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Optimization and validation of bioanalytical method for quantitative determination of Valsartan in rat plasma samples using HPLC-ESI-MS/MS

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ABSTRACT

The validated protein precipitation method was applied for estimation of Valsartan (VL) in rat plasma with VLD_9 as an internal standard (ISTD) by using HPLC-ESI-MS/MS. The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v) using the C_{18} column Ascentis Express (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and flow rate was set to 0.6 ml/min. The mass transitions of VL, VLD_9 obtained were m/z 436.3→234.9 and m/z 445.3→234.9. The standard curve shows correlation coefficient (r^2) greater than 0.9993 with a range of 5-10000 ng/ml using the linear regression model. The validated method was applied to bioavailability study of ten male wistar rats through intravenous administration of 1.88 mg/kg of body weight of test formulation.

KEY WORDS: Valsartan, Wistar rats, HPLC-ESI-MS/MS, rat plasma, Bioanalysis.

1. INTRODUCTION

Valsartan (VL) is N-(1-oxopentyl)-N-[[2´-(1H-tetrazol-5-yl)[1,1´-biphenyl]-4-yl]methyl]-L-valine with chemical formula $C_{24}H_{29}N_5O_3$ (Fig.1) and its molecular weight is 435.5. Valsartan was used for the treatment of hypertension.

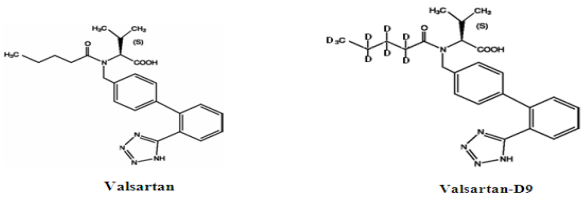

Fig.1.Chemical structures of Valsartan and ValsartanD_9.

The literature survey reveals that, a variety of methods were reported on the pharmacokinetics of valsartan in rat plasma (Yan, 2012), human plasma (Jones, 2012; Habtemariam, 2009; Iqbal, 2010; Zaid, 2011; Sechand, 2011). Several methods reported for quantification of Valsartan by using HPLC - MS/MS (Mehta, 2010; Kesting, 2010; Raman, 2009; Lu CY, 2009; Zhang, 2008), UPLC (Krishnaiah, 2010), HPLC (Koseki, 2007; Sharma and Pancholi, 2012; Rao, 2010; Piao, 2008; Iriarte, 2006; Macek, 2006). From the literature review it was concluded that the reported methods used highly expensive extraction process (SPE), long run time and lack of deuterated internal standard by using HPLC-ESI-MS/MS methods. There is no method reported for estimation of Valsartan using deuterated internal standard in biological samples.

The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged and reproducible analytical method for quantitative determination of VL in rat plasma by HPLC-ESI-MS/MS with a small amount of sample volume. The developed method would be applied in the bioavailability VL test formulation in rat plasma samples.

2. MATERIALS AND METHODS

2.1. Chemical Resources: Valsartan (VL) and ValsartanD_9 (VLD_9) (VARDA Biotech, Mumbai, India), methanol and acetonitrile (J.T Baker, USA), formic acid (Merck, Mumbai, India), Ultra-pure water (Milli-Q system, Millipore, Bedford, MA, USA), wistar rats and rat plasma (Bioneeds, Bangalore, India). The chemicals and solvents were used in this study were analytical and HPLC grade.

2.2. Instrument Resources: An API 4000 HPLC-ESI-MS/MS system (Applied Biosystems), 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), data acquisition and processing were accomplished using Analyst® Software 1.4.1.

2.3.Chromatographic conditions: The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v), gave the best peak shape and low baseline noise was observed using the Ascentis Express C_{18} (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and flow rate was set to 0.6 ml/min. The temperature was set to 40°C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

2.4. Detection: The pure drug of VL and VLD_9 were prepared in methanol (10 ng/ml) and injected with a flow rate of 5 µl/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra-high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM (multiple reaction mode) positive ion mode with mass transitions of m/z (amu) 436.3→234.9 and 445.3→234.9 for VL and VLD_9. The mass fragmentation pattern of parent and product ions mass spectras were depicted in fig 2.

