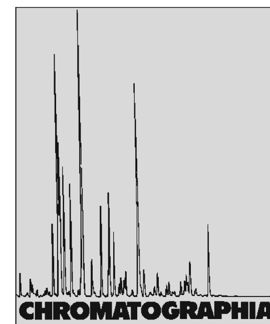
Simultaneous LC–MS–MS Analysis of Valsartan and Hydrochlorothiazide in Human Plasma



2009, *69*, 1055–1060

Hiten Janardan Shah[1,2,✉], Naresh B. Kataria[1], Gunta Subbaiah[1], Chagan N. Patel[2]

[1] Bioanalytical Laboratory, Torrent Research Center, Gandhinagar 382428, Gujarat, India;
E-Mail: hitenshah@torrentpharma.com; hiten12345@hotmail.com
[2] Shri Sarvajanik Pharmacy College, Mehsana 384001, Gujarat, India

Received: 30 July 2008 / Revised: 24 November 2008 / Accepted: 15 December 2008
Online publication: 8 February 2009

Abstract

A rapid and specific liquid chromatographic–tandem mass spectrometric method is described for simultaneous analysis of valsartan (VAL) and hydrochlorothiazide (HCTZ) in human plasma. VAL and HCTZ were chromatographed on a C_8 column with 75:15:10 (*v*/*v*) acetonitrile–methanol–0.001% aqueous ammonia as mobile phase. VAL and HCTZ were eluted at 0.69 min and 1.22 min, respectively, and, after electrospray ionization (ESI), detected in selected-reaction-monitoring mode. The precursor to product-ion transitions *m*/*z* 434.32 → 179.22 and *m*/*z* 295.85 → 204.86 were used to quantify VAL and HCTZ, respectively. Recovery by solid-phase extraction was >90% for both analytes and the internal standard. The method was suitable for application to a pharmacokinetic study after oral administration of tablet containing 160 mg VAL and 25 mg HCTZ to 18 healthy volunteers.

Keywords

Column liquid chromatography–tandem mass spectrometry
Valsartan and hydrochlorothiazide
Selective reaction monitoring

Introduction

Hypertension is a major risk factor for cardiovascular morbidity and mortality. Monotherapy of hypertension is often ineffective, because it enables control of approximately 50% only of the blood pressure of hypertensive patients [1]. HCTZ is a thiazide diuretic often prescribed in combination with other anti-hypertensive drugs, for example beta blockers, angiotensin-converting enzyme inhibitors, or angiotensin receptor blockers II [2]. VAL is a new angiotensin II receptor antagonist anti-hypertensive drug [3]. The combination of VAL and HCT is more effective than either drug alone and is effective for patients not responding to monotherapy with either agent [4].

It is desirable to develop and validate a bioanalytical method to enable both drugs to be quantified simultaneously in human plasma after a therapeutic dose. Several methods have been reported for quantification of VAL [5–10] or HCTZ [11–16] either individually or in combination with other antihypertensive drugs in biological matrixes. Individual determination is not favored because of its high cost and low efficiency.

A liquid chromatographic–tandem mass spectrometric method for simultaneous analysis of VAL and HCTZ in human plasma is reported in this paper. Few LC–MS–MS methods are available for simultaneous quantification of these drugs in human plasma [17, 18]. One has a matrix effect of 79.0–83.8% for VAL and 30.4–35.5% for HCTZ, resulting in major ion suppression [17]; this is not acceptable during method validation. This method also has a run time of 3.0 min per sample. Another LC–MS–MS method [18] has recovery of 76.20–78.35% for VAL and 75.55–86.88 for HCTZ, which is low compared with the method reported in this paper. The method also has a run time of 6.5 min per sample, which is also more than that of the method reported in this

Full Short Communication
DOI: 10.1365/s10337-009-0950-1
0009-5893/09/05

© 2009 Vieweg + Teubner | GWV Fachverlage GmbH

paper (2.0 min). In the previous methods extraction is based on protein precipitation by acetonitrile [17] or methanol [18] whereas the method reported in this paper uses solid-phase extraction (SPE) without evaporation and reconstitution steps.

