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Novel LCMS Method for Simultaneous Determination of Irbesartan and Hydrochlorothiazide in Human plasma

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Abstract

The present study describes a novel liquid chromatographic-tandem mass spectrometric (LC- MS/MS) method for the simultaneous estimation of Irbesartan (IRB) and hydrochlorothiazide (HCTZ) in human plasma using liquid-liquid extraction technique. This method made use of electro spray ionization in negative mode for IRB and HCTZ using triple quadrupole mass spectrometry where Irbesartan D4 and Hydrochlorothiazide 13C 15N2 D2 were used as an internal standard (IS) for IRB and HCTZ respectively. Analytes were recovered by di ethyl ether: dichloromethane (70:30) subsequently separated on an Ace 5 C_{18} column (100 mm × 4.6 mm, 5µm) using methanol:0.1% formic acid in water (70:30) as a mobile phase, at a flow rate of 1.0 mL/min. Quantification of IRB, HCTZ and Irbesartan D4 and Hydrochlorothiazide 13C 15N2 D2 was performed using multi-reaction monitoring mode (MRM) where transition of m/z 427.1 → 193.0 (IRB), 431.1 → 193.0 (Irbesartan D4), 295.8 → 269.0 for HCTZ and 300.9 → 271.1 for Hydrochlorothiazide 13C 15N2 D2 in negative mode. The calibration curve was linear (r^2 > 0.99) over the concentration range of 10-5000 ng/mL for IRB and 1-500 ng/mL for HCTZ. The intra- day and inter-day precisions were less than 15% and the accuracy was all within ±15% (at LLOQ level ±20%). The LC-MS/MS method was fully validated for all the other parameters such as selectivity, matrix effect, recovery and stability as well. In conclusion, the findings of the present study revealed the selectivity and sensitivity of this method for the simultaneous estimation of IRB and HCTZ in human plasma.

Keywords: Bioanalytical method, Irbesartan, Hydrochlorothiazide, LC-MS/MS

Full-length article *Corresponding Author, e-mail: dkumaraswamygandla@gmail.com

1. Introduction

By 2035, Irbesartan (IRB), an Angiotensin II receptor antagonist with chemical name 2-butyl-3-({4-[2- (2H-1,2,3,4-tetrazol-5-yl)phenyl]phenyl}methyl)-1,3-dia-zaspiro [4.4] non- 1-en-4-one is being used in treatment of hypertension. Irbesartan is a nonpeptide tetrazole derivative and an angiotensin II antagonist that selectively blocks the binding of angiotensin II to the AT1 receptor. The selective antagonism of the angiotensin-II receptors results in increase in plasma renin levels and angiotensin-II levels, and a decrease in plasma aldosterone concentration [1,2]. Irbesartan, by blocking the binding of angiotensin II to the AT1 receptor, promotes vasodilation and decreases the effects of aldosterone [2]. Chemically hydrochlorothiazide (HCTZ) (is 6-chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulphon-amide-1, 1-dioxide. Hydrochlorothiazide is one of the oldest thiazide diuretics used to treat hypertension. It is often prescribed in com- bination with other antihypertensive drugs such as β- blockers, angiotensin converting enzyme inhibitors or angiotensin II receptor blockers [3]. Many researchers demonstrated several techniques for qualitative and quantitative determination of IRB and HCTZ in the biological fluids using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography (HPLC) [4,9]. Further, stability testing of IRB and HCTZ in the pharmaceutical preparations has been studied using high performance thin layer chromatography (HPTLC) [10]. Irbesartan (IRB), 2-butyl- 3-({4-[2-(2H-1,2,3,4- tetrazol-5-yl) phenyl]phenyl}methyl)-1,3- diazaspiro [4.4] non-1-en-4-one an angiotensin II receptor antagonist used mainly for the treatment of hypertension. It acts by selectively blocks the binding of angiotensin II to the AT1 receptor. Hydrochlorothiazide (HCTZ), 6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7- sulfonamide, is an-anti-diuretic and anti- hypertensive drug. It acts via inhibiting Na^+-Cl^- symporter in the distal convoluted tubule and thereby reducing extracellular fluid volume, leading to fall in cardiac output which produces vasodilatation [9, 10]. IRB and HCTZ has been evaluated in biological fluids and pharmaceutical preparations using LC-MS/MS [4-9] and HPTLC, respectively [10].

