Simple and sensitive high performance liquid chromatographic (HPLC) method for the determination of the selegiline in human plasma

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Abstract: Selegiline is best known as an irreversible inhibitor of monoamine oxidase (MAO), an intracellular enzyme associated with the outer membrane of mitochondria. The purpose of this study is to establish a reliable and quick method for the assignment of selegiline in human plasma by high performance liquid chromatography with ultraviolet detection. A rapid and sensitive high performance liquid chromatographic (HPLC) method has been developed for determination of selegiline in human plasma. The method uses a protein precipitation step with methanol. Mobile phase was composed of phosphate buffer.0.1 M (pH 6.5)-acetonitrile (70:30 v/v) with a flow rate of 1 ml/min. The eluted peaks were detected by a UV detector was set at wavelength of 205 nm. The method was validated in the range of selegiline concentrations from 10 to 100 ng/ml. The limits of detection and quantitation of the method were 5 and 10 ng/ml, respectively. The average drug recovery from plasma was 98.20% throughout the linear concentration range. The average within-run and between-run accuracy values of 99.24 and 98.78, respectively. The method is quick, easy, very steady, and precise for the screen, assignment, and evaluation of selegiline in human plasma by HPLC.

Subjects: Health & Society; Health Conditions; Social Work and Social Policy

Keywords: selegiline; HPLC; human plasma

ABOUT THE AUTHOR
Hossein Danafar is Assistant Professor of Medicinal Chemistry and Dean of Department of Medicinal Chemistry at the School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran. He received his PhD (2013) degree in Medicinal Chemistry from Tabriz University of Medical Sciences, Tabriz, Iran. His current research interests are the analytical chemistry, pharmaceutical analysis, design, synthesis, and characterization of polymeric drug delivery systems, including micelles, nanogels and molecularly imprinted carriers, bioconjugates and magnetic targeted drug delivery systems. The research study titled is (Simple and sensitive high performance liquid chromatographic (HPLC) method for the determination of the selegiline in human plasma). The future study is analysis of other formulation drugs by HPLC, LC-MS, and GC-MS in human plasma.

PUBLIC INTEREST STATEMENT
Selegiline is an intracellular enzyme associated with the outer membrane of mitochondria. The purpose of this study is to establish a reliable and quick method for the assignment of selegiline in human plasma by high performance liquid chromatography with ultraviolet detection. A rapid and sensitive high performance liquid chromatographic (HPLC) method has been developed for determination of selegiline. The method uses a protein precipitation step with methanol. Mobile phase was composed of phosphate buffer-acetonitrile. The eluted peaks were detected by a UV detector was set at wavelength of 205 nm. The procedure will successfully apply to the determination of the selegiline in human plasma without any interference from the additives and endogenous substances. The method is quick, easy, and precise for the assignment, and evaluation of selegiline in human plasma. Our goal is to expand and validate a simple HPLC method for the validation of selegiline in human plasma.
1. Introduction

Selegiline (Figure 1) is best known as an irreversible inhibitor of monoamine oxidase (MAO), an intracellular enzyme associated with the outer membrane of mitochondria (Birkmayer, Riederer, Youdim, & Linauer, 1975; Cedarbaum, Silvestri, Clark, Harts, & Kutt, 1990; Elsworth, Glover, Reynolds, & Sandler, 1978; Knoll, 1978, 1980; Lees et al., 1977; Riederer et al., 1978). Parkinson’s disease is a progressive neurodegenerative disorder characterized by tremors, muscle rigidity, changes in speech and gait caused due to reduction of dopamine level in brain. At low doses, selegiline hydrochloride selectively and irreversibly inhibits monoamine oxidase, type B which is responsible for the metabolism of dopamine. Selegiline therefore acts as a neuroprotective and enhances the action of dopamine by preventing its metabolism. (Mary, Richard, & Pamela, 1997) It is used as an adjunct to levodopa/carbidopa in treating early-stage Parkinson’s disease. When administered orally (5 mg twice a day), selegiline metabolizes to L-amphetamine and L-methamphetamine that causes various side effects such as dizziness, dry mouth, insomnia, muscle pain, rash, nausea, and constipation. Oral bioavailability of selegiline is 4.4% due to extensive first pass metabolism. Transdermal delivery of selegiline can provide benefits of improved patient compliance, controlled release, avoidance of hepatic first pass effect, and reduction in side effects. Literature reports that selegiline has been analyzed by HPLC with UV/fluorescence detection and by spectrophotometric method in pharmaceutical dosage forms (Chad, 2012; Gupta & Paliwal, 2013). Analytical methods such as LCMS, fluorimeters, and gas chromatography have been used for the estimation of selegiline or its metabolites in human plasma (Krishnaiah et al., 2003; Kumble & Narayana, 2014; La Croix, Pianezzola, & Strolin Benedetti, 1994). However, some of this methods have high retention time (which causes consumption of large amount of mobile phase) and by using costly columns and mobile phases. So, the aim of this proposed work is the systemic approach to documenting the new process used to perform the analytical method development and its validation for estimation of selegiline by HPLC, respectively. Our previous works was determination of drugs by HPLC and LC–MS method in human plasma (Danafar, 2015; Danafar & Hamidi, 2013; Danafar & Hamidi, 2015a; Danafar & Hamidi, 2015b; Danafar & Hamidi, 2015c; Danafar & Hamidi, 2015d; Danafar & Hamidi, 2016a; Danafar & Hamidi, 2016b). In the present study, we describe a simple, selective, and stable method using high performance liquid chromatography (HPLC) for the determination of selegiline in human plasma.

