DEVELOPMENT AND VALIDATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRIC METHOD FOR QUANTIFICATION OF CLONIDINE IN HUMAN PLASMA

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ABSTRACT

A simple, sensitive and selective method for the determination of Clonidine by using rapid high-performance liquid chromatography/positive electroscopy ionization tandem mass spectroscopy. The method consists of Liquid-Liquid extraction with 80:20 Diethyl ether: Dichloromethane followed by the analysis of the Extracted sample by liquid chromatography-mass spectroscopy (LC-MS/MS) in selective reaction monitoring mode using electrospray ionization mode (ESI). Chromatography was performed on a C_{18} reverse phase column, 80:20 (50:50 Methanol: Acetonitrile):10mM ammonium acetate (pH 6.8) as a mobile phase. The assay exhibited a linear dynamic range of 50 to 2000 pg/ml for Clonidine in human plasma. Stability assessment was also included. A run time of 2.1 min for each sample made it possible to analyse healthy volunteers participating in pharmacokinetics drug-drug interaction studies.

KEY WORDS: LC-MS/MS, Human Plasma, Bioanalytical, Clonidine, Validation.

1.INTRODUCTION

Clonidine, *N*-(2, 6-dichlorophenyl)-4,5-dihydro-1*H*-imidazol-2-amine, is a direct-acting adrenergic agonist prescribed historically as an anti-hypertensive agent. It has found new uses, including treatment of some types of neuropathic pain, opioid detoxification, sleep hyperhydrosis and off-label, to counter the side effects of stimulant medications such as methylphenidate or amphetamine. It is becoming a more accepted treatment for insomnia, as well as for relief of menopausal symptoms. Clonidine is increasingly used in conjunction with stimulants to treat attention-deficit hyperactivity disorder (ADHD), where it's given in late afternoon and/or evening for sleep, and because it sometimes helps moderate ADHD-associated impulsive and oppositional behavior and may reduce tics. Clonidine can also be used in the treatment of Tourette syndrome (Natalie,2002; Schapiro,2002; Anavekar,1989; Jorg,1997; Guang,2005).

Several methods to determine Clonidine with HPLC or gas chromatography-mass spectrometry have been previously described (Mary,2002;Walters andStonys,1983;Chu,1978 ;Edlumdand Paalzow,1977; Muller,2007;Gary,2005). The assays used relatively large plasma sample volumes (up to 2 mL) and either multiple-step liquid or solid-phase extraction procedures. In this report, we describe a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method developed and validated for the quantification of Clonidine in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying Clonidine at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Clonidine.

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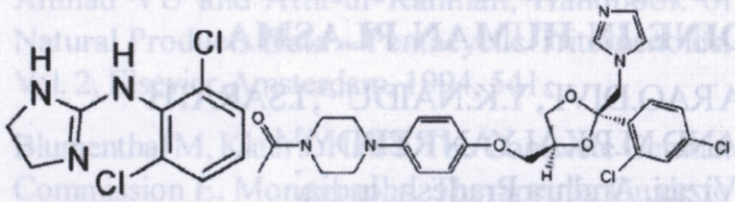
2.EXPERIMENTAL

I. Chemicals and Solvents

Clonidine drug substance and Ketoconazole (Internal Standard) was obtained from Inventis Drug Delivery Systems (Hyderabad, India). The chemical structures are represented in Fig.1. HPLC-grade Methanol, Acetonitrile, Diethyl ether and Dichloromethane was purchased from JT Bakers. Ammonium Acetate was purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q water system (Millipore, Bedford, MA, USA) was

used. All other chemicals were of analytical grade.



