

Method validation of clonidine hydrochloride in human plasma by LC-MS technique

Hossein Danafar*

¹Zanjan Pharmaceutical Nanotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran

²Department of Medicinal Chemistry, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

Received: Dec 5, 2015, Revised: Jan 4, 2016, Accepted: Jan 9, 2016

Abstract

A simple and sensitive high performance liquid chromatography-electrospray ionization mass spectrometry method has been evaluated for the assignment of clonidine hydrochloride in human plasma. The mobile phase composed of acetonitrile–water 60:40 (v/v), and 0.2% formic acid 20 μ L of sample was chromatographically analyzed using a repacked ZORBAX-XDB-ODS C₁₈ column (2.1 mm \times 30 mm, 3.5 micron). Detection of analytes was achieved by tandem mass spectrometry with electrospray ionization (ESI) interface in positive ion mode was operated under the multiple-reaction monitoring mode (m/z 230.0 \rightarrow 213). Sample pretreatment involved in a one-step protein precipitation (PPT) with methanol and perchloric acid (HClO₄) of 0.15 mL plasma. Standard curve was linear ($r = 0.998$) over the concentration range of 0.01-10.0 ng/ml and showed suitable accuracy and precision. The limit of quantification (LOQ) was 0.01 ng/ml. The method is rapid, simple, very steady and precise for the separation, assignment, evaluation of clonidine healthy in human plasma.

Keywords: Clonidine hydrochloride, LC-MS, human plasma

Pharm Biomed Res 2015; 1(4): 48-58

DOI: 10.18869/acadpub.pbr.1.4.48

Introduction

Clonidine (Fig. 1), 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 (1). Clonidine belongs to a class of drugs called central alpha-adrenergic agonists. Clonidine has been in clinical use for over 40 years, which is used to treat hypertensive disorders, hyperactivity disorder, anxiety disorders, migraine, menopausal flushing and certain pain conditions (1). It has been proven by clinical studies that fluctuation of clonidine plasma concentration is

responsible for its side effects. Hence a transdermal patch formulation of clonidine was developed (1) to maintain clonidine plasma concentration in a steady state over a prolonged period of time and reduce the risk of adverse effects. Then a sensitive and reliable analytical method needed to be established to describe the determination of clonidine in human plasma. Several bioanalytical methods are reported to determine clonidine in different biological matrices like plasma (2,3), serum (4,5,7-8), urine (4), and cerebrospinal fluids (2). Sensitive and selective methods based on LC-MS/MS

* E-mail: danafar@zums.ac.ir

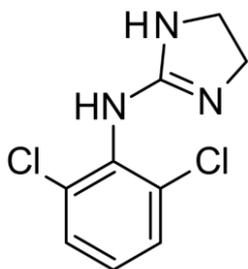


Figure 1 Chemical structure of clonidine

(3, 5, 6, 8), HPLC (2, 9), GC-MS (7) and capillary isotachopheresis (4) methodologies are reported. Muller *et al.* (10) developed an LC-MS/MS method in human serum with a lower limit of quantitation (LOQ) of 0.1 ng/ml while Li *et al.* (11) developed a 2D strong cation exchange reversed-phase LC-MS/MS method in dried blood spots with an LLOQ of 0.1 ng/ml. Although these methods were sufficiently sensitive, they were not suitable for most laboratories to perform studies involving samples in high through-put for therapeutic monitoring. Meanwhile, a high-throughput validated analytical method (12) for the quantitation of clonidine using LC-MS/MS has been published with a calibration range of 0.05–2.50 ng/ml using 500 μ L plasma. However, this study involved a solid-phase extraction, which was high costly. Most often these methods utilize solid phase extraction for sample cleanup. Since most of these methods were developed for human samples, relatively large volumes [i.e. > 0.1 mL] of plasma were needed to achieve the desired sensitivity limit. There are a limited number of methods reported for the quantitation of clonidine in preclinical samples. Measurement of clonidine in human plasma by

LC/MS/MS has yielded improved sensitivity limits of 0.5 ng/ml, and a liquid-liquid extraction procedure, and 5 ng/ml, a solid-phase extraction procedure. Therefore, it is valuable to describe the pharmacokinetic properties of clonidine to support in vivo pharmacokinetic study of transdermal clonidine patches. Previous our work was determination of ezetimibe by LC-MS method in human plasma (13-18). In the present study, we describe a simple, selective and high-through put method using high performance liquid chromatography which coupled with electrospray ionization triple quadruple mass spectrometry for the estimation of clonidine in human plasma.