Although, IRB and HCTZ have been studied in pharmaceutical preparations as well as in biological fluids using diverse analytical techniques, simultaneous estimation of these two drugs has got little attention. Therefore, the present study was designed to develop and validate a method for simultaneous determination of IRB and HCTZ in human plasma using LC- MS/MS.

2. Materials and Methods

2.1. Chemicals and reagents

IRB was purchased from LGC Promochem, Bangalore, India. IRB D4 and HCTZ were purchased from Vivan Life Sciences, Mumbai, India and Hydrochlorothiazide 13C 15N2 D2was purchased from Clearsynth Labs, Mumbai, India. Human Plasma was purchased from Supratech Laboratory, Ahmedabad, Gujarat, India. Methanol of HPLC grade was purchased from Spectrochem Pvt. Ltd., Mumbai, India. Formic acid, Hydrochloric acid was purchased from Merck Millipore, Mumbai, India. Dichloromethane and Di ethyl ether were of HPLC grade were purchased from Spectrochem Pvt. Ltd., Mumbai, India. Water was purified using a Milli-Q water purification system, Millipore Pvt. Ltd., Ahmedabad, Gujarat, India.

2.2. Instrumentation

The LC-MS/MS system consisted of a Shimadzu liquid chromatography system (Shimadzu Corporation, Japan) which consisted of a LC-20AD solvent delivery system, a DGU-20A5R vacuum degasser, a CTO-20AC thermos-stated column oven and SIL-20AC autosampler and coupled with a triple quadrupole mass spectrometer LCMS- API 3000 (MDS Sciex, USA). Data acquisition and processing were performed using Analyst software (version 1.4.2) from MDS Sciex, USA and Watson LIMS (version 7.3) from Watson, USA.

2.3. Chromatographic conditions

Chromatographic separations were achieved on an ACE 5 C_{18} column (100 × 4.6 mm, 5 µm pore size) placed in thermostated column oven at 40 °C using mobile phase consisting of 70% of methanol and 30% formic acid in water (0.1% v/v), at a flow rate of 1.0 mL/min. Sample injection volume was 5 µL. Analytical run time was 3.5 min.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in negative ion mode using electrospray ionization (ESI) source. Tuning parameters were optimized for IRB, HCTZ and IS by infusing a solution containing 250 ng/mL of each analyte. The source dependant parameters maintained for both IRB and HCTZ were Gas 1 (Nebuliser Gas): 10 psig; ion spray voltage (ISV): -3500V, turbo heater temperature (TEM): 400°C; interface heater (Ihe): ON; collisional activation dissociation (CAD): 6 psig and curtain gas (CUR): nitrogen: 10 psig. Quantification of analytes performed using multiple reaction monitoring of the transition's m/z 427.1 —› m/z 193.0 for IRB, m/z 295.8 —› m/z 269.0 for HCTZ, m/z 431.1 —› 193.0 for IRB D4 and m/z 300.9 —› 271.1 for HCTZ 13C 15N2 D2, with the dwell time of 200 ms per transition.

Optimized collision energies of -25 was used for IRB and its ISTD whereas Optimized collision energies of -28 was used for HCTZ and its ISTD.

2.5. Preparation of standard and quality control (QC) samples

The standard stock solutions of IRB, IRB D4, HCTZ and HCTZ 13C 15N2 D2 were prepared by dissolving 5 mg of analytes in methanol to give final concentration of 1000 µg/mL for each analyte. These solutions were further diluted with methanol to give final concentration of 500 and 5µg/mL of IRB and HCTZ, respectively. Further, solutions were diluted with methanol to achieve working standard solutions at the concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 20.0, 50.0, 125.0 and 250.0µg/mL for IRB and 0.05, 0.1, 0.2, 0.4, 0.8, 2.0, 5.0, 12.5 and 25.0 µg/mL for HCTZ.

A working solution of IS was prepared by diluting the standard stock solution of IRB D4 and HCTZ 13C 15N2 D2 in methanol to achieve a final concentration of 10.0 µg/mL for IRB D4 and 5.0 µg/mL for HCTZ 13C 15N2 D2. The working standard solutions (20 µL) were used to spike blank human plasma sample (980 µL) to build up the calibration curve of both the analytes and for quality control in validation studies.