Experimental

Chemicals and Reagents

VAL, HCTZ, clonazepam (used as internal standard (IS) for VAL), and gliclazide (used as IS for HCTZ) (99.85, 99.70, 99.14, and 99.40% pure, respectively) were obtained from Hetro Drugs (Hyderabad, India), Unichem Laboratories (Mumbai, India), Ray Chemical (Bangalore, India), and Bal Pharma (Bangalore, India), respectively. Suprapur grade orthophosphoric acid and ammonia were from Merck (Darmstadt, Germany). Methanol and acetonitrile were of gradient grade (Rankem, New Delhi, India) and water was from Millipore (MA, USA) Milli Q equipment. SPE was performed with 1-cm^3, 30 mg, HLB cartridges from Waters (MA, USA).

Preparation of Standard and Quality-Control Solutions

Stock solutions of VAL (5 mg mL^{-1}), HCTZ, clonazepam, and gliclazide (1.0 mg mL^{-1}) were prepared in 50:50 (v/v) methanol–water. Other stock solution of VAL (500 µg mL^{-1}), HCTZ (40 µg mL^{-1}), clonazepam (10 and 100 µg mL^{-1}), and gliclazide (300 ng mL^{-1} and 100 µg mL^{-1}) were also prepared in 50:50 (v/v) methanol–water. VAL (500.0 µg mL^{-1}) and HCTZ (40 µg mL^{-1}) were used to spike blank human plasma to furnish calibration standards containing 50–4,500 ng mL^{-1} VAL and 2–400 ng mL^{-1} HCTZ. Quality-control (QC) solutions at three concentrations 150 ng mL^{-1} (low QC, or LQC), 1,700 ng mL^{-1} (medium QC, or MQC), and 3,375 ng mL^{-1} (high QC, or HQC) for VAL and 6 ng mL^{-1} (LQC), 150 ng mL^{-1} (MQC), and 300 ng mL^{-1} (HQC) for HCTZ were also prepared in blank plasma.

Sample Preparation

After thawing, 500 µL plasma containing the drugs was vortex mixed with 25 µL working IS solution in 1.7-mL microtubes. Orthophosphoric acid (2%, 500 µL) was added and vortex mixing was repeated. The plasma mixture was then applied to an HLB cartridge (30 mg, 1 cm^3) previously conditioned with 1 mL methanol then 2 mL water. The loaded cartridge was washed with 2 mL water then 1 mL 5% methanol in water, then vacuum was applied to remove the aqueous solvent mixture. The cartridges were then eluted with 1.0 mL methanol–acetonitrile 90:10 (v/v) and the recovered solution was loaded into an autosampler vial.

LC–MS–MS Analysis

HPLC was performed with a Shimadzu (Kyoto, Japan) system comprising LC-10ADvp pump, SIL-HTc autosampler, DGU-14A solvent degasser, and CTO 10Avp column oven. Compounds were separated on a 50 mm × 4.6 mm i.d., 5-µm particle, Betabasic C_8 analytical column maintained at 50 °C. The mobile phase was 75:15:10 (v/v) acetonitrile–methanol–0.001% aqueous ammonia at a flow rate of 0.5 mL min^{-1}, without split. The injection volume was 5 µL and the run time 2.0 min. The sample-tray temperature was 10 °C. The injector needle was rinsed with 1.0 mL acetonitrile–methanol 50:50 (v/v) before and after each injection.

Mass spectrometric detection was performed with a TSQ Quantum Discovery triple-quadrupole mass spectrometer manufactured by Thermo Finnigan (San Jose, CA, USA). The detector settings are listed in Table 1.

Validation of the Bioanalytical Method

The method was validated for specificity/selectivity, linearity, precision and accuracy (intra and inter-day), recovery, matrix effects, dilution integrity, and stability, in accordance with USFDA guidelines.