Materials and methods

Materials

Clonidine hydrochloride extended release test tablets (batchno. 88001, Tolyd Daroo), clonidine hydrochloride reference tablets (batch no. 819609, EC) and clonidine hydrochloride reference standard (99.9% purity) were supplied and identified by (Boehringer Ingelheim, United Kingdom) amended by EC (European Community) pharm. Acetonitrile was HPLC grade and was purchased from Merck (Merck company, Germany). Other chemicals and solvents were from analytical or chemical lab purity grades, as needed, and purchased.

Instrumentation and operating conditions

Liquid chromatography

Liquid chromatography was carried out using a high performance liquid chromatography (HPLC) system (Agilent Technologies, model LC-1200, Englewood, USA) equipped by an auto sampler. The analytical column

used was a C₁₈ column (company, ZORBAX- XDB-ODS, USA) (2.1 × 100 mm, 3.5 micron) and was operated at 25 °C. The mobile phase consisted of acetonitrile–water 60:40 (v/v), and 0.2% formic acid was set at a flow rate of 0.2 ml/min.

Mass spectrometry

Mass spectrometric (MS) detection was performed using a triple-quadrupole mass spectrometer (Agilent Technologies, model LCMS-6410, Englewood, USA) with an electro spray ionization (ESI) interface. The ESI source was set at positive ionization mode. The [(M + H)⁺ m/z 230] for clonidine was selected as detecting ions, respectively. The MS operating conditions were optimized as follows: Ion spray voltage was set to 4000V, temperature of the ion transfer capillary was 250 °C, nebulizer gas (NEB) was 10, and curtain gas (CUR) was 8. The quantification was performed via peak-area. Data acquisition and processing were accomplished using Agilent LC-MS solution software for LC-MS-6410 system.

Preparation of stock solutions

Stock solutions of clonidine hydrochloride was prepared in HPLC mobile phase at concentrations of mg/ml and were stored at 4 °C. Working solutions of clonidine were prepared daily in HPLC mobile phase by appropriate dilution at 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 7 and 10 ng/ml.

Sample preparation and extraction procedure

Sample pretreatment involved in a one-step protein precipitation (PPT) with methanol perchloric acid (HClO₄). A 0.15 mL aliquot of the collected plasma

sample from a human volunteer was pipetted into a 1 mL centrifuge tube 0.1 mL methanol and 0.1 mL HClO₄ was added and then were vortexed for 2 min. After centrifugation of the sample at 15400 rpm for 20 min, the organic layer was transferred to another 1 mL centrifuge tube and an aliquot of 20 µL was injected into the LC-MS system.

Standard curves

Proper volume of one of the above-mentioned working solutions to produce the standard curve point's equivalent to 0.05, 0.20, 1.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/ml of clonidine hydrochloride and each sample was processed as described. Finally, the nominal known plasma concentrations were plotted against the corresponding peak areas to construct the standard curve.

Preparation of quality control samples

Quality control samples were prepared daily by spiking different samples of 0.15 mL plasma each with proper volume of the corresponding standard solution to produce a final concentration equivalent to low level (0.01 ng/ml), middle level (1.0 ng/ml) and high level (10.0 ng/mL) of clonidine hydrochloride. The following procedures were the same as describe above.

Method validation:

Assay specificity

To evaluate the matrix effect on the ionization of analytes, five different concentrations of clonidine (0.05, 0.20, 5.00, 10.00, and 100.00 ng/ml) were prepared in the drug-free blank plasma as five sample series using five different lots of the drug-free plasma and the samples were processed, as described, and injected to LC-MS. The

same concentrations were prepared in mobile phase instead of plasma and analyzed for drug concentration using the same procedure. A comparison of the matrix effects of the two variants was made as an indicator of the method specificity.

Linearity

Standard curves of ten concentrations of clonidine ranged 0.01–10.0 ng/ml were assayed. Blank plasma samples were analyzed to ensure the lack of interferences but not used to construct the calibration function. The limit of detection (LOD) was estimated from the signal-to- noise ratio. This parameter was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The limit of quantification (LOQ) was defined as the lowest concentration level that provided a peak area with a signal-to- noise ratio higher than 5.

Within-run variations

In one run, three samples with concentrations of 0.01, 1, and 10 ng/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed LC-Mass method. Then, the coefficient of variations (CV%) of the corresponding determined concentrations were calculated in each case.

