Journal of Chromatography B, 867 (2008) 172–178

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of clonidine in human plasma

Sagar A. Parekh[a,b,*], Ashutosh Pudage[b], Santosh S. Joshi[b], Vikas V. Vaidya[a], Noel A. Gomes[b]

[a] Department of Chemistry, Ruia College, Mumbai University, Matunga, Mumbai, India
[b] Accutest Research Laboratories (I) Pvt. Ltd., Khairane, Navi Mumbai 400709, India

ARTICLE INFO

Article history:
Received 17 July 2007
Accepted 26 March 2008
Available online 11 April 2008

Keywords:
Clonidine
Nizatidine
LC–MS/MS
Multiple reaction monitoring (MRM)

ABSTRACT

A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the estimation of clonidine in human plasma. Clonidine was extracted from human plasma by using solid-phase extraction technique. Nizatidine was used as the internal standard. A Hypurity C18 (50 mm × 4.6 mm i.d., 5 μm particle size) column provided chromatographic separation of analyte followed by detection with mass spectrometry. The method involves a rapid solid-phase extraction from plasma, simple isocratic chromatography conditions and mass spectrometric detection that enables detection up to picogram levels with a total run time of 3.0 min only. The method was validated over the range of 50–2500 pg/mL. The absolute recoveries for clonidine (71.86%) and IS (69.44%) achieved from spiked plasma samples were consistent and reproducible.

1. Introduction

Clonidine, an imidazoline derivative, is a centrally acting hypotensive agent but its use is being explored in a number of other indications such as anesthesia and the management of opiate withdrawal. Clonidine belongs to a class of drugs called central alpha-adrenergic agonists. Clonidine ((2-[2,6-dichlorophenyl] amino)-2-imidazoline) [1] preferentially stimulates central alpha (2)-adrenoceptors, which leads to inhibition of sympathetic tone, resulting in a lowering of arterial pressure and of heart rate. Additionally, many other desirable and undesirable effects are described, including analgesia, sedation and withdrawal reactions, which consist of a sudden rise in arterial pressure, nervousness, agitation and increased heart rate.

Several bioanalytical methods are reported to determine clonidine in different biological matrices like plasma [2–5], serum [6,7,11–13], urine [6], and cerebrospinal fluids [2]. Sensitive and selective methods based on LC–MS/MS [3–5,7,8,13], HPLC [2,9,10,12,14], GC–MS [11] and capillary isotachophoresis [6] methodologies are reported. Most of the assays reported for clonidine employ liquid–liquid extraction, where as we are presenting a solid phase extraction (SPE) procedure for sample extraction which is very simple, less time consuming and gives high throughput.

The aim of this study was to develop a sensitive, selective and high-throughput method for estimation of clonidine in human plasma for therapeutic drug monitoring and pharmacokinetic studies. As a part of our ongoing research in this area, we have developed and validated a LC–MS/MS assay for this drug in human plasma. Special emphasis was given to optimize the extraction step in order to get quantitative and reproducible recovery for the analyte. The method presents a simple and clean SPE procedure with drying and reconstitution steps. The positive ion ESI mode selected for this study, gave high response for the analyte as they possess amino groups which can be readily protonated by the mobile phase consisting of 2 mmol/L ammonium acetate in water:acetonitrile (20:80, v/v). The analyte and IS were well separated with minimum matrix interference in a run time of 3.0 min under isocratic conditions. The lower limit of quantitation (LLOQ) for clonidine was 50 pg/mL.

2. Experimental

2.1. Chemicals and materials

Working standards of clonidine were provided by Unichem Laboratories Ltd. (Goa, India) having purity greater than 99%. Nizatidine (IS) was supplied by Shasun Chemicals and Drugs Ltd. (Pondicherry, India). HPLC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade ammonium acetate was procured from Qualigens Ltd. (Mumbai, India). Purified water was obtained from Milli-Q A10 gradient water purification system

* Corresponding author at: Accutest Research Laboratories (I) Pvt. Ltd., Khairane, Navi Mumbai 400709, India. Tel.: +91 9833546270.
E-mail address: parekh.sagar@rediffmail.com (S.A. Parekh).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.03.030

(Millipore, Bangalore, India). Blank human plasma was collected in house with heparin as an anticoagulant and stored at −20 °C. Orochem, 30 mg; 1 mL DVB HL SPE cartridges were procured from Orochem (Mumbai, India).