Specificity/selectivity was checked by analysis of ten different samples of blank plasma comprising four samples of blank plasma in CPD (citrate, phosphate, and dextrose), four samples of heparinized blank plasma, one samples of lipemic blank plasma, and one sample of hemolyzed blank plasma. All were processed by the same extraction procedure and analyzed to determine the extent to which endogenous plasma components contributed to interference at the retention time of the analytes and the internal standard. The results obtained from these samples of blank plasma were compared with those from plasma samples spiked at the LLOQ levels of 50.0 and 2.0 ng mL^{-1} for VAL and HCTZ, respectively.

The linearity of the method was determined by analysis of nine-point calibration plots. Concentrations of VAL and HCTZ were calculated from the simple linear equations $y = mx + c$ obtained by regression analysis of spiked plasma calibration standards with the reciprocal of the drug concentration as a weighting factor (1/concentration, i.e. $1/x$).

Intra-day and inter-day precision and accuracy experiments were conducted at four levels, by analysis of the LLOQ, LQC, MQC, and HQC samples in replicates of six for both analytes. Mean and standard deviation (SD) were obtained for calculated analyte concentration at each level. Accuracy and precision were evaluated as relative error (RE, %) and coefficient of variation (CV, %), respectively, relative to the nominal concentration.

A dilution integrity experiment was performed with the objective of validating the dilution of higher drug concentrations (more than upper limit of quantification, or ULOQ), which may be encountered during analysis of real samples. This was to ensure that dilution of samples with the same matrix did not affect the results obtained. To perform this experiment, twice the ULOQ concentration was diluted two and four times with blank human plasma. Their back calculated concentrations were obtained by applying the dilution factors two and four for half and quarter dilutions, respectively.

Recovery (extraction efficiency) of VAL, HCTZ, clonazepam, and gliclazide from human plasma by the extraction procedure described above was determined at the LQC, MQC, and HQC levels by comparing the peak area obtained from the extracted analyte with those from unextracted samples (spiked externally) which represented 100% recovery. Absolute recovery was calculated by comparing the peak area obtained from extracted samples with those obtained from aqueous samples at the equivalent concentration.

To study the effect of the plasma matrix on analyte quantification with regard to consistency in signal suppression/enhancement, the matrix effect was checked for six different plasma samples at the LQC and HQC levels. These plasma samples comprised four samples of normal control heparinized plasma, one sample of lipemic control heparinized plasma, and one sample of hemolyzed control heparinized plasma. Three samples at each of the LQC and HQC levels were prepared from different plasma samples (a total of 36 QC samples) and the relative error for all the QC samples was checked.

Experiments were performed to evaluate the stability of the analyte in stock solutions and in plasma samples under different conditions, by simulating the conditions which occurred during sample analysis. Stock solution stability was assessed by comparing peak-area response obtained from stability samples of the analytes and IS with the peak-area response of samples prepared from fresh stock solutions. Bench-top stability (room-temperature stability), refrigerated stability of extracted samples, and freeze–thaw stability were assessed at the LQC and HQC levels. Long-term stability was assessed at the LQC, MQC, and HQC levels by analysis of six replicates at each level.

Study Design

The study design was an open-label, randomized, two-period, two-treatment, two-sequence cross-over, single-dose bioequivalence study of VAL + HCTZ tablet containing 160 mg VAL and 25 mg HCTZ test and reference in 18 healthy human volunteers under fasting and fed conditions. An ethics committee approved the study protocol. The studies were conducted strictly in accordance with guidelines laid down by USFDA.