Between-run variations

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by LC-Mass method. Then, the corresponding CV% values were calculated.

Extraction recovery

Three samples with concentrations of 0.01, 1, and 10 ng/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed LC-Mass method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of clonidine with the same concentrations were determined as percentage in each case.

Repeatability test

To test the method repeatability, six independent spiked plasma samples with a drug concentration of 1 ng/mL were prepared as described. A single injection of each preparation was made to LC/MS and the RSD% between the results was determined as the repeatability of the method.

Intermediate precision

On a different day to that of the repeatability study, a second analyst executed analysis of a further six samples prepared as described in repeatability test procedure. The analysis was carried out using fresh reagents and a different HPLC column. The RSD% between six measurements was determined along with the RSD% between the total of 12 measurements from the repeatability and intermediate precision tests.

Reproducibility

Mean results for the same sample analysis between our laboratory and two different test facilities were obtained and the percentage of difference between content measurements was calculated using the equation:

$$[(\text{highest value} - \text{lowest value}) / \text{mean value}] \times 100.$$

Stability:

Freeze and thaw stability

Three concentration levels of QC plasma samples were stored at the storage temperature (-20°C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw Cycle were repeated twice, then the samples were tested after three freeze (-20°C)-thaw (room temperature) cycles.

Short-term temperature stability

Three concentration levels of QC plasma samples were kept at room temperature for a period that exceeded the routine preparation time of samples (around 6 h).

Long-term stability

Three concentration levels of QC plasma samples kept at low temperature (-20°C) were studied for a period of 4 weeks.

Post-preparative stability

The auto sampler stability was conducted reanalyzing extracted QC samples kept under the auto sampler conditions (4°C) for 12 h.

Standard curve and quality control sample in each batch

A standard curve in each analytical run was used to calculate the concentration of clonidine in the unknown samples in the run. It was prepared at the same time as the unknown samples in the same batch and analyzed in the middle of the run. The QC samples in five duplicates at three concentrations (0.010, 1.0 and 10.0 ng/ml) were prepared and were analyzed with processed test samples at intervals per batch.

Results

Method development

Considering the complex biological matrix of the samples to be analyzed and the nature of the method to be used for drug assay, the method development efforts were made in two different areas of sample preparation and analyte separation which are discussed in detail in the following sections:

Sample preparation

Methanol and perchloric acid were finally adopted because of its high extraction efficiency and less interference.

Analyte separation

LC-MS/MS with positive ESI was selected to detect clonidine in human plasma. A prominent fragment with m/z 230.0 was observed in the product ion scan with positive ESI. The multiple-reaction monitoring (+) chromatograms extracted from supplemented plasma are depicted in figure 2. The retention time of clonidine was 2.4 min (Fig. 2). The total HPLC-MS analysis time was 4 min per sample. A representative chromatogram of a plasma sample with concentration of 4 ng/ml is shown in figure 2.C. No interferences of the analytic were observed. Figure 2.A shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of clonidine.

Method validation

Assay specificity

As it is clearly evident from the typical chromatograms of the developed method shown in figure 2, there are no discernible interferences between the matrix factors and the analyst. This, in