2.2. Liquid chromatography and mass spectrometric condition

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-10AD prominence pump, autosampler (SIL-HTc) and solvent degasser (DGU-14) were used for all the analysis. For separation, the samples were applied without any guard column to Hypersil Hypurity C18 (50 mm × 4.6 mm i.d., 5 μm particle size) analytical column from Thermo (I) Pvt. Ltd. (Mumbai, India). The flow rate of the mobile phase under isocratic condition was kept at 0.4 mL/min. The autosampler temperature was set at 4 °C and the injection volume was 10 μL. The mobile phase consisted of 2 mM ammonium acetate:acetonitrile (20:80, v/v). The total LC run time was 3.0 min. Detection of analyte and IS was performed on a triple quadrupole mass spectrometer, API-3000 (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray ionization source and operating in the positive ion mode. Analyst software version 1.4 was used to control all parameters of LC and MS. Q1 full scan spectrum of both analyte and internal standard were recorded (refer to Figs. 1 and 3). In the Q1 full scan spectrum, clonidine showed a clusterof ions at 230, 232, 234 which correspond to M^+, M_2H^+, M_4H^+ owing to the presence of two chlorine atoms in the structure. Peak at m/z 230 corresponds to chlorine 35 isotopes (for both the atoms), peak at 232 corresponds to 1 chlorine 35 and another 37 isotope and peak at 234 corresponds to both chlorine 37 isotopes. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion (m/z) transitions for clonidine (231.9 → 44.1), and IS (332.1 → 155.1), respectively. Figs. 1–4 show the mass spectra's of parent and product ions for analyte and IS, respectively. Source dependent parameters optimized were gas 1 (nebuliser gas): 6 psi; gas 2 (heater gas flow): 8000 cm^3/min; ion spray voltage (ISV): 1250 V (response was evaluated at different ion spray voltages such 2500, 3000 and 4500 V); temperature (TEM): 350 °C. Compound dependent parameters like declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) were 60 V, 10 V, 320 V, 45 eV and 8 V for clonidine. Nitrogen was used as collision activated dissociation (CAD) gas and was set at 6 psi. Both quadrupoles 1 and 3 were maintained at unit resolution and dwell time was set at 200 ms. Product ion spectrum was evaluated at different collision energies and it was observed that clonidine showed only single fragment ion at m/z 44.1.

2.3. Analytical data processing

Chromatographic data were collected and integrated using Analyst software version 1.4. Peak area ratio of the analyte to IS was utilized for the construction of calibration curve. A weighing of $1/x$ (least-squares linear regression analysis, where x is the analyte concentration) was used for curve fitting. Concentration in unknown samples were calculated from the best-fit equation ($y = mx + c$), where y is the peak area ratio. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each quality control (QC) level.

2.4. Standard and quality control preparation

Standard stock solution of clonidine (100 μg/mL) and IS (100 μg/mL) were separately prepared in methanol. Working solutions in the required concentration range were prepared by appropriate dilution of their stock solutions in acetonitrile:water