2. Materials and methods

2.1. Materials

Selegiline reference standard (99.9% purity) was abounding and branded by Orion Pharma. Other chemicals and solvents were from chemical lab or HPLC purity grades, whenever needed, and were purchased. Drug-free human plasma was provided by Iranian Blood Transfusion Organization after routine safety evaluations.

2.2. Instrument and HPLC method

The HPLC system to include of pump (KNAUER, model 1000, Germany), wavelength UV detector (KNAUER, model 2800 (DAD), Germany) used at a wavelength of 205 nm with the outputs to record
and analyze using with a software (Chrom Gate, KNAUER, Germany). The drug analization was performed using a C18 analytical column (250 mm × 4.6 mm, particle size 5 μm; Perfectsill, MZ-Analysen technik, Germany) equipped by a guard column of the same packing. The mobile phase was composed of phosphate buffer 0.1 M (pH 6.5)-acetonitrile (70:30 v/v) with a flow rate of 1 ml/min. Sample injection to system (20 μl) was made by a loop injector (Rheodyne®7725i, Cotati, CA, USA).

2.3. Preparation of stock solutions
Stock solutions of selegiline were prepared in HPLC mobile phase at concentrations of 1 mg/ml and were stored at 4°C. Working solutions of selegiline were prepared daily in HPLC mobile phase by appropriate dilution at 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1,000, and 1,500 ng/ml.

2.4. Sample preparation and extraction procedure
A 0.1 ml aliquot of the collected plasma sample from a human volunteer was pipetted into a 1 ml centrifuge tube. 0.1 ml methanol was added and then were vortexes for 2 min. After centrifugation (Heidolph, Germany) of the sample at 15,400 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 HPLC system.

2.5. Standard curves
Proper volume of one of the above-mentioned working solutions to produce the standard curve point’s equivalent to 10, 20, 30, 40, 50, and 100.0 ng/ml of selegiline 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.

2.6. Preparation of quality control samples
Quality control samples were prepared daily by spiking different samples of 0.1 ml plasma each with proper volume of the corresponding standard solution to produce a final concentration equivalent to low level (10 ng/ml), middle level(50.0 ng/ml), and high level (100.0 ng/ml) of selegiline. The following procedures were the same as described above.

2.7. Method validation
The method was validated for selectivity, linearity, accuracy, precision, recovery, stability, detection limit, and quantitation limit according to the principles of the FDA industry guidance.

2.7.1. Assay specificity
To evaluate the matrix effect on the ionization of analytes, five different concentration levels of selegiline (10, 20, 30, 40, 50, and 100.0 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 HPLC. 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.

2.7.2. Linearity
Standard curves of ten concentrations of selegiline ranged 10–100.0 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 five, with precision (CV %) within ±20% and accuracy (% recovery) between 80 and 120%.

2.7.3. Precision and accuracy
2.7.3.1. Within-run variations. In one run, three samples with concentrations of 10, 50, and 100 ng/ml (from high, middle, and low regions of the standard curve) were prepared in
triplicate and analyzed by developed HPLC method. Then, the coefficient of variations (CV %) of the corresponding determined concentrations were calculated in each case.

2.7.3.2. 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 HPLC method. Then, the corresponding CV % values were calculated.

2.7.3.3. Repeatability test. To test the method repeatability, six independent spiked plasma samples with a drug concentration of 50 ng/ml were prepared as described. A single injection of each preparation was made to HPLC and the % RSD between the results was determined as the repeatability of the method.