(a) Clonidine **(b) Ketoconazole**

Figure 1. Chemical Structures for Clonidine and the ISTD (Ketoconazole)

II.LC/MS/MS instrument and conditions

The high-performance liquid chromatography (HPLC) SILHTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-20 AD VP binary pump, a DGU20A3 Degasser and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermostated column. The chromatography (Chatwal,2007; Lloyd,2004;Murray,2005) was on Cohesive PropelC18, (5 µm, 3.0 x 50mm) at a temperature of 20°C. The isocratic mobile phase composition was a mixture of 80:20 (50:50 Methanol: Acetonitrile):10mM ammonium acetate (pH 6.8), which was pumped at a flow rate of 0.35 mL/min. Mass spectrometric detection was performed on a TSQ Quantum Discovery MAX triple quadrapole instrument (Thermo Finnigan, USA) using the Selective reaction monitoring (SRM) mode. A turbo electrospray ionization (ESI) interface in positive mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on LC Quan 2.5.6. Software package (Thermo).

Table 1. Main working parameters of the tandem mass spectrometer

Parameters	Value
Spray voltage	5000
Sheath gas pressure	40
Auxiliary gas pressure	15
Capillary temperature	350
Tubelens offset	30 & 18(Analyte and IS)
Skimmer offset	-14(Analyte) and -12 (IS)
Collision energy	20(Analyte) and 37 (IS)
Polarity	Positive
Mode of analysis	SRM
Ion transition for Clonidine, m/z	230.00±0.5/ 213.939±0.5
Ion transition for Ketoconazole, m/z	531.100±0.5/ 243.391±0.5

III. Sample preparation

Standard stock solutions of Clonidine (5 mg/mL) and the IS (5 mg/mL) were separately prepared in Methanol. Working solutions for calibration and controls were prepared by appropriate dilution in 80:20 Methanol: water. The IS working solution (500 ng/mL) was prepared by diluting its stock solution with Diluent (80:20) methanol: water. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain Clonidine concentration levels of 50.520, 101.041, 252.602, 400.956, 549.255, 704.173, 1005.962, 1378.030, 1680.525 and 2000.625 pg/mL, as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 61.634(LLOQ), 133.987(Low), 858.889(medium-1), 343.556(medium-2) and 1431.481ng/mL (high), as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in Ria Vials (Tarson, 5 mL) and stored in a freezer at below -80°C until analyses. Plasma samples (0.500 mL) were pipetted into a 5-mL Ria vials, 50 µL of IS working solution (500 ng/mL) and 100µL 10mM Ammonium Acetate pH 6.8 were added. After vortex mixing for 1 min's, a 2.0-mL 80:20 Diethyl ether: Dichloromethane was added and the samples were vortex-mixed for 10 min's. Centrifuge the Ria vials at 4000 rpm at 10°C for 10 min, transferred approximately 1.6mL of supernatant to prelabelled glass vials and evaporated to dryness using nitrogen evaporator maintained at 37°C. After completion of evaporation reconstituted the Ria vials containing drug using 150µL of 80:20 (50:50 Methanol: Acetonitrile):10mM Ammonium Acetate (pH 6.8), vortexed, transferred to HPLC vials and a 10-µL aliquot was injected into the chromatographic system.

IV.Bioanalytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and Ten non-zero samples covering the total range 50–2000 pg/mL, including the lower limit of quantitation (LLOQ). The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression (US FDA,2000) on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification. The within-batch precision and accuracy were determined by analyzing six sets of QC samples in a batch. The between batch precision and accuracy were determined by analyzing six sets of QC

