after an oral administration.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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