2.5. Standard calibration and quality control samples preparation: Stock solutions of VL (1000 µg/ml) and VLD_9 (1000 µg/ml) were prepared in methanol. The internal standard (VLD_9) spiking solution (500 ng/ml) was prepared in 75% methanol from VLD_9 stock solution. Stock solutions of VL, VLD_9 and intermediate spiking solutions were stored in refrigerated conditions (2-8°C) until analysis.

Calibration standards (5, 10, 50, 100, 500, 1000, 2000, 4000, 6000, 8000 and 10000 ng/ml), quality control samples of lower limit QC, low QC, mid QC, high QC (5, 15, 3000, 7000 ng/ml) were used by spiking the appropriate amount of standard solution in the drug free rat plasma and stored at -30 °C till analysis.

2.6. Sample extraction: The protein precipitation method was applied to extract VL and VLD_9. To each labelled polypropylene tube 50 µl of VLD_9 (500 ng/ml) was mixed with the 100 µl plasma sample, then 0.25 ml of acetonitrile were added, vortexed for 5 min and centrifuged at 4000 rpm for 10 min at 20°C. The organic phase was transferred to auto sampler vials containing 100 µl of 0.1% formic acid and injected into the HPLC-ESI-MS/MS for analysis.

2.7. Method validation: The developed method was validated over a linear concentration range of 5–10000 ng/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section.

2.8. Selectivity and Specificity: Ten lots of blank rat plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of VL retention time and less than 5% for VLD_9 retention time.

2.9. Limit of Quantification (LOQ): Six LLOQ standards were prepared in screened plasma lot along with IS (500 ng/ml) and signal to noise ratio (S/N) was calculated using analyst software.

2.10. Linearity: Calibration standards were prepared to obtain linearity range of 5, 10, 50, 100, 500, 1000, 2000, 4000, 6000, 8000 and 10000 ng/ml and assayed in five replicates on five different days.

2.11. Precision & Accuracy: One set of calibration standards and one set contains four different concentrations of quality control standards of Lower limit QC (5 ng/ml), Low QC (15 ng/ml), Mid QC (3000 ng/ml) and High QC (7000 ng/ml) concentrations were prepared in screened rat plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intraday) and five different days (Inter day).

2.12. Matrix Effect: Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (3000 ng/ml) and compared with un-extracted standards of the same concentration.

2.13. Recovery: The recovery of samples was performed by protein precipitation method. The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (15 ng/ml), medium (300 ng/ml), high (7000 ng/ml).

2.14. Stability studies

2.14.1. Bench top Stability (Room Temperature Stability, 24 h): Six replicates of spiked low and high concentrations (BT stability samples) were set aside at ambient temperature up to 24 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

2.14.2. Freeze and thaw stability (after 3rd cycle at -30°C): Six replicates of low and high concentrations (FT stability samples) were frozen at -30°C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-30°C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

2.14.3. Autosampler stability (2-8°C, 65 h): Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 65 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

2.14.4. Long-term Stability (-30°C, 45 Days): After completion of the stability period stored at -30 °C (45 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

2.15. Sample Analysis: Before conducting in-vivo study in wistar rats, the study was approved by the local Institutional ethics committee (IEC). The validated method has been successfully applied to analyze VL concentrations in rat plasma samples of six male wistar male rats (n=10, weight range 200-210 g) were procured from bioneeds, Bangalore, India. These rats were acclimatized to laboratory conditions for a week before the start of the experiment. After overnight fasting of animals, administrated intravenously to the rats with a dose of 1.88 mg/kg. A volume of 0.2 ml blood was collected by retero-orbital puncture into a EDTA coated glass tubes at a time

intervals of 0, 0.1, 0.5, 1, 2, 4, 10, 20, 28 and 60 h after drug administration. Blood was centrifuged at 3000 rpm for 10 min and plasma was separated and stored at -30°C until analysis.

2.16. Pharmacokinetic analysis: The pharmacokinetics parameters from rat plasma samples were calculated by a non-compartmental statistic model using WinNon-Lin5.0 software (Pharsight,USA). Blood samples were collected for a period of 3-5 times the terminal elimination half-life ($t_{1/2}$).

Valsartan plasma concentration-time profile was visually inspected and C_{max} and T_{max} values were determined. The AUC_{0-t} was obtained by the trapezoidal method and $AUC_{0-\infty}$ was calculated up to the last measurable concentration and extrapolations were obtained by the last measurable concentration. The terminal elimination rate constant (K_{el}) was estimated from the slope of the terminal exponential phase of the plasma of the VL concentration-time curve using linear regression method. The terminal elimination half-life ($t_{1/2}$) was then calculated as $0.693/K_{el}$. All the plasma concentrations of Valsartan were within the standard curve region and remained above the 5.00 ng/ml (LOQ) for the entire sampling period.