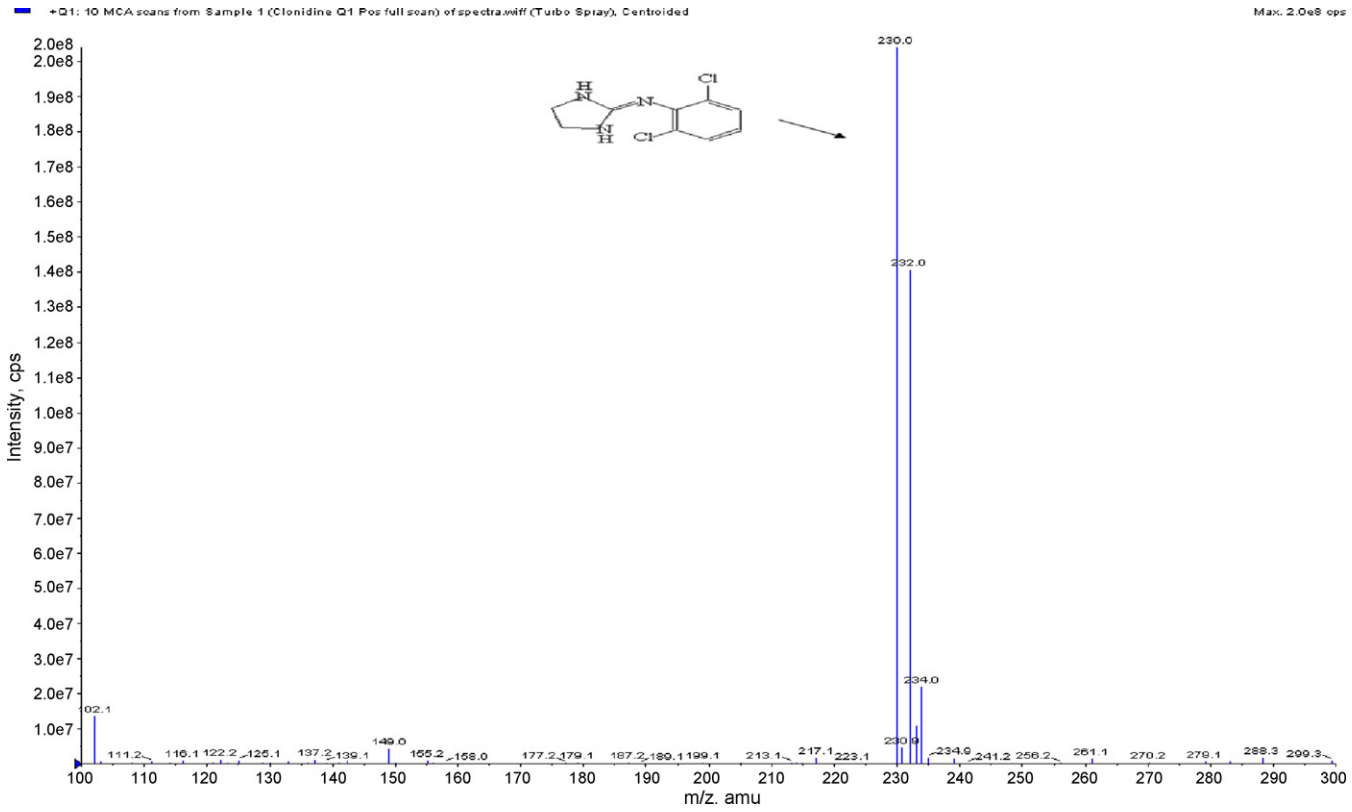

Fig. 1. Representative spectra of parent ion of clonidine (500 ng/mL in methanol).

Fig. 2. Representative spectra of product ion of clonidine (500 ng/mL in methanol).