The final concentrations in standard plasma samples were 10.0, 20.0, 40.0, 80.0, 160, 400, 1000, 2500 and 5000 ng/mL for IRB and 1.0, 2.0, 4.0, 8.0, 16.0, 40.0, 100, 250 and 500 ng/mL for HCTZ. The QC samples were prepared in the same way as the calibration samples. The plasma concentrations of QC samples were 10.0, 30.0, 2000 and 4000 ng/mL for IRB and 1.0, 3.0, 200 and 400 ng/mL for HCTZ. All samples were stored at 2-8 °C until analysis.

2.6. Plasma sample preparation

Aliquots of 500 µL of unknown plasma, blank, calibration curve standard (CCs) and quality control standard (QCs) samples were prepared in 5 mL eppendorf centrifuge tubes and mixed with 50 µL of buffer solution (0.5 N HCl) to which 50 µL of IS solution was added. Subsequently, 2.5 mL of liquid extraction mixture (Di ethyl ether: dichloromethane; 70:30) was added to the above solutions and samples were rotated on extractor for 15 minutes at 40 rpm. The resultant mixture was then centrifuged at 4000 rpm for 5 min at 10°C and 2.0 mL of supernatant thus obtained was transferred to glass vials, evaporated to dryness in a evaporator at 40 °C under the gentle stream of nitrogen. The dried samples were reconstituted by addition of 100 µL of mobile phase, loaded into autosampler and then 5 µL of reconstituted samples were injected into LC-MS/MS system.

2.7. Quantification

Quantitative analysis of IRB and HCTZ was performed using IRB D4 and HCTZ 13C 15N2 D2 as an IS respectively. Calibration curves were established with CCs prepared in plasma. Nine- point CCs constructed using peak area ratio of analytes to IS. Concentration of analytes in QCs and unknown samples were calculated by interpolation from the calibration curves.

2.8. Method validation

Method validation protocol was based on the recommendations of the United States Food and Drugs Administration (USFDA) guidelines [11].

2.8.1. Selectivity

The selectivity of method toward endogenous plasma matrix components was assessed by comparing the interfering signals in ten different batches of plasma (seven were of K_3EDTA, and one each of lipidemic, haemolysed and heparinized plasma) with the signals of analytes and IS. Aliquots of plasma samples were used to prepare lower limit of quantification (LLOQ) and blank samples. Baseline noise should be < 20% of analyte response at this concentration level.

2.8.2. Linearity, accuracy and precision

The linearity of the method was assessed by processing a nine-point calibration curve over the concentration range of 10-5000 ng/mL for IRB and 1-500 ng/mL for HCTZ in three consecutive runs. Calibration curves were constructed by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte to IS. Each calibration curve was analyzed individually by least square weighted ($1/x^2$) liner regression. The inter- or intra-batch accuracy and precision were evaluated using six replicates of QC samples at LLOQ, lower (LQC), middle (MQC) and higher (HQC) concentration levels for three consecutive analytical days. The concentration of QC samples was selected from the calibration curve range. The criteria for acceptability of the data included precision within 15% coefficient of variance (% CV) and an accuracy within ±15% relative error (% RE) of the nominal values. Lower limit of quantification was determined with acceptable precision and accuracy (six replicates on three consecutive validation days with RSD < 20% and accuracy within ±20%).

2.8.3. Recovery

Recovery of the analytes after liquid-liquid extraction was estimated at low, middle, and high concentration levels by comparing mean peak- area of the extracted samples with mean peak- area of post-spiked extracted samples, which represent 100% recovery. Extraction recovery of IS was determined in the similar way using QC samples at medium concentration as a reference.

2.8.4. Matrix effect

Matrix effect was evaluated by comparing the mean peak area of analytes spiked in blank extracted plasma samples (respective analyte working solution was spiked at the time of reconstitution) (A) with corresponding mean peak area of analytes prepared in mobile phase (B).

Matrix effect = (A/B) × 100

For a method to be free from relative matrix effect, the % coefficient of variance (CV) of normalized matrix effect should be less than 15%.