Table 1. Ion source and analyte-dependent MS conditions

Ion source				
Spray potential				3,500 V
Capillary temperature				350 °C
Sheath gas				40 U (arbitrary)
Auxiliary gas				20 U (arbitrary)
Polarity mode				Negative
Analyte-dependent	**VAL**	**HCTZ**	**Clonazepam**	**Gliclazide**
Precursor ion (*m*/*z*)	434.25	295.85	314.00	322.13
Product ion (*m*/*z*)	179.22	204.86	278.27	170.13
Tube lens offset (V)	127	97	69	106
Collision energy	27	29	22	29
Q1 Pw[a] (amu)	0.30	0.70	0.70	0.50
Q3 Pw[b] (amu)	0.30	0.70	0.70	0.50

[a] Quadrupole 1 peak width
[b] Quadrupole 3 peak width

Results and Discussion

Method Development

The objective of this work was to develop and validate a rapid, rugged, and adequately sensitive method for quantitative extraction and simultaneous analysis of VAL and HCTZ at therapeutic concentrations for analysis of routine samples, with a short run time per sample. To achieve this, sample extraction, chromatographic conditions, and MS detection settings were optimized. To obtain better method specificity and reproducibility, mass spectrometry was performed using negative-ion electrospray ionization (ESI) in selective-reaction-monitoring (SRM) mode. The response for all compounds was much better in negative-ion mode than in positive-ion mode. Full-scan mass spectra of the analytes and IS are shown in Fig. 1.

Use of methanol in the mobile phase resulted in better selectivity, but the response was not adequate for the analytes and the IS. Acetonitrile was therefore tested in different proportions. It was observed that a mobile phase with high acetonitrile content and a low methanol content gave sufficient response with adequate selectivity. It was also observed that a basic mobile phase was necessary to obtain a reproducible and consistent response, so to increase the ion response and improve the peak shape of the analytes and IS, 0.001% aqueous ammonia was added. The mobile phase 10:15:75 (*v*/*v*) 0.001% aqueous ammonia –methanol–acetonitrile was eventually found to be the most suitable. C_8 stationary phases with particle size 5 μm were most appropriate for achieving high selectivity, suitable compound retention, and separation from the plasma matrix. Several trials were conducted with different C_8 columns; finally a 50 mm × 4.6 mm, 5.0-μm particle size, Betabasic C_8, column, with a mobile phase flow rate of 0.5 mL min^{-1}, was selected for optimum results. A column-oven temperature of 50 °C resulted in symmetrical analyte peaks. Total LC run time per sample was only 2.0 min. To prevent unwanted matrix components from entering the MS, a divert valve was set in waste mode up to 0.25 min. The small injection volume of 5 μL reduced overloading of the column with analytes (maximum on-column loading of VAL and HCTZ was 250 and 10 pg per injection), thereby ensuring more analyses on the same column.

For simultaneous extraction of the analytes and IS from plasma, liquid–liquid extraction (LLE) using different solvents was investigated. All efforts resulted in inconsistent recovery, long