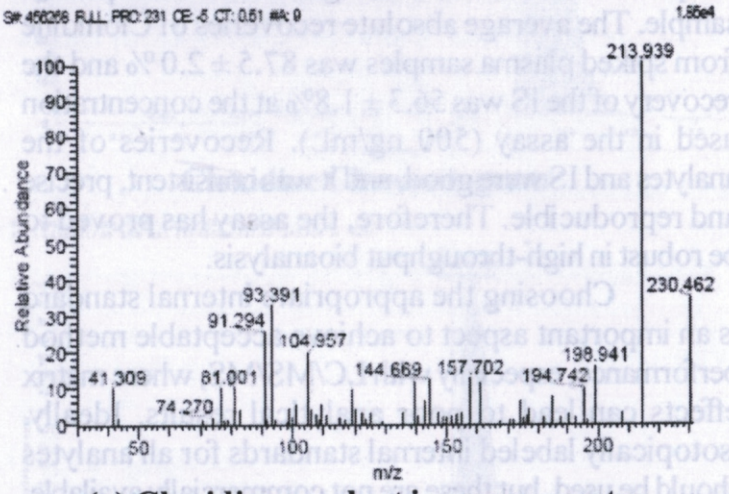
samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations. Recovery of Clonidine from the extraction procedure was determined by a comparison of the peak area of Clonidine in spiked plasma samples (six each of low, medium and high QCs) with the peak area of Clonidine in samples prepared by spiking extracted drug-free plasma samples with the same amounts of Clonidine at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 6) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography. The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (-80°C) and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (61.634 and 1430.481 pg/mL as low and high values) with six determinations for each.

3.RESULTS AND DISCUSSION

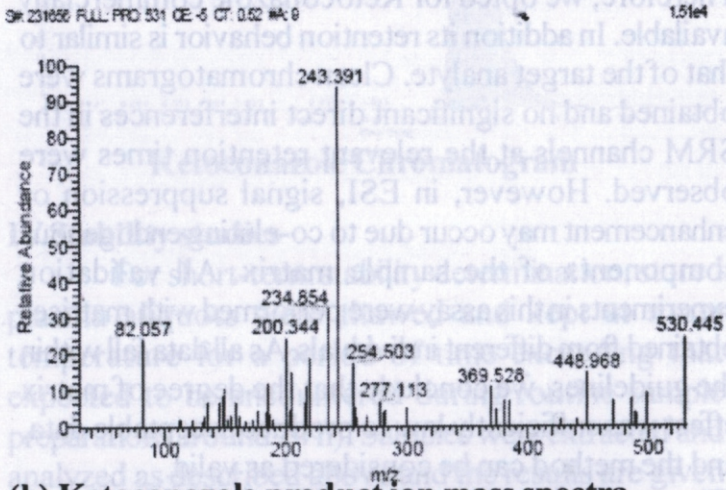
I.Mass spectrometry

The analysis of Clonidine from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC/MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The product ion mass spectra of Clonidine and the IS are shown in Fig. 2. $[M-H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The collisionally induced dissociation (CID) mass spectrum of Clonidine shows the formation of characteristic product ions at m/z 213.939. The CID mass spectrum of the IS shows the formation of characteristic product ions at m/z 243.391. The most sensitive mass transition was from m/z 230.000 to 213.939 for Clonidine and m/z 531.100 to 243.391 for the IS. LC/SRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the SRM technique was chosen for the assay development. The SRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

Figure 2. Full- scan product ion mass spectra's for analyte and ISTD



(a) Clonidine product ion mass spectra



(b) Ketoconazole product ion mass spectra

Figure 2. Full- scan product ion mass spectra's for analyte and ISTD

II. Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 80:20 (50:50 Methanol: Acetonitrile):10mM Ammonium Acetate (pH 6.8) could achieve this purpose and was finally adopted as the mobile phase. The proportion of organic solvent eluted the analyte and the IS at retention times of 1.5 and 1.3 min, respectively. A flow rate of 0.35 mL/min produced good peak shapes and permitted

a run time of 2.1 min. Liquid-Liquid Extraction was used for the sample preparation in this work. An Extraction with 80:20 Diethyl ether: Dichloromethane can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses. An Extraction with 80:20 Diethyl ether: Dichloromethane was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of Clonidine from spiked plasma samples was 87.5 ± 2.0 % and the recovery of the IS was 56.3 ± 1.8% at the concentration used in the assay (500 ng/mL). Recoveries of the analytes and IS were good, and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Therefore, we opted for Ketoconazole commercially available. In addition its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the SRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. All validation experiments in this assay were performed with matrices obtained from different individuals. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