2.7.4. Extraction recovery
Three samples with concentrations of 10, 50, and 100 ng/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed HPLC method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of selegiline with the same concentrations were determined as percentage in each case.

2.7.5. 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.

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

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

2.7.7. Stability

2.7.7.1. 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).

2.7.7.2. 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).

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

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

3. Results and discussion
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.

Methanol, perchloric acid, and acetonitrile were all attempted and methanol was finally adopted because of its high extraction efficiency and less interference. The chromatograms for plasma sample (concentration of selegiline = 100 ng/ml) extraction by different solvents was showed to Figure 2. Typical chromatograms produced from the developed method are shown in Figure 2. The HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of selegiline was shown in Figure 2(A) and (B) shows the spiked plasma sample with selegiline concentration of 100 ng/ml, the retention times of selegiline was 4.96 min and the total HPLC analysis time was 5.5 min per sample, no interferences of the analyte were observed. Figure 2(C) LOQ of the method.

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 analyte. This, in turn, ensures obtaining reliable results from the method for determination of biological concentrations of selegiline.

The method produced linear responses throughout the selegiline concentration range of 10–100 ng/ml, which is suitable for intended purposes. A typical linear regression equation of the method was: y = 36689x + 49747, with x and y representing selegiline concentration (in ng/ml) and peak area (in arbitrary units), respectively, and the regression coefficient (r) of 0.998. The lower LOQ for selegiline was proved to be 10 ng/ml and the LOD was 5 ng/ml. Figure 2(C) shows the chromatogram of an extracted sample that contained 10 ng/ml (LOQ) of selegiline.
The within-run variations of the developed HPLC 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.

The between-run variations of the developed HPLC 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. The repeatability of the method is shown in Table 3. As shown, the method has a remarkable repeatability for the drug assay in plasma.

The extraction recovery determined for selegiline was shown to be consistent, precise, and reproducible. Data were shown in Table 4. These data indicate that there is no significant matrix effect on the outputs of the assay method.

The results of the intermediate precision test are shown in Table 5. As indicated, the developed method shows an acceptable intermediate precision for selegiline assay. The highest test result of the spiked plasma with 50 ng/ml selegiline was 133,546 and the lowest value was 132,867 with the mean value of 133,188. Therefore, the % difference was 0.50% which means a high reproducibility for the method. Table 6 summarizes the freeze and thaw stability, short-term stability, long-term stability, and post-preparative stability data of selegiline. 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.

Selegiline is best known as an irreversible inhibitor of monoamine oxidase (MAO), an intracellular enzyme associated with the outer membrane of mitochondria. Several bioanalytical methods are reported to determine selegiline in different biological matrices like plasma (10–13), serum, urine, and cerebrospinal fluids. 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 for the estimation of selegiline in human plasma. For this method, the plasma preparation for analysis consists of protein precipitation method. Protein precipitation was necessary and important because this technique can not only purify but also concentrate the sample. Methanol, per choleric acid, and acetonitrile were all attempted and methanol was finally adopted because of its high extraction efficiency and less interference. Precipitation with and without adding 0.1 M NaOH (100 μL) were both tried, and obvious differences were not observed, so the precipitation using methanol without adding 0.1 M NaOH was used at last. The validation tests

| Nominal added concentration (ng/ml) | Sample number | Measured concentration (ng/ml) | Mean (SD) | CV % | Accuracy | Mean (SD) |
|-----------------------------------|---------------|-------------------------------|-----------|------|----------|-----------|
|                                   | 1             | 10.12                         | 10.04 (0.07) | 0.7 | 95.23 | 95.54 (1.37) |
|                                   | 2             | 9.98                          |           |      |         | 94.36     |
|                                   | 3             | 10.03                         |           |      |         | 97.05     |
| 50                                | 1             | 49.51                         | 50.22 (1.7) | 3.39 | 99.24 | 97.12 (1.84) |
|                                   | 2             | 52.17                         |           |      |         | 95.89     |
|                                   | 3             | 48.99                         |           |      |         | 96.23     |
| 100                               | 1             | 99.46                         | 100.33 (0.93) | 0.93 | 100.02 | 99.23 (0.70) |
|                                   | 2             | 101.32                        |           |      |         | 99.02     |
|                                   | 3             | 100.23                        |           |      |         | 98.65     |
### Table 2. Between-run variations and accuracy of the HPLC method for quantitation of selegiline (n = 3)