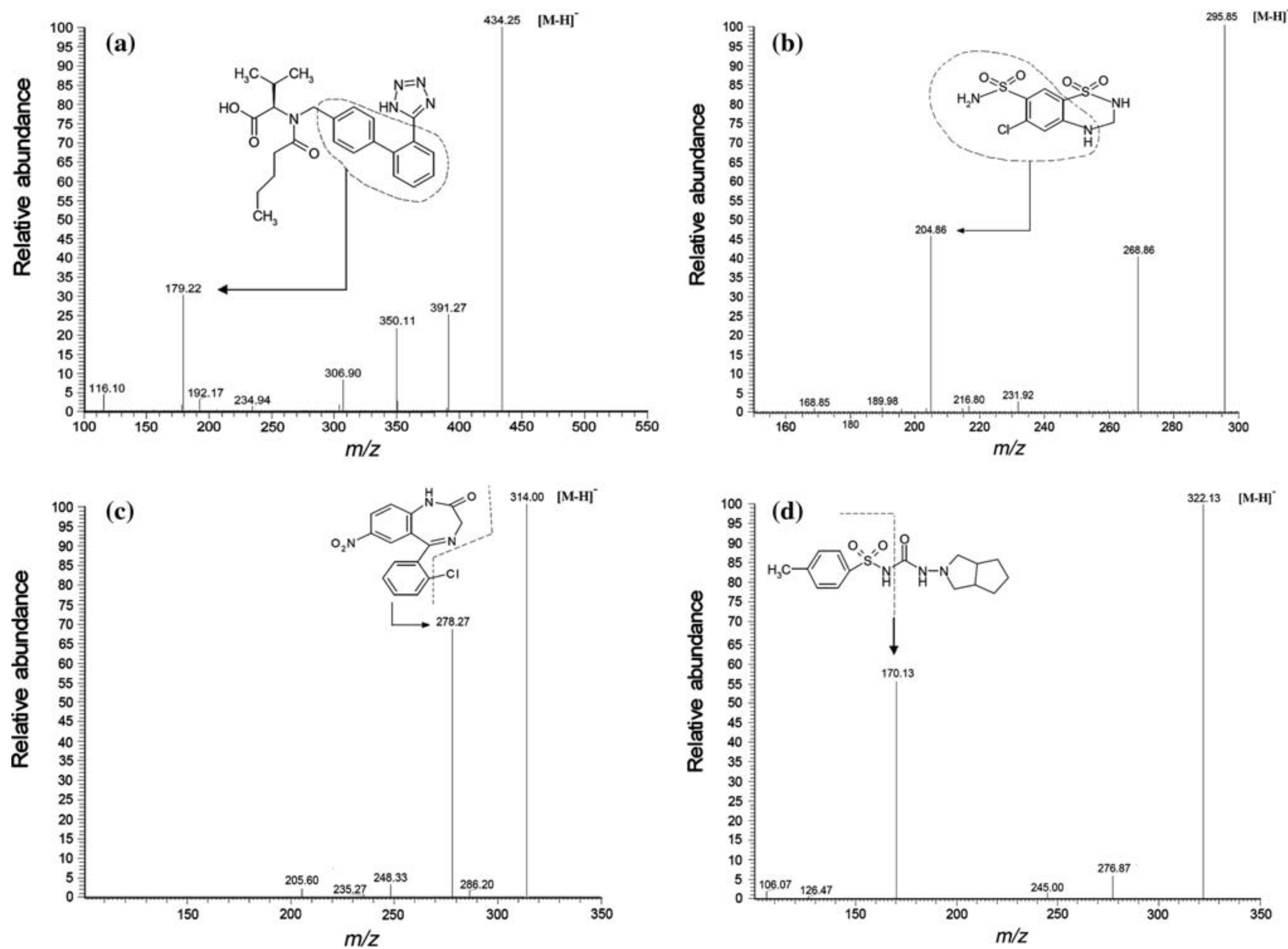


Fig. 1. Full-scan mass spectra of the [M–H]$^-$ ions of **a** Val **b** HCTZ **c** clonazepam, and **d** gliclazide

extraction time, and high background, however. To obtain cleaner extracts with minimum matrix and quantitative recovery, SPE was performed with Oasis HLB cartridges (1 cm^3, 30 mg). A plasma volume of 0.5 mL resulted in quantitative recovery of the analytes. Pretreatment of the plasma samples with 2% orthophosphoric acid helped to convert the drugs to the un-ionized forms. The eluate (1 mL) was submitted directly to LC–MS–MS analysis without previous evaporation and reconstitution. The SPE method resulted in quantitative recovery and greater selectivity for the analytes than LLE.

Specificity/Selectivity and Sensitivity

Analysis of ten different samples of drug-free human plasma resulted in no significant interfering peaks at the retention times of the analytes or internal standards. Representative chromatograms obtained from extracted blank plasma, drug-free plasma fortified with IS, and blank plasma fortified with 50.0 ng mL^{-1} VAL and 2.0 ng mL^{-1} HCTZ (LLOQ) and the IS are shown in Fig. 2a–c, respectively. The areas obtained from blank plasma samples at the retention times of VAL and HCTZ were less than 20% of the LLOQ areas; those at the retention times of the IS were less than 5% of the IS peak areas.