III. Assay performance and validation

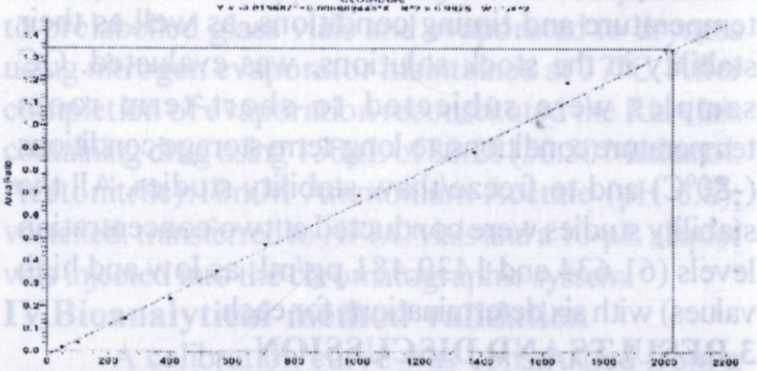
The ten-point calibration curve was linear over the concentration range 50–2000 pg/mL. The calibration model was selected based on the analysis of the data by linear regression with intercepts and weighting factors (1/x, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighting factor. Linear regression equation for the calibration curve is $y = mx + c$ here y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9997_0.0004; Table 2 summarizes the calibration curve results. The Calibration curve obtained for Clonidine depicted in Fig. 3.

The selectivity of the method was examined by analyzing six blank human plasma extract, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Excellent sensitivity was observed for a 10-µL injection volume. The SRM chromatograms obtained for an extracted plasma sample are depicted in Fig. 4.

Table 2. Precision and accuracy data of back-calculated concentrations of calibration samples for Clonidine in human plasma (SD: standard deviation)

Concentration Added (pg/mL)	Concentration found (mean±SD, n=6) (pg/mL)	Precision (%)	Accuracy (%)
50.525	52.7493± 1.7990	3.4	104.4
101.051	90.4985± 5.4157	6.0	89.6
252.627	244.7430 ± 1.5599	0.6	96.9
400.995	373.3150 ± 10.2589	2.7	93.1
549.308	547.4340 + 22.6935	4.1	99.7
704.241	715.6450± 34.4821	4.8	101.6
1006.059	1003.2297 ± 62.6496	6.2	99.7
1378.162	1388.3100 ± 42.07766	3.0	100.7
1680.686	1782.5215 ± 33.8457	1.9	106.1
2000.816	2116.2747 ± 110.9741	5.2	105.8

Figure. 3. Calibration curve obtained for Clonidine



The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision and was found to be 50.525 pg/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (50.525 pg/mL) was ten-fold greater than the Mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The between-batch precision at the LOQ QC was 15.9% and the between-batch accuracy was 98.0% (Table 3). The within batch precision was 10.7% and the accuracy was 99.3 for Clonidine. The Lower and upper quantification levels of Clonidine ranged from 135.165 to 1444.067 pg/mL in human plasma. For the between-batch experiments the precision ranged from 10.0 to 12.2 % and the accuracy from 98.4 to 106.7 % (Table 3). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria (=15%). The upper concentration limits can be extended with acceptable precision and accuracy by a fourfold dilution with control human plasma.