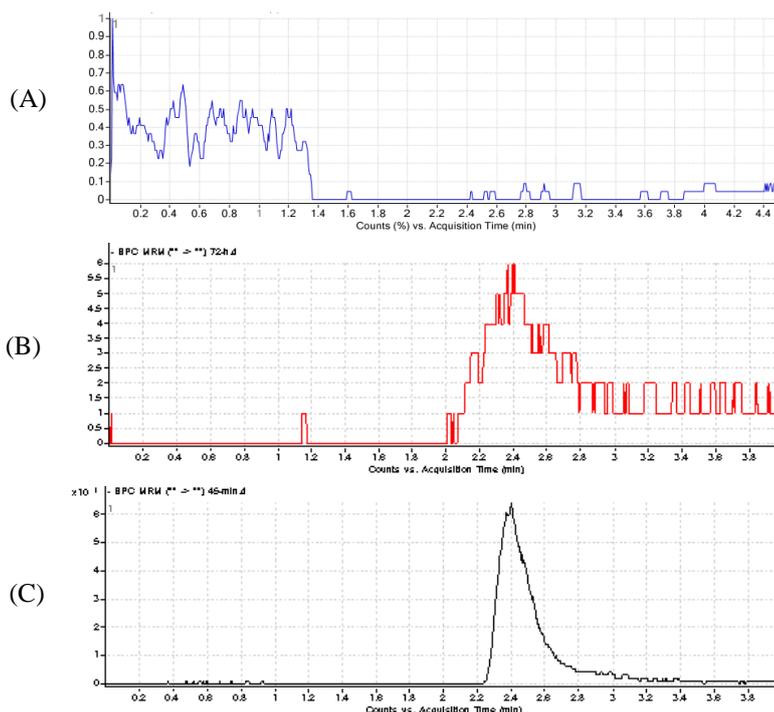


Figure 2 The MRM (+) chromatograms of clonidine. (A) Blank plasma, (B) LOQ (concentration of clonidine= 0.01ng/mL). (C) Supplemented plasma (concentration of clonidine = 4 ng/mL)

turn, ensures obtaining reliable results from the method for determination of biological concentrations of clonidine.

Linearity and LOQ

The method produced linear responses throughout the clonidine concentration range of 0.01-10 ng/ml, which is suitable for intended purposes. A typical linear regression equation of the method was: $y = 1444.3x - 16740$, with x and y representing clonidine concentration (ng/ml) and peak height (arbitrary units), respectively, and the regression coefficient (r) of 0.998. The lower limit of quantification for clonidine hydrochloride was proved to be 0.010 ng/ml. Figure 2.B shows the chromatogram of an extracted sample that contained 0.01 ng/ml (LOQ) of clonidine hydrochloride.

Within-run variations and accuracy

The within-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries are shown in table 1. These data clearly show that the developed method has an acceptable degree of repeatability and accuracy within an analytical run.

Between-run variations and accuracy

The between-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries are shown in table 2. As stated for the previous test, these data clearly show that the developed method has an acceptable degree of reproducibility and accuracy between different analytical runs.

Relative recovery (Matrix effect)

The extraction recovery determined for clonidine hydrochloride was

Table 1 Within-run variations and accuracy of the LC-Mass method for quantitation Of clonidine hydrochloride (n = 3).

| Nominal added concentration (ng/ml) | Sample number | Measured concentration (ng/ml) | Mean ± SD | CV% |
|-------------------------------------|---------------|--------------------------------|----------------|------|
| 0.01 | 1 | 0.012 | 0.0109 ± 0.001 | 9.57 |
| | 2 | 0.0099 | | |
| | 3 | 0.011 | | |
| 1 | 1 | 1.03 | 1.05 ± 0.072 | 6.86 |
| | 2 | 0.99 | | |
| | 3 | 1.13 | | |
| 10 | 1 | 10.3 | 10.05 ± 0.219 | 2.18 |
| | 2 | 9.96 | | |
| | 3 | 9.89 | | |

Table 2 Between-run variations and accuracy of the LC-Mass method for quantitation of clonidine hydrochloride (n = 3)

| Nominal added concentration (ng/ml) | Run number | Measured concentration (ng/ml) | Mean ± SD | CV% |
|-------------------------------------|------------|--------------------------------|----------------|------|
| 0.01 | 1 | 0.012 | 0.011 ± 0.0009 | 8.61 |
| | 2 | 0.099 | | |
| | 3 | 0.014 | | |
| 1 | 1 | 0.99 | 1.03 ± 0.078 | 7.58 |
| | 2 | 0.98 | | |
| | 3 | 1.12 | | |
| 10 | 1 | 9.96 | 10.06 ± 0.13 | 1.31 |
| | 2 | 10.21 | | |
| | 3 | 10.01 | | |

shown to be consistent, precise and reproducible. Data was shown below in table 3. These data indicate that there is no significant matrix effect on the outputs of the assay method.

Repeatability test

The repeatability of the method is shown in table 4. As shown, the method

has a remarkable repeatability for the drug assay in plasma.

Intermediate precision

The results of the intermediate precision test are shown in table 5. As indicated, the developed method shows an acceptable intermediate precision for clonidine hydrochloride assay.