Linearity, Precision, Accuracy, and Dilution Integrity

The calibration plots obtained for VAL were linear from 50 to 4,500 ng mL^{-1} with correlation coefficients, r, $\geq$0.9989 for five calibration plots. For HCTZ the linear dynamic range was from 2 to 400 ng mL^{-1} with correlation coefficients $\geq$0.9997. Observed mean back-calculated concentrations at each level were from 97.58 to 101.92% of the nominal concentration for VAL and from 97.38 to 101.70% for HCTZ.

Intra-day precision and accuracy for VAL and HCTZ were evaluated by analysis of five replicates at four concentrations, viz. LLOQ, LQC, MQC, and HQC, in the same analytical run. Inter-day precision and accuracy were calculated after replicate analysis in three different analytical runs. Intra-day and inter-day precision was less than 10.0% at the four QC levels for both analytes. Intra-day and inter-day accuracy, expressed as RE (%), were within −10.0 to 10.0% for both analytes. The mean back-calculated concentrations for the half and quarter-diluted samples were within 85–115% of the nominal

concentrations for both analytes. The coefficient of variation (CV, %) for the half and quarter-diluted samples of VAL and HCTZ was less than 3.96%.

Recovery and Matrix Effect in Bioanalysis

Recovery reveals the efficiency of the extraction procedure, which may not be 100% but should be consistent, precise, and reproducible. Six replicate analyses at the LQC, MQC, and HQC levels were performed for determination of recovery. Mean recovery was 90.28%, CV 0.12%, for VAL, 96.63%, CV 1.15%, for HCTZ, 90.66%, CV 2.51%, for clonazepam, and 95.43%, CV 2.06%, for gliclazide.

No significant matrix effect was observed in analysis of six different samples of drug-free human plasma containing VAL and HCTZ at the LQC and HQC levels. Assessment of the matrix effect by the post-column analyte infusion method also led us to conclude there was no significant effect ($<2\%$) for VAL and HCTZ.

Stability Study

Exhaustive experiments were performed to evaluate the stability of VAL and HCTZ in stock solutions and plasma samples. Observed and acceptable percentage changes for the stability experiments are compiled in Table 2.

Application of the Method to Human Subjects

Mean C_{max} (maximum plasma concentration) under fed conditions was 3,354.280 ng mL^{-1} (at 5 h) for VAL and 90.083 ng mL^{-1} (at 2.5 h) for HCTZ. Under fasted conditions mean C_{max} was 4,360.564 ng mL^{-1} (at 5 h) for VAL and 148.637 ng mL^{-1} (at 2.5 h) for HCTZ. The chromatographic run time of 2.0 min makes it possible to analyze 500 samples in a day.