2.8.5. Stability

The stability studies of stock solutions were performed for IRB (1 mg/mL), HCTZ (1 mg/mL), IRB D4 (1 mg/mL) and HCTZ 13C 15N2 D2 (1mg/mL) at 2-8 °C for ten days and at room temperature for 22 hr. The mean peak area of freshly prepared solutions was compared with that of stability solutions and expressed as % mean change. The bench top (at ambient temperature), freeze-thaw (at -20 °C and at - 78°C), process stability (2 hr at room temperature and 48 hr at 2-8 °C) and long term stability (at -20°C & -78°C) study of each analyte was evaluated at LQC and HQC concentration level using six replicates at each concentration. Analyte was considered as stable if the % change is less than 15% as per USFDA guidelines. The bench top stability, process stability and freeze-thaw stability for IRB and HCTZ were assessed at different conditions of temperature and time. Frozen samples were allowed to thaw at room temperature for 60 min and then refrozen at -20°C and -78°C. Concentrations of stability samples and freshly prepared samples were calculated and stability was shown as the percentage mean change in calculated concentration. Long term stability was performed for 118 days. Samples were kept at -20 °C and at -78°C for 118 days, processed and then compared with new freshly prepared solutions.

3. Results and Discussion

3.1. Optimization of the mass spectrometric condition

For optimum detection and simultaneous quantification of IRB and HCTZ with IS in human plasma, it was necessary to adjust chromatographic conditions and mass parameters as well. The mass parameters were tuned in both the positive and negative ionization mode for all analytes. IRB, HCTZ and IS showed prominent peak in the negative ionization mode. Optimization of heat block temperature, interface temperature, DL temperature and drying gas flow are of utmost importance in order to minimize ion suppression and to increase sensitivity. The results of the present study showed that increase in heat block temperature, interface temperature, DL temperature and drying gas flow above 400°C, 350°C, 250°C and 15 L/min, respectively, augmented the intensity of the analyte. Further, minor changes in ion spray voltage and nebulizer gas flow did not alter the signal intensity and were maintained at 4.5 kV and 3 L/min, respectively. A dwell time of 0.200 s for IRB, HCTZ, and IS was sufficient and no cross talk was found between all MRMs.

3.2. Optimization of the sample preparation and chromatographic condition

One of the key fundamental steps in the development of an analytical method is sample preparation. Sample preparation procedure should be quick, easy to proceed and should require least amount of reagents with maximum recovery of analytes. In this regard, literature review revealed the use of solid phase extraction technique for the extraction of IRB and HCTZ.

Table 1: Calibration standard data detailing slope, intercept, correlation-coefficient (R^2) for drugs (n=15)

Analyte		**Slope**	**Intercept**	**R^2**
Irbesartan	Mean	0.0012	0.0017	0.9979
	% CV	8.33	41.18	0.0011
Hydrochlorothiazide	Mean	0.0037	0.0005	0.9979
	% CV	8.11	40.00	0.0011

Table 2: Accuracy and precision performance data for the quantification of Irbesartan and hydrochlorothiazide in human plasma

Analyte	**Nominal conc. (ng/mL)**	**Intra batch precision (n = 6) (% CV)**	**Inter batch precision (n = 18) (% CV)**	**Intra batch accuracy (n = 6) (%)**	**Inter batch accuracy (n = 18) (%)**
Irbesartan	10	4.36	3.58	96.30	98.50
	30	1.93	4.25	95.33	99.67
	2000	1.42	2.09	96.50	98.00
	4000	1.48	2.44	96.25	99.00
Hydrochlorothiazide	1	6.59	9.67	87.20	94.60
	3	3.69	4.53	95.67	98.67
	200	1.93	2.35	97.00	99.50
	400	1.47	2.86	105.75	102.25

Table 3: Recovery performance data of Irbesartan, hydrochlorothiazide (at three QC level), Irbesartan D4 and Hydrochlorothiazide 13C 15N2 D2

Analyte (ng/ml)	**Mean peak area ($\times 10^4$, n = 6)**		**(% Mean Recovery)**	**% CV**
	Post-spiked Extracted Samples[a]	**Extracted Samples[b]**		
Irbesartan				
30	12.680	7.504	59.2	6.71
2000	658.389	429.396	65.2	
4000	1203.111	811.913	67.5	
Hydrochlorothiazide				
3	1.950	0.700	35.9	4.56
200	122.397	44.675	36.5	
400	250.590	97.933	39.1	
Irbesartan D4 10000	273.063	175.741	64.4	
Hydrochlorothiazide 13C 15N2 D2 5000	149.161	53.525	35.9	

[a] Peak area of analytes solution spiked in mobile phase.