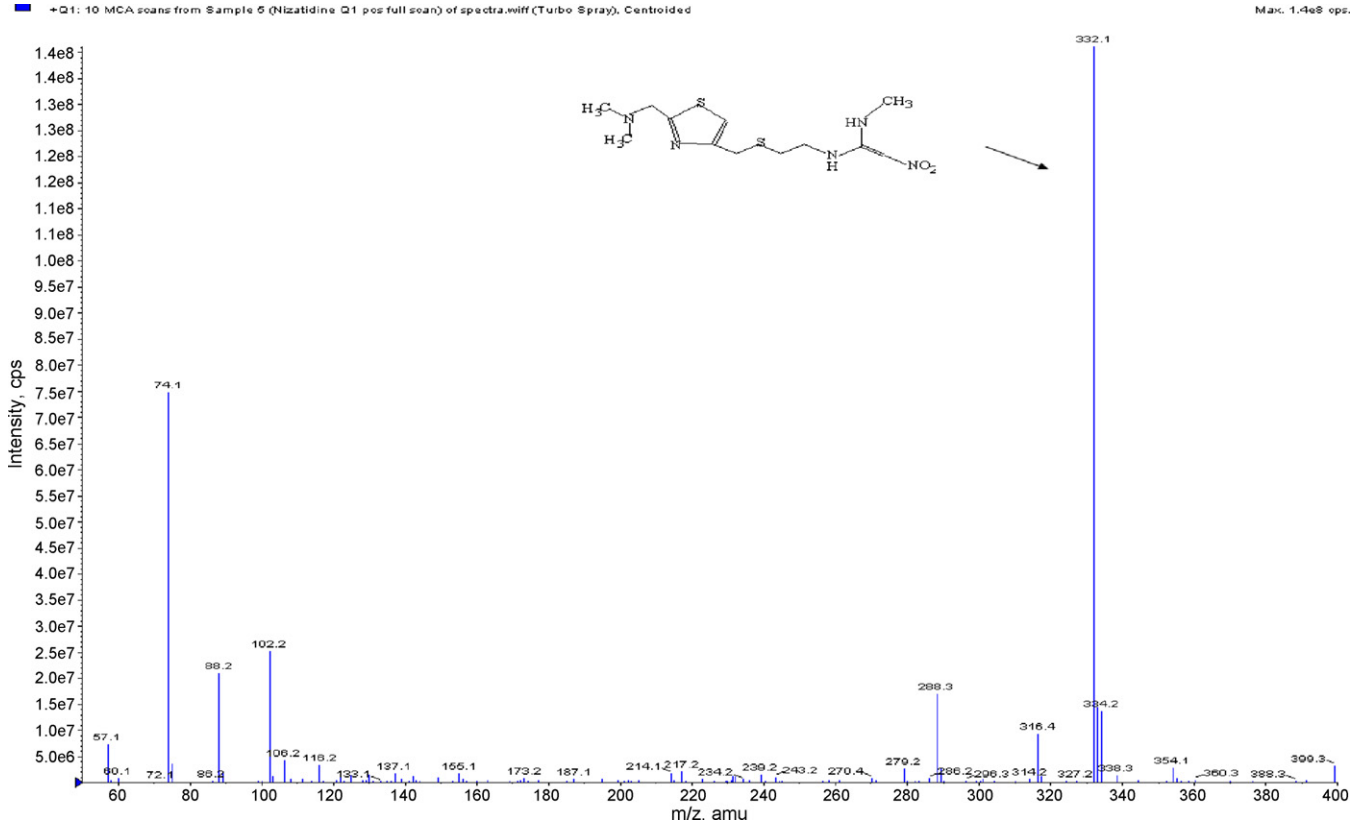

Fig. 3. Representative spectra of parent ion of nizatidine (500 ng/mL in methanol).

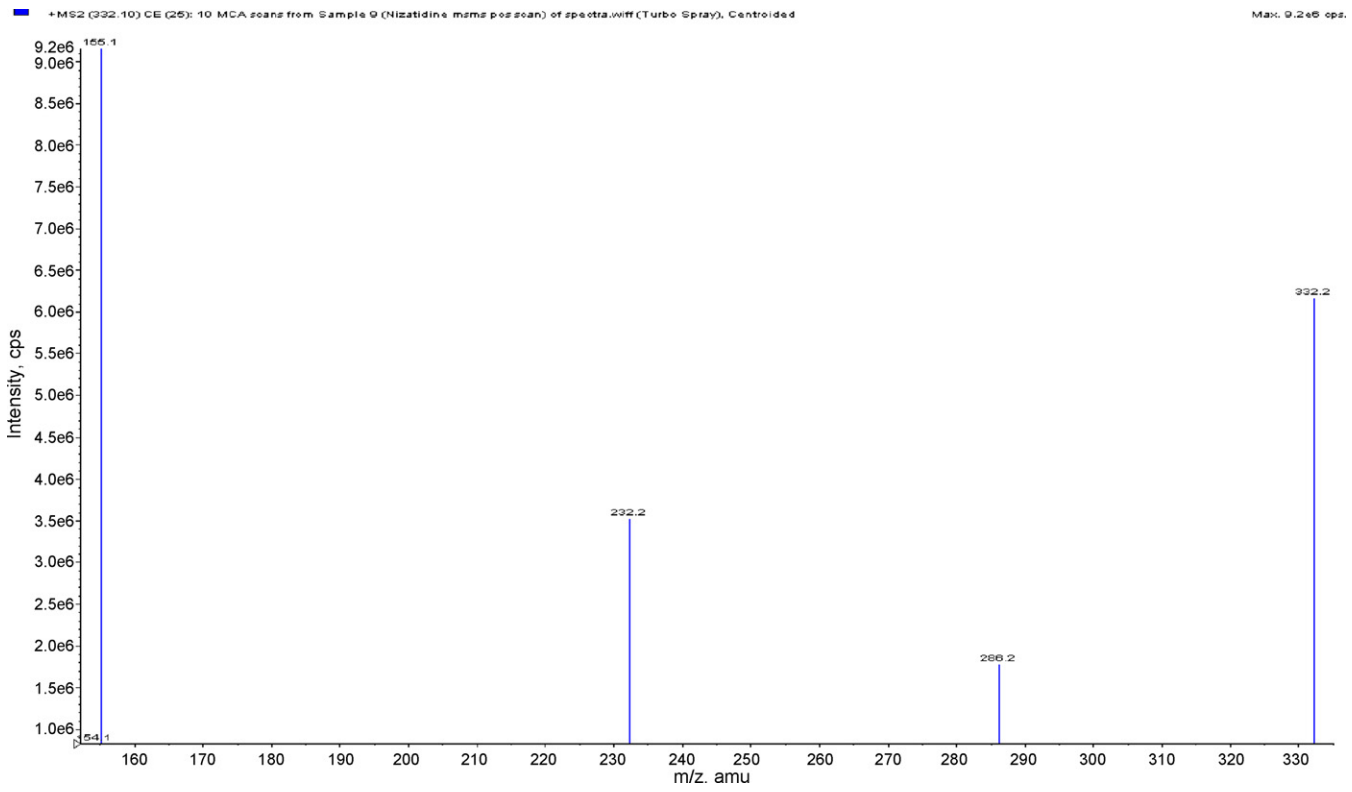

Fig. 4. Representative spectra of product ion of nizatidine (500 ng/mL in methanol).

(80:20, v/v). All the solutions were stored at 2–8 °C and were brought to room temperature before use.