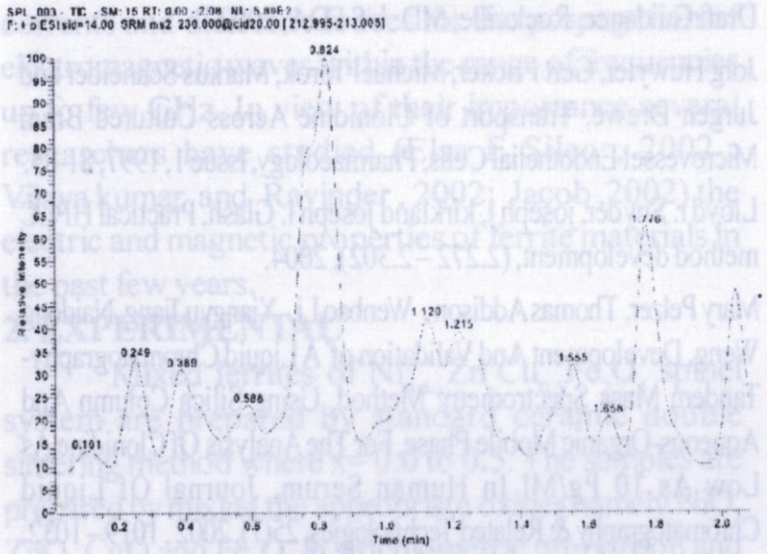
These results suggest that samples with concentrations greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

Table 3. Precision and accuracy of the method for determining Clonidine concentrations in plasma samples

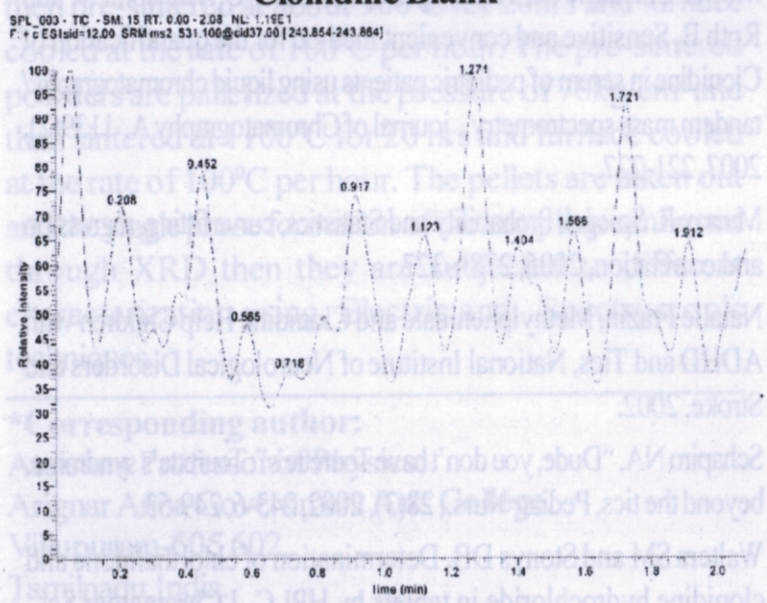
Concentration added (pg/mL)	Concentration found) (mean±SD) (pg/mL)	Precision (%)	Accuracy (%)	Concentration found (mean±SD) (pg/mL)	Precision (%)	Accuracy (%)
1444.067	1465.43733 ± 178.774347	12.2	101.5	1304.1345± 90.744498	7.0	90.3
866.440	924.32237 ± 92.753877	10.0	106.7	817.4563 ± 79.92275	9.8	94.3
346.576	352.80093± 39.986351	11.3	101.8	319.3828 ± 43.22907	13.5	92.2
135.165	133.0190 ± 15.037232	11.3	98.4	125.4325 ± 17.44787	13.9	92.8
62.176	60.93370 ± 9.658517	15.9	98.0	61.7115 ± 6.59145	10.7	99.3

(SD: standard deviation), Between- batch precision (n=6) with in batch precision (n=6)