[b] Peak area of analytes extracted from spiked human plasma.

[c] Recovery was expressed as the percentage of the mean peak area of the analytes prepared in the mobile phase relative to that of analytes extracted from human plasma.

Table 4: Assessment of matrix effect of Irbesartan, hydrochlorothiazide (at three QC level), Irbesartan D4 and Hydrochlorothiazide 13C 15N2 D2

Analyte (ng/mL)	Mean Peak Area ($\times 10^4$, n = 6)		Matrix Effect[c]	% CV
	Neat Solution[a]	Post-spiked extracted samples[b]		
Irbesartan				
30	12.574	12.951	1.03	2.69
4000	1197.541	1173.590	0.98	3.10
Hydrochlorothiazide				
3	1.931	1.970	1.02	3.01
400				
Irbesartan D4 10000	276.782	274.014	0.99	2.54
Hydrochlorothiazide 13C 15N2 D2 5000	145.796	147.254	1.01	1.97

[a] Peak area of analytes solution without plasma extracts.

[b] Peak area of analytes spiked in plasma extracts.

[c] Matrix effect was expressed as the percentage of the mean peak area of the analytes spiked in blank extracted plasma samples relative to that of analytes prepared in the mobile phase.

Table 5: Stability data of Irbesartan and hydrochlorothiazide at low and high QC level (n=6)

Stability study	Irbesartan		Hydrochlorothiazide	
	Concentration (ng/mL)	% Mean stability[a]	Concentration (ng/mL)	% Mean stability[a]
Bench top (17 hours at ambient temperature)	30	1.23	3	0.63
	4000	6.04	400	6.58
Process stability[b]	30	0.61	3	-0.95
	4000	13.12	400	12.91
Process stability[c]	30	-2.55	3	1.97
	4000	-1.54	400	-0.17
five freeze-thaw stability	30	-0.35	3	-2.69
	4000	0.27	400	0.00
Dry extract stability	30	2.54	3	-1.24
	4000	-0.24	400	2.31
Long term stability (118 days)	30	6.64	3	6.48
	4000	7.27	400	3.50

[a] % Mean stability = % Mean change in the concentration of the stability samples when compare to the freshly spiked samples

[b] Process stability evaluated after 48 hr storage at 5 °C.

[c] Process stability estimated after 2 hr at room temperature.