The calibration standards and QC samples were prepared by spiking working solutions (5%) with blank plasma. Calibration samples were made at concentrations of 50, 75, 100, 300, 800, 1400, 2000 and 2500 pg/mL and quality control samples were prepared at 150 pg/mL (lower quality control, LQC), 1000 pg/mL (medium quality control, MQC) and 2200 pg/mL (higher quality control, HQC) for clonidine. Spiked plasma samples were stored at −20 °C.

2.5. Sample preparation

All frozen calibration standards and quality control samples were thawed at room temperature. The samples were adequately vortexed to mix. 0.5 mL of plasma sample was dispensed into eppendorf tubes and 50 μL of 30 ng/mL of internal standard was added, followed by vortexing for 10 s. The samples were centrifuged at 15,000 rpm at 10 °C for 5 min and then loaded on Orochem DVB-HL cartridges preconditioned with 1 mL of methanol followed by 1 mL of 2 mM ammonium acetate. Further, plasma was drained out under nitrogen pressure and cartridges were washed with 1 mL 10% methanol in water to clean up the samples. After proper drying of the cartridge, elution was carried out using 1 mL methanol. The eluate was evaporated to dryness under nitrogen at 50 °C. The residue was reconstituted by 300 μL mobile phase and transferred into vials, capped and placed in an autosampler rack for injection.

2.6. Method validation

A thorough and complete method validation of clonidine in human plasma was done following the USFDA guidelines [15]. The method was validated for selectivity, sensitivity, linearity, accuracy and precision, recovery, stability, matrix effect and dilution integrity.

The selectivity towards endogenous and exogenous plasma matrix components was assessed in 12 different batches (6 normal, 3 haemolysed and 3 lipemic) of human plasma samples by analyzing blank and spiked samples at LLOQ level. It was performed in two sets, in the first set, plasmas were extracted and directly injected for LC–MS/MS detection and in the second set, blank plasmas spiked with LLOQ working solution of clonidine were extracted and analyzed. The second set was also used for sensitivity determination.

The linearity of the method was determined by analysis of standard plots associated with an eight-point standard calibration curve. Five linearity curves containing eight non-zero concentrations were analyzed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes was calculated from the simple linear equation using regression analysis of spiked plasma calibration standard with reciprocate of the drug concentration as a weighting factor (1/concentration, i.e. $1/x$). The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the range tested.

Inter-batch and intra-batch accuracy and precision was evaluated at five different concentrations (LLOQ quality controls (LLOQ-QCs), LQC, MQC, HQC and upper limit of quantitation (ULOQ)). Mean and standard deviation (SD) were obtained for calculated drug concentration at each level. Accuracy and precision were evaluated in terms of relative error (RE) and %CV, respectively.

Recovery presents the extraction efficiency of a method. It was performed at LQC, MQC and HQC levels. The absolute recoveries were evaluated by comparing peak area of extracted samples to that of aqueous samples (quality control working solutions spiked in mobile phase).

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions. Stock solution stability was performed by comparing area response of stability sample of analyte and internal stan-

dard with the area response of sample prepared from fresh stock solutions. Bench top stability, extracted sample stability (process stability), freeze thaw stability, Dry extract and long-term stability were performed at LQC and HQC level using six replicates at each level.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with six different lots of heparinised plasma. Two replicates each of LQC and HQC were prepared from different lots of plasma (total 24 QC samples).

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above ULOQ, which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 1.6 times the ULOQ concentration for all the analytes. Six replicates each of 1/2 and 1/4th concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 against the freshly prepared calibration curve.

3. Result and discussion

3.1. Method development

For optimum detection up to picogram level of clonidine with IS in human plasma, it was necessary to adjust not only the chromatographic conditions and mass parameters but also to develop an efficient extraction method that gives consistent and reproducible recovery of analytes from plasma. Parent ions and product ions were optimized by infusing 500 ng/mL solutions of clonidine and nizatidine into mass spectrometer in 5–500 m/z range, in positive polarity mode using electro spray ionization technique. Best intensity for $[M+H]^+$ ions was found in positive mode for the analyte including IS as they have an ability to accept protons. Most abundant product ions were obtained at m/z 44.1 by applying sufficient CAD gas and CE. Optimization of source temperature and heater gas flow is important as they play an important role in minimizing ion suppression and altering the sensitivity. Increase in source temperature and heater gas above 350 °C and 8000 cm^3/mL, respectively, augmented the intensity for the analyte. Minor changes in ion spray voltage and nebuliser gas did not have a marked effect on the signal intensity and were maintained at 1250 V and 6 psi, respectively. A dwell time of 0.2 s was sufficient and no cross-talk was found between all MRMs.