| Nominal added concentration (ng/ml) | Run number | Measured concentration (ng/ml) | Mean (SD) | CV % | Accuracy | Mean (SD) |
|-----------------------------------|------------|--------------------------------|-----------|------|----------|-----------|
| 10                                | 1          | 9.85                           | 10.06 (0.19) | 1.94 | 97.90    | 99.23 (1.62) |
|                                   | 2          | 10.23                          |           |      |          |           |
|                                   | 3          | 10.12                          |           |      |          |           |
| 50                                | 1          | 50.32                          | 49.68 (0.75) | 1.51 | 96.55    | 98.79 (2.34) |
|                                   | 2          | 49.87                          |           |      |          |           |
|                                   | 3          | 48.85                          |           |      |          |           |
| 100                               | 1          | 100.16                         | 99.86 (0.3) | 0.3  | 101.02   | 99.63 (1.25) |
|                                   | 2          | 99.87                          |           |      |          |           |
|                                   | 3          | 99.56                          |           |      |          |           |

### Table 3. Repeatability of the test results for spiked plasma containing 50 ng/ml selegiline

| Sample | Peak area | Mean (SD) | CV % | Retention time (min) | Mean (SD) | CV % |
|--------|-----------|-----------|------|----------------------|-----------|------|
| 1      | 133546    | 133188.2 (326.98) | 0.26 | 4.98 | 4.99 (0.014) | 0.28 |
| 2      | 133054    |           | 5.01 | 4.99 |           |      |
| 3      | 132987    |           | 4.99 | 5.00 |           |      |
| 4      | 133654    |           | 4.97 | 4.99 |           |      |
| 5      | 132867    |           | 5.00 | 5.00 |           |      |

### Table 4. Relative recovery of selegiline by the HPLC method (n = 3)

| Nominal added concentration (ng/ml) | Sample number | Recovery (%) | Mean (SD) |
|------------------------------------|---------------|--------------|-----------|
| 10                                 | 1             | 95.21        | 98.77 (3.41) |
|                                   | 2             | 102.01       |           |
|                                   | 3             | 99.11        |           |
| 50                                 | 1             | 96.18        | 96.62 (4.30) |
|                                   | 2             | 101.14       |           |
|                                   | 3             | 92.56        |           |
| 100                                | 1             | 101.25       | 99.21 (4.22) |
|                                   | 2             | 102.04       |           |
|                                   | 3             | 94.36        |           |

### Table 5. Intermediate precision of the test results for spiked plasma containing 50 ng/ml selegiline

| Sample | Peak area | Mean (SD) | CV % | Retention time (min) | Mean (SD) | CV % |
|--------|-----------|-----------|------|----------------------|-----------|------|
| 1      | 132956    | 133190.8 (351.77) | 0.28 | 4.97 | 4.99 (0.014) | 0.28 |
| 2      | 132863    |           | 4.96 | 4.96 |           |      |
| 3      | 133065    |           | 5.01 | 5.01 |           |      |
| 4      | 133620    |           | 4.96 | 4.96 |           |      |
| 5      | 132987    |           | 4.99 | 4.99 |           |      |
| 6      | 133654    |           | 4.98 | 4.98 |           |      |
on the developed method showed acceptable degree of linearity, sensitivity, precision, accuracy, and recovery for the method.

4. Conclusion
It was concluded that a simple and sensitive HPLC method has developed and validated by using C18 column. Statistical analysis of the results shows that all the proposed procedures have good precision and accuracy shown in table. The procedure will successfully apply to the determination of the studied compound in pharmaceutical formulation without any interference from the additives and endogenous substances. This method offers several advantages such as a rapid and simple extraction scheme and a short chromatographic run time, the method is suitable for and applicable to pharmacokinetic and pharmacodynamics studies and for routine applications in the quality control laboratories because of the simplicity, economic, accuracy, sensitivity, and reproducibility.

Table 6. Data showing stability of selegiline in human plasma at different QC levels (n = 5)

| Stability            | 15 (ng/ml) | 50 (ng/ml) | 100 (ng/ml) |
|----------------------|------------|------------|-------------|
| Short-term stability | 95.45      | 94.32      | 91.01       |
| Freeze and thaw stability | 97.85      | 96.54      | 98.12       |
| Long-term stability  | 96.32      | 98.21      | 96.32       |
| Post-preparative stability | 98.12      | 92.30      | 94.12       |

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Competing Interests
The author declares that no competing interests.

Author details
Hossein Danafar
E-mail: danafar@zums.ac.ir
1 Zanjan Pharmaceutical Nanotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran.
2 Department of Medicinal Chemistry, School of Pharmacy, Zanjan University of Medical Sciences, P.O. Box 45195-1338, Zanjan 45139-56184, Iran.

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