3. RESULTS AND DISCUSSIONS

3.1. Method development: On the way to develop a simple and easy applicable method for determination of VL in rat plasma for conducting pharmacokinetic study, HPLC-MS/MS was selected as the method of choice. During method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results.

Separation of the VL was performed with different branded RP-HPLC C_{18} columns. Initial separation was performed with isocratic elution of 10mM ammonium formate and acetonitrile was selected as a mobile phase in varying combinations were tried, but a low response was observed. A mobile phase consisting of 0.1% acetic acid: acetonitrile (20:80 v/v) and 0.1%acetic acid: methanol (20:80 v/v) gave the best response, but poor peak shape was observed.

After a series of trials a mobile phase consisting of 0.1% formic acid in combination with methanol and acetonitrile in varying combinations were tried. Using a mobile phase containing 0.1% formic acid in combination with methanol (25:75 v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Ascentis Express C_{18} (50 mm × 4.6 mm, 2.7 µm) analytical column with a flow rate of 0.6 ml/min and reduced runtime to 3 min. The column oven temperature was kept at a constant temperature of about 40 °C and temperature of auto sampler was maintained at 4°C. Injection volume of 10 µl sample was adjusted for better ionization and chromatography. For selection of internal standard, sumatriptan, zolmitriptan and naratriptan were tried with optimized mobile phase and column conditions. Finally valsartanD_9 (VLD_9) was selected as internal standard in terms of better chromatography and extractability.

The retention times of analyte (VL) and internal standard (VLD_9) were eluted at 1.42 ± 0.2 min and 1.44 ± 0.2 min respectively with 3 min total runtime. Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the PPT was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard.

Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at a flow rate of 20 µl/min. Valsartan gave more response in positive ion mode as compare to the negative ion mode.

To get high intense productions source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 35, 25, 10, 20, 12 eV for valsartan and valsartanD_9, respectively. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 200 ms for valsartan and valsartanD_9.

The predominant peaks in the primary ESI spectra of VL and VLD_9 correspond to the MH^+ ions at m/z 436.3 and 445.3 respectively. Productions of VL and VLD_9 scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a m/z of 234.9 for both respectively. The parent and productions mass spectrums of VL and VLD_9 were shown in Figure 2.

3.2. Method validation

3.2.1. Selectivity and Specificity, Limit of Quantification (LOQ): No significant response was observed at retention times of VL and VLD_9 in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 5.0 ng/ml. Represent chromatograms were shown in Figure 3.

3.2.2.Linearity: Linearity was plotted as a peak area ratio(VL peak area/VLD_9 peak area) on the y-axis against VL concentration (ng/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for VL over a linearity range of 5 to 10000 ng/ml. The correlation coefficient was greater than 0.9993 for VL. The %CV was less than 15% and mean % accuracy was ranged between 99.7-102.3%. Results were presented in Table 1.

3.2.3. Precision & Accuracy: Intra and inter batch % accuracy for VL was ranged between 98.9% - 102.4% and % CV is 1.46% - 5.68%. Results are presented in Table 2.

3.2.4. Recovery: The mean % recovery for LQC, MQC, HQC samples of VL were 99.85%, 95.30% and 93.54% respectively. The overall mean % recovery and % CV of VL across QC levels is 96.23% and 3.38%. For the VLD_9 (internal standard) the mean % recovery and % CV is 91.68% and 7.09%.

3.2.5. Matrix Effect: No significant matrix effect found in different sources of rat plasma tested for VL, VLD_9. The % CV was found to be 3.71.

3.2.6. Stability (freeze–thaw, auto sampler, bench top, long term): Quantification of the VL in plasma subjected to three freeze–thaw cycles (−30°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in Table 3.

3.2.7. Application to biological samples: The validated method was characterized with an adequate rapid, exact, specific, responsive, rugged and stable. The validated method was applied to bioavailability study of ten male wistar rats through intravenous administration of 1.88 mg/kg of body weight of test formulation. Typical plasma concentration versus time profile is shown in Figure 4. All the plasma concentrations of VL were within the standard curve region and retained above the 5.0 ng/ml (LOQ) for the entire sampling period (Table 4).