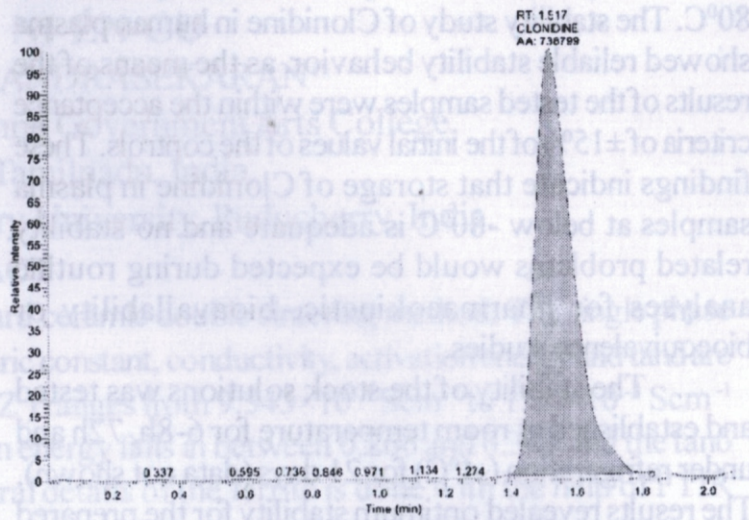
Figure 4. SRM chromatograms for Blank (with out Analyte and ISTD), Clonidine and the ISTD resulting from analysis



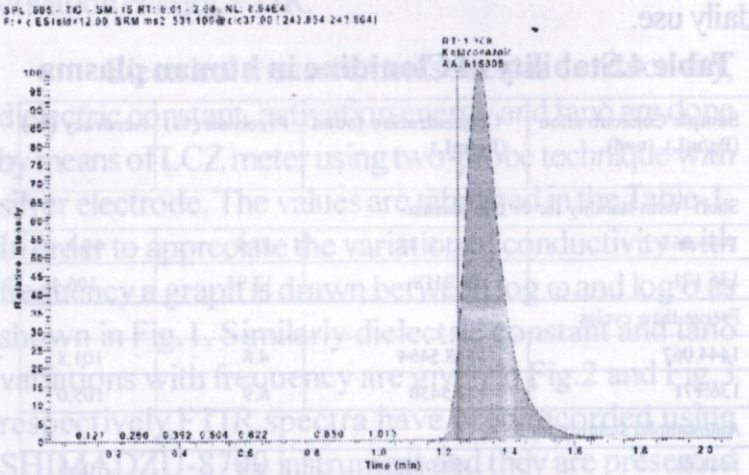
Clonidine Blank



Ketoconazole Blank



Clonidine Chromatogram



Ketoconazole Chromatogram

IV.Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results are given in Table 4. These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the auto sampler for 35 h was also assessed. The results indicate that solutions of Clonidine and the IS can remain in the auto sampler for at least 35 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 4). The data representing the stability of Clonidine in plasma at two QC levels over three freeze/thaw cycles are given in Table 4. These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below 80^{0}C and thawed to room temperature.. Table 4 also summarizes the long-term stability data for Clonidine in

plasma samples stored for a period of 24 days at below 80°C. The stability study of Clonidine in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of Clonidine in plasma samples at below -80°C is adequate and no stability related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 6-8h, 72h and under refrigeration (-4°C) for 21 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Table 4.Stability of Clonidine in human plasma

Sample Concentration (Pg/mL) (n=6)	Concentration found (Pg/mL)	Precision (%)	Accuracy (%)
Short –term stability for 08 h in plasma			
1444.067	1655.5185	13.5	99.9
136.171	59.9418	13.9	106.4
Freeze-thaw cycles			
1444.067	1538.5464	4.8	101.8
136.171	127.5430	6.9	105.0
Autosampler stability for 35 h			
1444.067	1581.9832	1.8	102.8
136.171	129.1327	10.2	101.2

4.CONCLUSION

In summary, a method is described for the quantification of Clonidine in human plasma by LC/MS/MS in negative ESI mode using multiple reaction monitoring and fully validated according to commonly accepted criteria. The current method has shown acceptable precision and adequate sensitivity for the quantification of Clonidine in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of Clonidine was achieved with an LLOQ of 50.525 pg/mL. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay and use of rapid extraction and sample turnover rate of 2.1 min per sample make it an attractive procedure in high-throughput bioanalysis of Clonidine. The validated method allows quantification of Clonidine in the 50–2000 pg/mL range.

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