Chromatographic analysis of the analytes and IS was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Separation was tried using various combinations of acetonitrile and buffer with varying contents of each component on variety of columns like C8 and C18 Hypersil, Hypurity; C18 advance high purity and Symmetry shield RP 18 to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape.

Use of buffer helped in achieving good response for MS detection operating in the positive mode. To get a good chromatographic separation with desired response it was observed that, mobile phase as well as selection of column is an important criterion. Thus, a mobile phase consisting of 2 mM ammonium acetate:acetonitrile (20:80, v/v) was found suitable as the analyte was protonated and well separated by this phase. High content of acetonitrile (80%) in the mobile phase helped in eluting the analyte and IS within 3 min at a flow rate of 0.4 mL/min. Hypersil Hypurity C18 (50 mm × 4.6 mm, 5 μm particle size) column gave good peak shape and response even at LLOQ level for all the analyte including IS. Low-injection volume of 10 μL reduced overloading of column with analytes, thereby ensuring more number of analyses on the same column.

For extraction of clonidine and IS with quantitative recovery and negligible matrix effect from plasma samples, an efficient extraction method was mandatory. A simple and rapid solid-phase extraction method was developed using Orochem, 30 mg; 1 mL DVB HL SPE cartridges. Extraction of analytes was carried out with 1 mL of methanol. The eluate was subjected for drying and reconstitution to increase the sensitivity (lower LLOQ) with improved response up to picogram level. No interference was observed from any endogenous or exogenous plasma matrix.

It was difficult to find a compound which could ideally mirror the analyte to serve as a good IS. Several compounds were investigated to find a suitable IS, and finally nizatidine belonging to a same class of compounds was found most appropriate for the present purpose. There was no significant effect of IS on analyte recov-

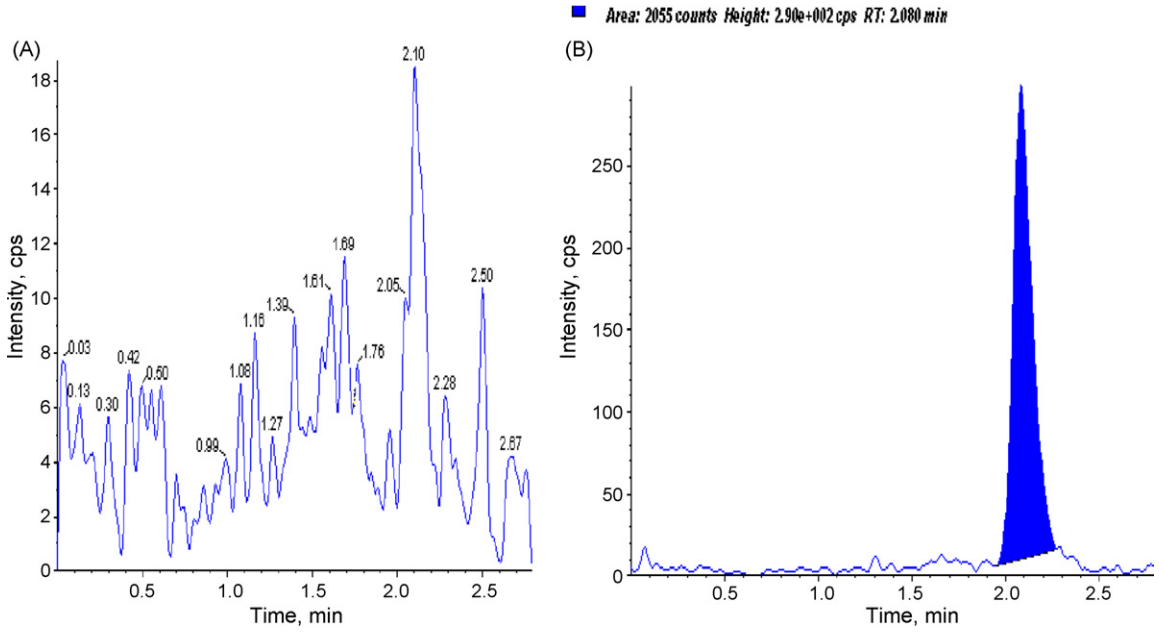

Fig. 5. Representative chromatogram of plasma blank (A) and plasma spiked with lower limit of quantification (50.00 pg/mL) (B).

Table 1
Summary of calibration curve standards ($n = 5$)

Nominal concentration (pg/mL)	Mean back calculated concentration (pg/mL)	%CV	RE (%)
49.850	49.466	7.09	−0.77
74.775	83.5415	1.06	11.72
99.700	98.0072	5.02	−1.70
299.100	311.3018	14.83	4.08
797.600	787.7328	7.43	−1.24
1395.800	1424.0242	7.37	2.02
1994.000	1960.4424	4.30	−1.68
2517.425	2537.546	4.73	0.80

CV, coefficient of variation; RE, relative error.

ery, sensitivity or ion suppression. The results of method validation using nizatidine as the IS were acceptable in this study based on FDA guidelines. Use of nizatidine as internal standard makes it mandatory that subjects participating in the study should not be under any treatment, which involves use of nizatidine.