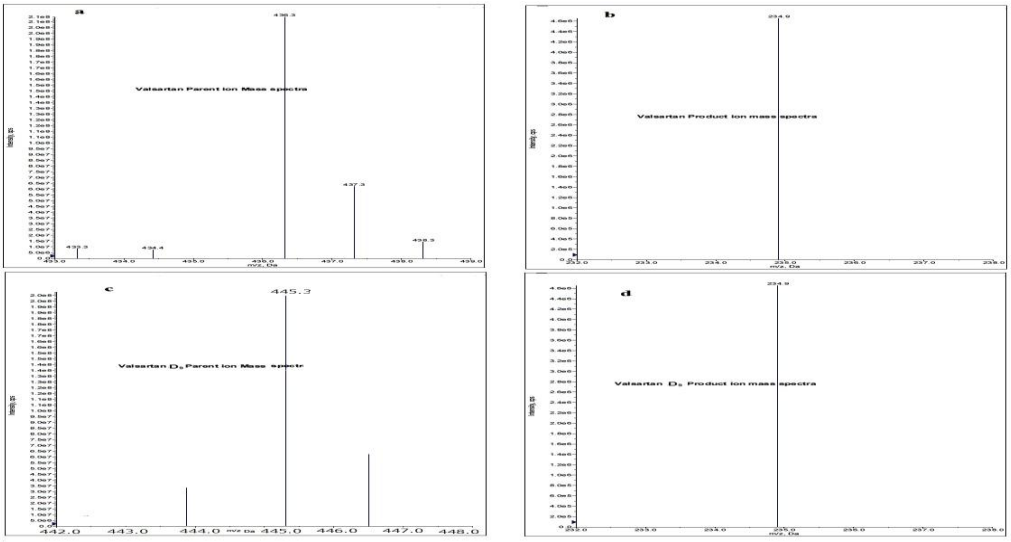

Fig.2.Mass fragmentation pattren of valsartan (VL) valsartanD_9 (VLD_9). a) Parent ion mass spectra (Q1) of VL b) Product ion mass spectra (Q3) of VL c) Parent ion mass spectra (Q1) of VLD_9 d) Product ion mass spectra (Q_3) of VLD_9

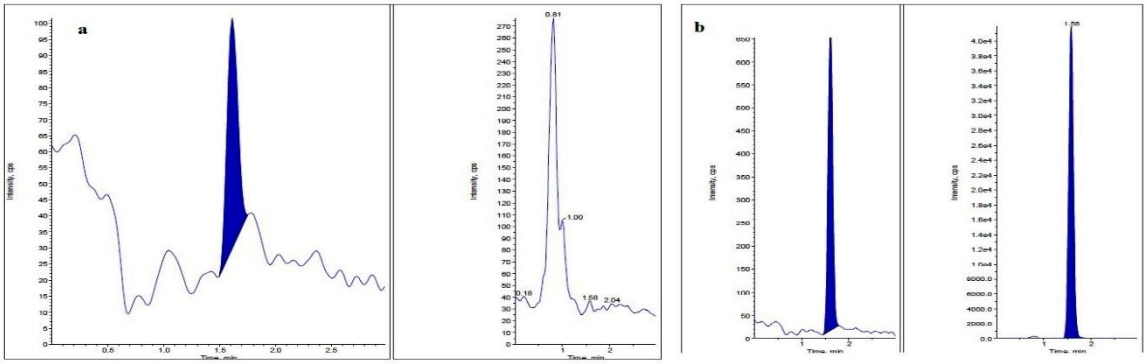

Fig.3.Representative chromatograms of valsartan in rat plasma a) Blank plasma chromatogram for interference free VL and VLD_9 b) Chromatogram of LLOQ sample (VL with VLD_9)