Table 3 Relative recovery of clonidine hydrochloride by the LC-Mass method (n = 3)

| Nominal added concentration (ng/ml) | Sample number | Percent recovery (%) | Mean \pm SD | CV% |
|-------------------------------------|---------------|----------------------|------------------|------|
| 0.01 | 1 | 99.25 | 96.80 \pm 2.5 | 2.62 |
| | 2 | 94.18 | | |
| | 3 | 96.98 | | |
| 1 | 1 | 98.02 | 98.12 \pm 1.06 | 1.08 |
| | 2 | 97.11 | | |
| | 3 | 99.23 | | |
| 10 | 1 | 97.32 | 96.76 \pm 2.22 | 2.29 |
| | 2 | 98.65 | | |
| | 3 | 94.31 | | |

Table 4 Repeatability of the test results for spiked plasma containing 1 ng/ ml clonidine hydrochloride

| Sample | Peak area | Mean \pm SD | CV% | Retention time (min) | Mean \pm SD | CV% |
|--------|-----------|---------------------|------|----------------------|------------------|------|
| 1 | 137235 | 137546 \pm 261.55 | 0.19 | 2.41 | 2.40 \pm 0.008 | 0.32 |
| 2 | 137653 | | | 2.40 | | |
| 3 | 137669 | | | 2.39 | | |
| 4 | 137211 | | | 2.40 | | |
| 5 | 137854 | | | 2.41 | | |
| 6 | 137654 | | | 2.39 | | |

Table 5 Intermediate precision of the test results for spiked plasma containing 1 ng/ ml clonidine hydrochloride

| Sample | Peak area | Mean \pm SD | CV% | Retention time (min) | Mean \pm SD | CV% |
|--------|-----------|--------------------|-------|----------------------|------------------|-----|
| 1 | 137698 | 137610 \pm 77.68 | 0.056 | 2.41 | 2.396 \pm 0.01 | 0.5 |
| 2 | 137542 | | | 2.39 | | |
| 3 | 137601 | | | 2.38 | | |
| 4 | 137612 | | | 2.41 | | |
| 5 | 137511 | | | 2.40 | | |
| 6 | 137699 | | | 2.39 | | |

Reproducibility

The highest test result of the spiked plasma with 1 ng/ml clonidine was 137854 and the lowest value was 137235 with the mean value of 137546. Therefore, the % difference was 0.45% which means a high reproducibility for the method.

Stability

Table 6 summarizes the freeze and thaw stability, short-term stability, long-term stability and post-preparative stability data of clonidine hydrochloride. All the results showed the stability behavior during these tests and there were no stability related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies. The stability of working solutions was tested at room temperature for 6 h. based on the results obtained; these working solutions were stable within 6 h.

Discussion

Clonidine belongs to a class of drugs called central alpha-adrenergic agonists. Clonidine has been in clinical use for over 40 years, which is used to treat hypertensive disorders, hyperactivity disorder, anxiety disorders, migraine, menopausal flushing and certain pain conditions (1). Several bioanalytical methods are reported to determine clonidine in different biological matrices like plasma (2,3), serum (4,5,7-8), urine (4), and cerebrospinal fluids (2). Although these methods were sufficiently sensitive, they were not suitable for most laboratories to perform studies involving samples in high through-put for therapeutic monitoring. In the present study, we describe a simple, selective and high-through put method using high performance liquid chromatography which coupled with electrospray ionization triple quadruple mass spectrometry for the estimation of

Table 6 Data showing stability of clonidine hydrochloride in human plasma at different QC levels (n = 5) *

| Stability | 0.01(ng/ml) | 1 (ng/ml) | 10(ng/ml) |
|----------------------------|-------------|-----------|-----------|
| Short-term stability | 95.69 | 96.12 | 97.89 |
| Freeze and thaw stability | 97.87 | 97.89 | 96.54 |
| Long-term stability | 96.32 | 93.21 | 96.21 |
| Post-preparative stability | 95.48 | 96.87 | 93.89 |

*Data are presented as the percentage of the remaining concentration to the initial starting concentration

clonidine in human plasma. For this method, the plasma preparation for analysis consist of a protein precipitation method. Protein precipitation was necessary and important because this technique can not only purify but also concentrate the sample. Methanol, perchloric acid and acetonitrile were all attempted and methanol and perchloric acid were finally adopted because of its high extraction efficiency and less interference. Precipitation with and without adding 0.1 M NaOH (100 μ L) were both tried, and obvious differences were not observed, so the precipitation using methanol and perchloric acid without adding 0.1 M NaOH was used at last. The validation tests on the developed method showed acceptable degree of linearity, sensitivity, precision, accuracy and recovery for the method.