3.2. Selectivity and sensitivity (LLOQ)

The selectivity of the method towards endogenous plasma matrix was evaluated in 12 different batches of human plasma by analyzing blanks and spiked samples at LLOQ levels. Endogenous peaks at the retention time of the analytes were not observed for any of the plasma batches. Fig. 5 demonstrates the selectivity results with the chromatograms of blank plasma and the peak response of analyte at LLOQ level having peak area 2055 and peak height 2.90e+002 cps. The response was calculated in terms of signal to noise (S/N) ratio for spiked and unspiked plasmas. The mean S/N ratio for 12 plasma samples found was 748.92. The mean accuracy (%) for back calculated concentration for normal, heamolysed and lipemic was within 101–117% with %CV between 4.28% and 6.50%. The retention times for clonidine and IS was 2.10 and 1.60 min, respectively.

3.3. Linearity, accuracy and precision, recovery

The peak area ratios of calibration standards were proportional to the concentration of analyte in each assay over the nominal concentration range of 50.0–2500.0 pg/mL. The calibration curves appeared linear and were well described by least squares lines with correlation coefficient ≥0.9983. A weighing factor of 1/concentration ($1/x$) was chosen to achieve homogeneity of variance. The observed mean back calculated concentration with accuracy (%) and precision (%CV) of five linearities are given in Table 1.

The precision and accuracy of the developed method was determined by analysis five quality control samples. Intra-day variation of the assay was assessed by injecting two batches containing these samples on the same day. Inter-day variation was assessed by injecting one batch on 3 different days (including one intra-day batch). Each run consisted of six replicates at five concentration levels (LLOQ-QC, LQC, MQC, HQC and ULOQ-QC). Intra-batch precision was less than 15% at lower level (LLOQ-QC) and less than 6% for remaining levels. Precision observed for inter-batch was also almost 15% at lower level (LLOQ-QC) and less than 14% for remaining levels. Accuracy was within 89–100% of their nominal concentration, respectively as given in Table 2.

Six replicates at LQC, MQC and HQC level were prepared for recovery determination. Mean absolute recovery found was 71.50%, 72.78% and 71.29% with a precision (%CV) of 4.17, 2.20 and 3.03 at LQC, MQC and HQC, respectively for clonidine. Recovery of IS was 71.09%, 71.94% and 65.29% with %CV of 1.74, 5.67 and 4.11 at LQC, MQC and HQC, respectively. This indicates that the extraction efficiency for the analyte as well as IS was consistent and reproducible.

3.4. Stability, matrix effect and dilution integrity

Stock solution of analyte and IS were stable at room temperature for 6 h and at 2–8 °C for 7 days. Clonidine in control human plasma at room temperature was stable at least for 4 h and for minimum of five freeze and thaw cycles. Process stability was of 46 h

Table 2
Intra-batch and inter-batch precision and accuracy

Level	Concentration added (pg/mL)	Intra-batch			Inter-batch		
		Mean concentration found (pg/mL)[a]	RE (%)	%CV	Mean concentration found (pg/mL)[b]	RE (%)	%CV
LLOQ	49.890	49.472	−0.84	14.73	47.577	−4.64	15.09
LQC	149.670	134.567	−10.09	5.59	139.422	−6.85	13.22
MQC	997.800	923.007	−7.50	4.11	946.019	−5.19	5.43
HQC	2195.160	2117.215	−3.55	3.41	2022.371	−7.87	5.14
ULOQ	2494.500	2324.182	−6.83	4.90	2345.837	−5.96	5.91

RE, relative error; CV, coefficient of variance.
[a] Mean of six replicates observations at each concentration.
[b] Mean of 24 replicates observations over 4 different analytical runs.