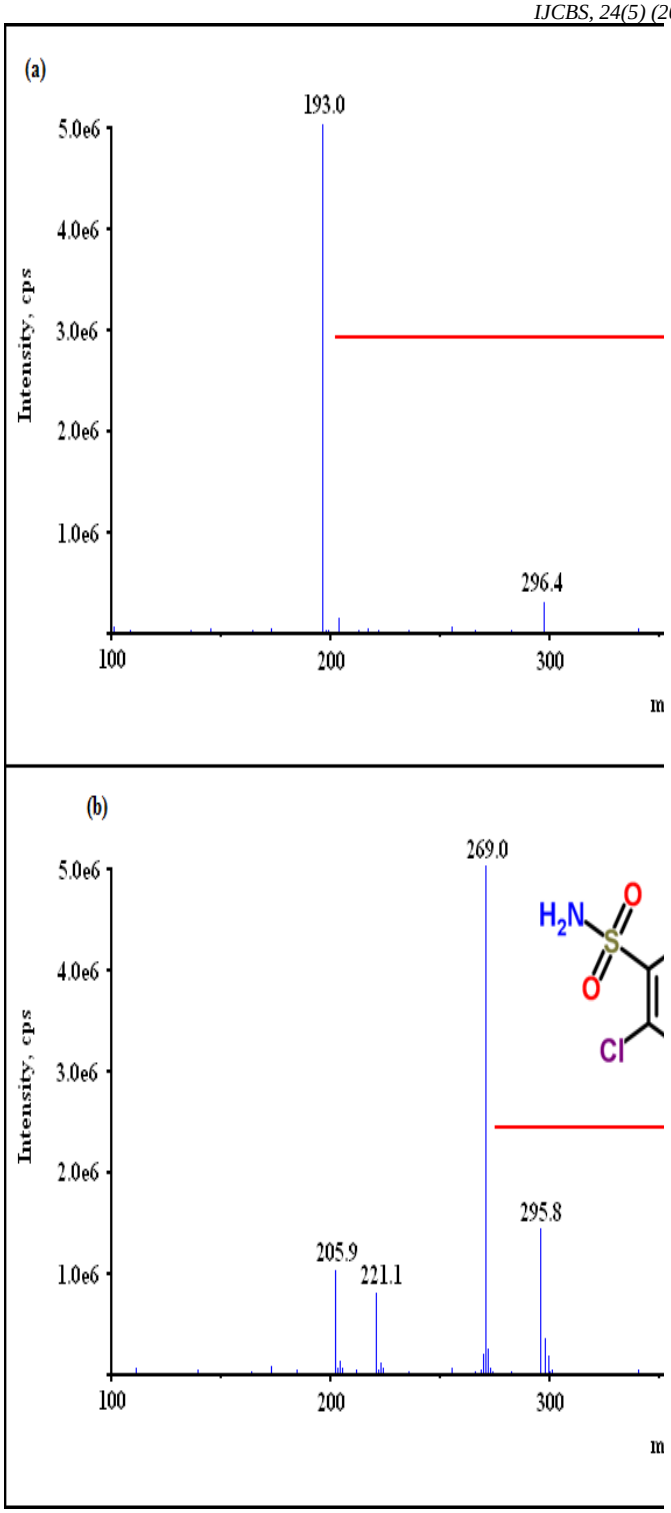

Figure 1: Spectrum of product ion scans of ramipril (a) and hydrochlorothiazide (b)

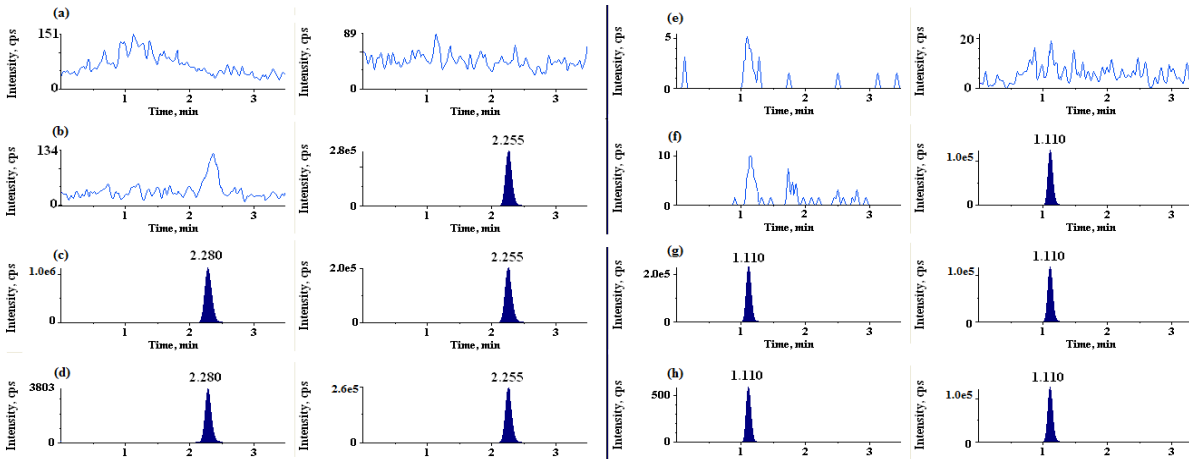

Figure 2: Representative MRM Chromatogram STD BL (a), STS Zero (b), ULOQ (c) LLOQ (d) of ramipril and STD BL (e), STS Zero (f), ULOQ (g) LLOQ (h) of hydrochlorothiazide in human plasma