Conclusion

A sensitive, selective, accurate and precise HPLC method by triple quadruple mass spectrometer with ESI interface was developed and validated for determination

References

1. Neil MJ. Clonidine: clinical pharmacology and therapeutic use in pain management. *Curr Clin Pharmacol* 2011;6:280-7.
2. Fujimura A, Ebihara A, Ohashi K, Shiga T, Kumagai Y, Nakashima H, Kotegawa T. Comparison of the pharmacokinetics, pharmacodynamics, and safety of oral (Catapres) and transdermal (M-5041T) clonidine in healthy subjects. *J Clin Pharmacol* 1994;34:260-5.
3. Zhao LH, Ding L and Wei X. Determination of moxonidine in human plasma by liquid chromatography–electrospray ionisation–mass spectrometry. *J Pharm Biomed* 2006;40:95-99.
4. Hercegova A, Sadecka J, Polonsky J. Isotachopheresis determination of bisoprolol and clonidine in human fluids *Acta Pol. Pharm.* 1998;55:167-71.
5. Larsson P, Nordlinder A, Bergendahl HT, Lönnqvist PA, Eksborg S, Almenrader N, Anderson BJ. Oral bioavailability of clonidine in children. *Pediatric Anesthesia* 2011;21:335-40.
6. Ghosh C, Singh RP, Inamdar S, Mote M, Chakraborty BS. Sensitive, selective, precise and accurate LC-MS method for determination of clonidine in human plasma. *Chromatographia* 2009;9:1227-32.
7. Arrendale RF, Stewart JT, Tackett RL. Determination of clonidine in human plasma by cold on-column injection capillary gas chromatography–selected-ion monitoring–mass spectrometry. *J Chromatogr* 1988;432:165-75.
8. Yamahata T, Dote S, Ozawa Y, Nishikawa H, Maeda S. Determination of clonidine in human plasma by gas chromatography–electron-impact mass spectrometry. *J Chromatogr B: Biomed Appl* 1994;653:92-7.
9. Wilezyska-Wojtulewicz I, Sadlej-Sosnowska N. Determination of clonidine hydrochloride in pharmaceutical preparations by high-performance liquid chromatography. *J. Chromatogr* 1986;367:434-7.

of clonidine hydrochloride in human plasma. This method offers several advantages such as a rapid and simple extraction scheme and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from study of clonidine hydrochloride.

Acknowledgement

The authors wish to thank Zahravi Pharmaceuticals (Tabriz, Iran) for kindly providing us by clonidine samples. We also would like to thank Zanjan University of Medical Sciences and Iranian Blood Transfusion Organization for providing the drug-free plasma.

Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

10. Muller C, Ramic M, Harlfinger S, Hunseler C, Theisohn M, Roth B. Sensitive and convenient method for the quantification of clonidine in serum of pediatric patients using liquid chromatography/tandem mass spectrometry. *J Chromatogr A* 2007;1139:221-7.
11. Li F, McMahon C, Li F, Zulkoski J. LC-MS/MS sensitivity enhancement using 2D-SCX/RPLC and its application in the assessment of pharmacokinetics of clonidine in dried blood spots. *Bioanalysis* 2011;3:1577-86.
12. Parekh SA, Pudage A, Joshi SS, Vaidya VV, Gomes NA. Rapid and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the determination of clonidine in human plasma. *J Chromatogr B* 2008;867:172-8.
13. Danafar H, Hamidi M. A Rapid and Sensitive LC–MS Method for Determination of ezetimibe Concentration in Human Plasma: Application to a Bioequivalence Study. *Chromatographia* 2013;76:1667-75.
14. Danafar H, Hamidi M. Pharmacokinetics and Bioequivalence Study of Amlodipine and Atorvastatin in Healthy Male Volunteers by LC-MS. *Pharm Sci* 2015;21:167-74.
15. Danafar H, Hamidi M. Simple and sensitive high-Performance liquid Chromatography (HPLC) method with UV detection for mycophenolic acid assay in human plasma. Application to a bioequivalence study. *Adv Pharm Bull* 2015;5:563-8.
16. Danafar H, Hamidi M. Liquid chromatography–tandem mass spectrometry (LC-MS) method for the assignment of enalapril and enalaprilat in human plasma. *Pharm Biomed Res* 2015;1:47-58.
17. Danafar H, Hamidi M. Simple and sensitive high performance liquid chromatographic method for the simultaneous quantitation of the phenylalanine in human plasma. *Pharm Biomed Res* 2015;1:12-20.
18. Danafar H. A quick and easy high performance liquid chromatography method for evaluation of cefixime in human plasma. *Pharm Biomed Res* 2015;1:29-39.