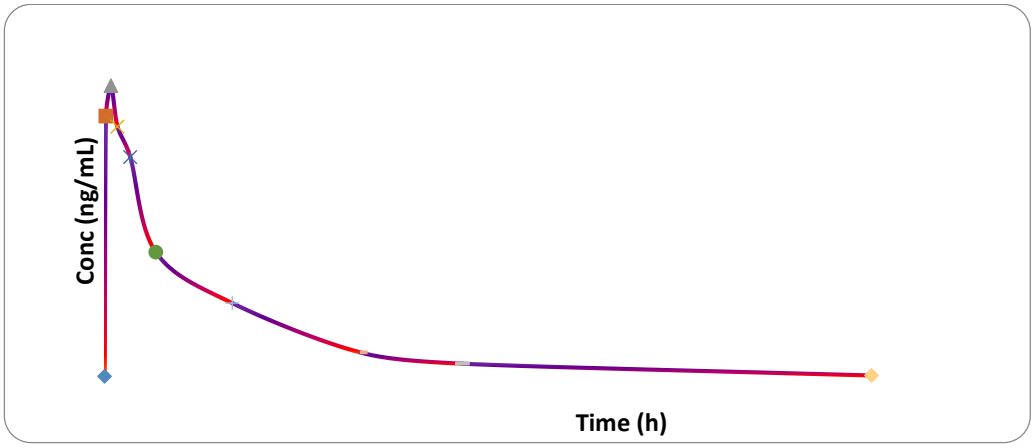

Fig.4.Mean plasma concentrations(ng/ml)-time(h) profile of VL in ten male wistar rats through intravenous administration with a dose of 1.88 mg/kg of body weight

Table.1.Calibration curve details

Spiked plasma Concentration (ng/ml)	Concentration measured (ng/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
5.00	4.99±0.01	1.4	99.9
10.00	10.24±0.02	3.6	101.7
50.00	49.89±0.15	2.7	101.3
100.00	100.24±0.22	2.5	100.1
500.00	501.6±0.27	3.8	100.1
1000.00	1004.22±0.21	2.6	101.7
2000.00	1999.18±1.02	3.1	99.4
4000.00	4001.35±1.10	3.4	101.7
6000.00	6003.76±1.11	1.7	102.6
8000.00	8001.12±1.96	3.8	101.5
10000.00	10000.07±1.23	2.5	100.5

Table.2.Precision and accuracy (Analysis with spiked samples at three different concentrations)

Spiked plasma concentration (ng/ml)	15.00	3000.00	7000.00
Within – run (Intra-day)			
Concentration measured (n=6;ng/ml;mean±S.D)	14.8±0.07	3002.34±1.23	6999.47±2.45
%CV	5.6	2.1	3.7
%Accuracy	98.9	102.4	99.8
Between-run (Inter-Day)			
Concentration measured (n=6;ng/ml;mean±S.D)	14.9±0.08	2999.78±2.56	7004.33±3.61
%CV	3.2	1.6	2.4
%Accuracy	99.93	99.45	103.45

Table.3. Stability studies of valsartan in rat plasma.

Spiked plasma concentration (ng/ml)	15.00	7000.00
Room temperature stability (24h)		
Concentration measured (n=6;ng/ml;mean±S.D)	14.9±0.12	7005.3±0.14
%CV (n=6)	7.8	8.9
Processed sample stability (65h)		
Concentration measured (n=6;ng/ml;mean±S.D)	15.5±2.16	7001.3±1.23
%CV (n=6)	5.3	9.5
Long term stability (45 days)		
Concentration measured (n=6;ng/ml;mean±S.D)	15.2±1.54	6999.563±0.12
%CV (n=6)	8.8	9.4
Freeze and thaw stability cycle (48h)		
Concentration measured (n=6;ng/ml;mean±S.D)	14.8±0.12	7001.4±2.55
%CV (n=6)	5.8	2.7

Table.4.Pharmacokinetic parameters of VL in ten male wistar rats through intravenous administration with a dose of 2.88 mg/kg of body weight.

Pharmacokinetic parameter	Calculated value
AUC_{0-t} (ng x h/ml)	56139.55
C_{max} (ng/ml)	7039.51
$AUC_{0-\infty}$ (ng x h/ml)	56365.29
T_{max} (h)	0.25
K_{el} (h^{-1})	0.090
$t_{1/2}$ (h)	7.78

4. CONCLUSION

The method described in this manuscript has been developed and validated over the concentration range of 5.0–10000.0 ng/ml in rat plasma. The intra and inter-batch precision (% CV) was less than 6.0% and % accuracy ranged from 98.9%–102.4%. The overall %recovery for VL,VLD_9 was greater than 90%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid protein precipitation extraction with less run time of 3.0 min per sample, make it an attractive procedure in high-through put bioanalysis of valsartan. The validated method was successfully applied in a bioavailability study of test formulation valsartan in ten male wistar rats through intravenous administration.

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