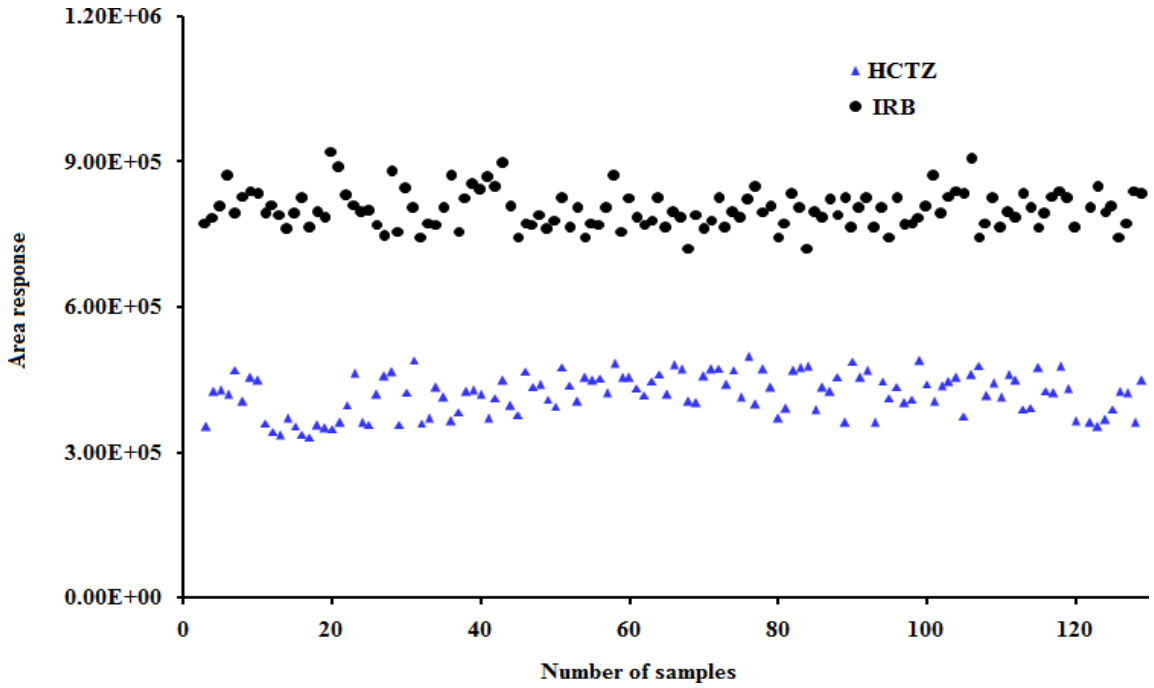

Figure 3: Internal standard variation in area response

However, solid phase extraction technique is time consuming and requires expensive materials when compared to liquid-liquid extraction method. Therefore, we used liquid-liquid extraction method as the sample preparation method to shorten processing time and to acquire desired recoveries of the analytes. Some reported methods also employed the higher plasma volume for sample preparation and injection volume for the chromatographic development. Whereas, proposed method was developed with less plasma volume and injection volume, leads to better acceptability of the method. To develop a simple and rapid liquid-liquid extraction method several extraction solvents such as dichloromethane, diethyl ether, n-hexane, ethyl acetate, methyl tert- butyl ether were used. Better response and recovery were obtained using extraction solvents methyl di ethyl ether: dichloromethane (85:15% v/v). There was no interference from any endogenous or exogenous plasma matrix and IS did not alter analyte recovery, sensitivity and/or ion suppression as well. IRB D4 and HCTZ 13c 15n2d2 were chosen as the IS for both IRB and HCTZ respectively. Chromatographic conditions were optimized to achieve good sensitivity and peak shape for both analytes and IS, as well as a short chromatographic run time. In this study, we tried Thermo, Atlantis, Ace, Gemini, Symmetry and Enable columns with various mobile phases such as methanol, acetonitrile, formic and acetic acid, ammonium acetate and aqueous ammonia. The ACE column was selected as it gave better separation between IRB and HCTZ, and the highest sensitivity was achieved with the acidic (formic acid in water, 0.1%v/v)-methanol mobile phase system. Low injection volume of 5 µL reduced overloading of column with analytes, thereby ensuring more numbers of analyses on the same column. The simultaneous quantification of RAM and HCTZ was done using LC-MS/MS. Chromatograms of STD BL, STD ZERO, STD1, and LLOQ of IRB and HCTZ are presented in Fig.2 and Fig. 3, respectively.