Table 3
Stability results

Stability	Level	*A*	%CV	*B*	%CV	Change (%)
Bench top (4 h at room temperature)	LQC ($n = 6$)	130.515	9.27	130.147	4.49	−0.28
	HQC ($n = 6$)	2219.081	6.08	1960.470	7.78	−11.65
Autosampler (46 h, 10 °C)	LQC ($n = 6$)	144.690	7.03	140.182	9.12	−3.12
	HQC ($n = 6$)	2133.062	3.13	2068.197	5.24	−3.04
Fifth freeze thaw cycle	LQC ($n = 6$)	144.690	7.03	141.738	6.17	−2.04
	HQC ($n = 6$)	2133.062	3.13	2030.848	4.72	−4.79
Long-term (133 days, −20 °C)	LQC ($n = 6$)	134.532	9.98	149.117	6.08	10.86
	HQC ($n = 6$)	2010.486	3.19	2062.007	5.16	2.56
Dry extract (19 h, 2–8 °C)	LQC ($n = 6$)	136.847	5.11	127.917	5.17	−6.53
	HQC ($n = 6$)	2281.442	2.04	2201.548	2.64	−3.50

A, mean comparison sample concentration (pg/mL); *B*, mean stability sample concentration (pg/mL); CV, coefficient of variation.

at 4 °C. Spiked plasma samples stored at −20 °C for long term stability experiment were stable for minimum 133 days. Dry extract samples at 2–8 °C were stable for a period of 19 h. Different stability experiments in plasma and the values for the precision and percent change are shown in Table 3. There was no significant degradation observed since the deviations in concentration was within 15% of their nominal values.

Matrix effect is due to co elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS/MS method for supporting pharmacokinetics studies. It was performed with the aim to see the matrix effect by processing six lots of different plasma samples in quadruplet ($n = 4$). LQC and HQC stock solutions were spiked post-extraction in duplicate. Aqueous recovery solutions of LQC and HQC along with internal standard were also prepared. The results found were well within the acceptable limits as the % difference of post-spiked samples and aqueous recovery samples at LQC and HQC levels were within 13% Moreover, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analytes and IS peak. Thus, the extraction method was rugged enough and gave accurate and consistent results when applied to subject sample analysis.

The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal. The coefficient of variation (%CV) for 1/2 and 1/4 dilution samples of clonidine were less than 3.0%.

4. Conclusion

The developed LC–MS/MS assay for clonidine is selective, rugged and suitable for routine measurement of subject samples. This method has significant advantages in terms of clean and reproducible SPE extraction procedure and a short chromatographic run time of 3.0 min. The extraction method gave consistent and reproducible recoveries for analytes and IS from plasma, with minimum matrix interference and ion suppression. The reconstituted residue after evaporation (10 μL) is directly submitted for LC–MS analysis to give high throughput. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with test formulation of clonidine.

Acknowledgements

The authors are indebted to the Directors of Accutest Research Laboratory for their continuous support and encouragement and for providing necessary facilities to carry out this work.

References

[1] C. Dollery (Ed.), Therapeutic Drugs, 2nd ed., Churchill Livingstone Publication, p. C294.
[2] N.D. Nader, T.A. Ignatowski, C.J. Kurek, P.R. Knight, R.N. Spengler, Anesth. Analg. 93 (3) (2001) 363.
[3] G.M. Ke, E.H. Zhang, L. Wang, Q. Zhang, H.G. Du, H.Y. Guo, Acta Pharm. Sin. 39 (5) (2004) 367.
[4] G.-M. Ke, L. Wang, H.-Y. Xue, W.-L. Lu, X. Zhang, Q. Zhang, H.-Y. Guo, Biol. Pharm. Bull. 28 (2) (2005) 305.
[5] L. Zhao, L. Ding, Xin Wei, J. Pharm. Biomed. Anal. 40 (2006) 95–99.
[6] A. Hercegova, J. Sadecka, J. Polonsky, Acta Pol. Pharm. 55 (3) (1998) 167.
[7] M. Pelzer, T. Addison, W. Li, X. Jiang, N. Weng, J. Liq. Chromatogr. Rel. Technol. 25 (2002) 1019.
[8] W. Naidong, H. Bu, Y.L. Chen, W.Z. Shou, X. Jiang, T.D. Halls, J. Pharm. Biomed. Anal. 15;28 (6) (2002) 1115.
[9] C.E. Wolf, A. Poklis, J. Anal. Toxicol. 29 (7) (2005) 711.
[10] S.M. Walters, D.B. Stonys, J. Chromatogr. Sci. 21 (1) (1983) 43.
[11] T. Wenzl, E.P. Lankmavr, R. Wintersteiger, A. Sadjak, K. Likar, D. Zakel, J. Biochem. Biophys. Meth. 53 (1–3) (2002) 131.
[12] L.M. Todesco, J.J. Thoma, R.D. Barth, N.J. Myers, R. White, R.M. Ward, Ther. Drug Monit. 9 (1) (1987) 78.
[13] C. Mueller, M. Ramie, S. Harlfinger, C. Huenseler, M. Theisohn, B. Roth, J. Chromatogr. A 1139 (2) (2007) 221.
[14] I. Wilezynska-Wojtulewiez, N. Sadlej-Sosnowaska, J. Chromatogr. 367 (2) (1986) 434.
[15] Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.