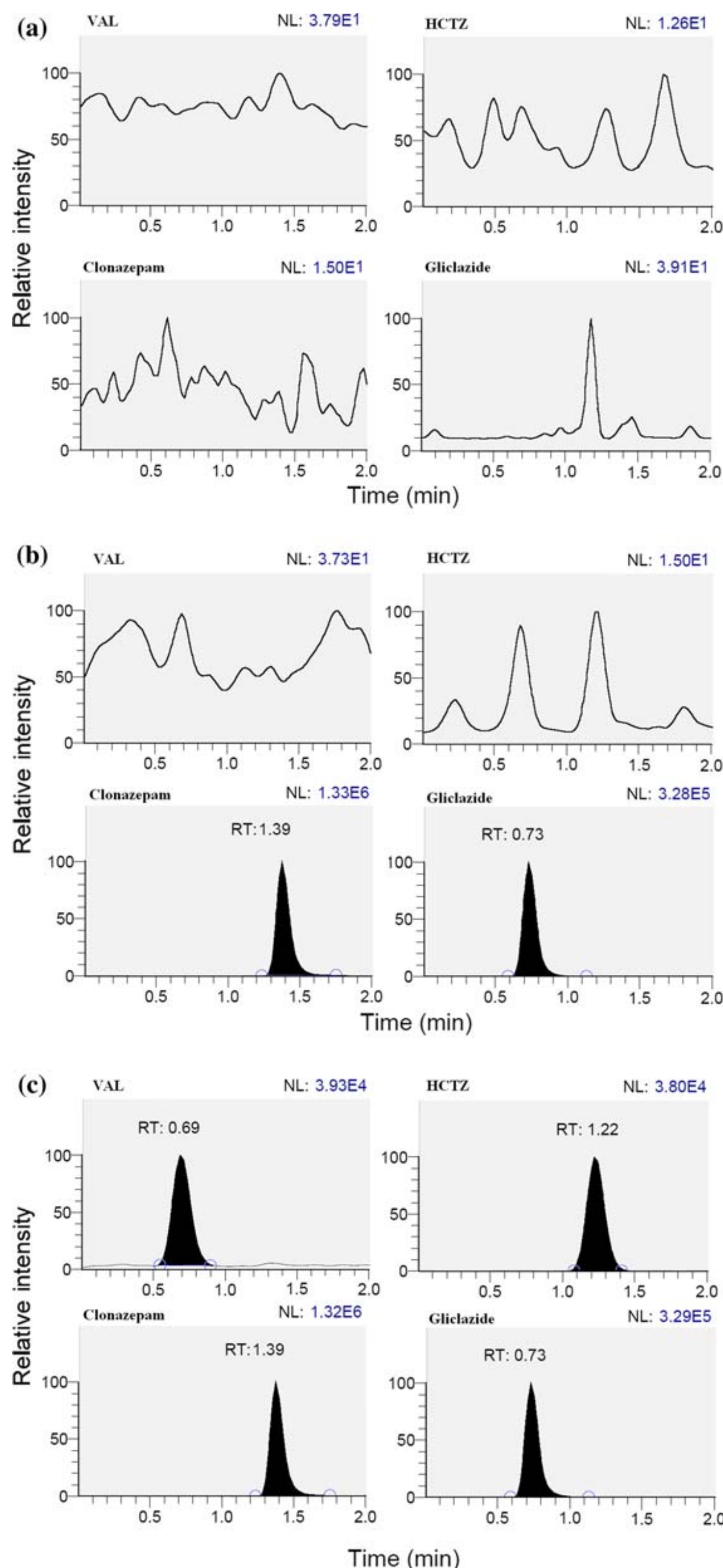


Fig. 2. Representative LC–SRM chromatograms obtained from **a** blank plasma (analytes and IS-free), **b** zero blank plasma (analytes-free, spiked with IS), **c** plasma spiked with 50 ng mL^{-1} Val, 2 ng mL^{-1} HCTZ (LLOQ), and the IS

Table 2. Stability of VAL and HCTZ in plasma

Stability experiment	Stability conditions	QC level	Mean comparison sample conc. found (ng mL^{-1})	% CV	Mean stability sample conc. found (ng mL^{-1})	% CV	Mean change (%)
VAL							
Bench-top stability	Room temperature (11 h)	LQC	146.483	8.12	141.908	3.97	−3.12
		HQC	3,563.941	2.13	3,355.051	2.43	−5.86
Process stability (extracted sample)	Autosampler (5 °C, for 43 h)	LQC	147.857	0.96	142.738	5.52	−3.46
		HQC	3,344.441	4.90	3,396.545	1.95	1.56
Freeze and thaw stability	After 3rd cycle at −70 °C	LQC	144.248	3.57	144.734	5.69	0.34
		HQC	3,450.527	2.65	3,446.892	3.17	−0.11
Long-term stability in human plasma	For 126 days at −70 °C	LQC	146.483	8.12	148.794	7.55	1.58
		MQC	1,826.545	3.97	1,775.904	2.63	−2.77
		HQC	3,563.941	2.13	3,421.444	2.96	−4.00
HCTZ							
Bench-top stability	Room temperature (11 h)	LQC	5.540	4.90	5.658	3.01	0.34
		HQC	298.349	1.87	288.888	1.59	−0.11
Process stability (extracted sample)	Autosampler (5 °C, for 43 h)	LQC	6.226	6.44	5.839	5.22	−6.21
		HQC	309.056	3.48	317.160	3.25	2.62
Freeze and thaw stability	After 3rd cycle at −70 °C	LQC	6.161	4.74	5.841	3.81	−5.19
		HQC	313.161	1.66	314.032	3.50	0.28
Long-term stability in human plasma	For 126 days at −70 °C	LQC	5.540	4.90	5.651	4.59	2.00
		MQC	153.123	2.69	150.466	3.07	−1.74
		HQC	298.349	1.87	292.200	2.20	−2.06