3.3. Selectivity

There was no interference peaks observed from endogenous compounds at retention time in any of the samples of IRB, HCTZ and IRB D4 and HCTZ 13C 15N2 D2, extracted from plasma as represented in Fig. 4. The response of drug substances in blank plasma were less than 1.94% and 4.20% in case of IRB at LLOQ of 10 ng/mL and HCTZ at LLOQ of 1 ng/mL, respectively. Typical retention time of IRB, HCTZ and IRB D4 and HCTZ 13C 15N2 D2 was 2.20, 1.10, 2.20 and 1.10 min, respectively.

3.4. Linearity, accuracy, and precision

Usually, the least square method can create relatively large errors at the levels with low concentrations, as in case with proposed developed method. The proposed method utilizes the concentration range for 10-5000 ng/mL for IRB and 1-500 ng/mL for HCTZ which indicates lower concentration range. To overcome this problem, the concept of weighted calibration curves was applied and calculation was made using weighting factors [none (unweighted), $1/x^2$] and the calibration curve is calculated. The results indicated that the weighted data for calibration curve is more accurate in the experimentation and the application of weighted factor was the best choice for proposed method. A typical equation of the calibration curve on a validation batch was as follows: $y = 0.0012x + 0.0017$ ($r^2 = 0.9979$) for IRB and $y = 0.0037x + 0.0005$ ($r^2 = 0.9979$) for HCTZ (Table 1).

The present bio-analytical method provided lower limit of quantitation and good range of linearity. The proposed method can detect lower concentration up to 5% C_{max} of both the drugs and upper concentration was more than two times of the C_{max} of drugs. Good linearity was obtained with aforementioned concentration ranges with a correlation coefficient (r^2) greater than 0.995. Table 2 summarizes the results for intra- and inter-day precision and accuracy for IRB and HCTZ measured by QCs. To validate the accuracy and precision of the developed method, three concentrations of QCs in six replicates were utilized. The results showed that the intra- and inter-day accuracy ranged from 95.33-99.00% for IRB and 87.20-105.75% for HCTZ. In context to this, the present LC-MS/MS method for simultaneous assessment of IRB and HCTZ was found to meet the accepted limits for accuracy and precision experiments.

3.5. Recovery

The % recovery was determined by comparing the mean peak area in extracted samples with freshly prepared un-extracted samples at three concentrations. The % recovery of IRB and HCTZ was found to be 59.2, 65.2, 67.5% and 35.9, 36.5, 39.1%, respectively at three different concentrations. Mean recovery for the IRB D4 was 64.4% and HCTZ 13C 15N2 D2 was 35.9% (Table 3). It has been documented that % recovery should be 80% in analytical methods. Whereas, in bioanalytical method development the extent of recovery is not considered as an issue provided that the method yields sensitivity, precision and accuracy.

3.6. Matrix effect

Two QC concentrations of each tested analyte and IS were utilized in the test with six different sources of human plasma. Table 4 depicts that there was no significant ion suppression or enhancement observed for all the analytes and IS under the present experimental conditions.

3.7. Stability

The stock solutions of IRB, HCTZ and IS in methanol were stable for 22 hr at room temperature (short term stock solution stability) and at least 10 days at 2-8 °C (long term stock solution stability). The bench top, freeze-thaw, and autosampler stability of IRB and HCTZ in human plasma samples were assessed by analyzing low and high QC samples (n=6) at each concentration which were exposed to different temperature and storage conditions. These QC samples were studied after storage at room temperature for 17 hr (bench-top), process samples for 2 hr at room temperature and for 48 hr at 2-8 °C (process stability). Further, QC samples were exposed to five freeze (-20 °C & - 78°C)-thaw (room temperature) cycles and stored at -20 °C and -78°C for 118 days (long term) to evaluate stability. The data are summarized in Table 5.

4. Conclusion

A simple, rapid, sensitive and selective LC-MS/MS method was developed and validated for the simultaneous estimation of IRB and HCTZ in human plasma by using IRB D4 and HCTZ 13C 15N2 D2 as an internal standard in the present study. The advantage of this method includes simple sample preparation procedure, low sample volume and reproducible recoveries of analytes and IS with minimum matrix effect. To the best of our knowledge, the findings of the present study provide strong scientific evidence for accuracy and precision of quantification of IRB and HCTZ in human plasma. This method might be applied to characterize the clinical pharmacokinetics study of IRB and HCTZ in humans.

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