Acknowledgments

The authors are indebted to Dr Chaitanya Dutt, Director, Torrent Research Centre, for his continuous support, motivation, and assistance during the course of development and validation of this method. The authors gratefully acknowledge Torrent Pharmaceutical Ltd for providing the facilities necessary to perform this work.

References

1. Chrysant SG (2003) Expert Rev Cardiovasc Ther 1(3):335–343. doi:10.1586/14779072.1.3.335
2. Wellington K, Faulds DM (2002) Drugs 62(13):1983–2005. doi:10.2165/00003495-200262130-00015
3. Markham A, Goa KL (1997) Drugs 54(2):299–311. doi:10.2165/00003495-199754020-00009
4. Wagstaff AJ (2006) Drugs 66(14):1881–1901. doi:10.2165/00003495-200666140-00011
5. Gonzalez L, Lopez JA, Alonso RM, Jimenez RM (2002) J Chromatogr A 949(1/2):49–60. doi:10.1016/S0021-9673(01)01496-0
6. Tatar S, Saglik S (2002) J Pharm Biomed Anal 30(2):371–375. doi:10.1016/S0731-7085(02)00360-6
7. Sechaud R, Graf P, Bigler H, Gruendl E, Letzkus M, Merz MF (2002) Int J Clin Pharmacol Ther 40(1):35–40
8. Daneshtalab N, Lewanczuk RZ, Jamali F (2002) J Chromatogr B Analyt Technol Biomed Life Sci 766(2):3449. doi:10.1016/S0378-4347(01)00507-2
9. Macek J, Klima J, Ptacek P (2006) J Chromatogr B Analyt Technol Biomed Life Sci 832(1):169–172. doi:10.1016/j.jchromb.2005.12.035
10. Koseki N, Kawashita H, Hara H, Niina M, Tanaka M, Kawai R, Nagae Y, Masuda N (2007) J Pharm Biomed Anal 43(5):1769–1774. doi:10.1016/j.jpba.2006.12.030
11. Vetuschi C, Giannandrea A, Carlucci G, Mazzeom P (2005) Farmaco 60(8):665–670. doi:10.1016/j.farmac.2005.04.013
12. Zendelovska D, Stafilov T, Milosevski P (2004) Biomed Chromatogr 18(2):71–76. doi:10.1002/bmc.293
13. Erk N (2003) J Chromatogr B Analyt Technol Biomed Life Sci 784(1):195–201. doi:10.1016/S1570-0232(02)00759-6
14. Ramakrishna NV, Vishwottam KN, Manoj S, Koteshwara M, Wishu S, Varma DP (2005) Biomed Chromatogr 19(10):751–760. doi:10.1002/bmc.510
15. Takubo T, Okada H, Ishii M, Hara K, Ishii Y (2004) J Chromatogr B Analyt Technol Biomed Life Sci 806(2):199–203. doi:10.1016/j.jchromb.2004.03.060
16. Liu F, Xu Y, Gao S, Zhang J, Guo Q (2007) J Pharm Biomed Anal 44(5):1187–1191. doi:10.1016/j.jpba.2007.04.020
17. Li H, Wang Y, Jiang Y, Tang Y, Wang J, Zhao L, Gu J (2007) J Chromatogr B Analyt Technol Biomed Life Sci 852(1/2):436–442. doi:10.1016/j.jchromb.2007.02.014
18. Liu F, Zhang J, Xu Y, Gao S, Guo Q (2008) Anal Lett 41(8):1348–1365. doi:10.1080/00032710802119186