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Liquid Chromatographic Assay for the Analysis of Atazanavir in Rat Plasma after Oral Administration: Application to a Pharmacokinetic Study

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Abstract

A new reverse phase liquid chromatographic method for the investigation of atazanavir in rat plasma was developed after oral administration. The chromatographic separation was achieved on Phenomenex C_{18} column (250 mm × 4.6 mm I.D., 5 μm), under isocratic conditions using UV detection at 249 nm. The optimized mobile phase consisted of a mixture of potassium dihydrogen phosphate (pH 3.5) and acetonitrile (58: 42, v/v) at a flow rate of 1ml/min. The system was found to produce sharp and well-resolved peak for atazanavir with retention time of 5.78 min. The linear regression analysis for the calibration curves showed a good linear correlation over the concentration range 0.050–2.0 μg/ml, with determination coefficients, R^2, exceeding 0.9989. The limits of detection (LOD) and quantitation (LOQ) were found to be 0.004 µg/ml and 0.012 µg/ml, respectively. The method was successfully applied for the pharmacokinetic in rats. Atazanavir concentration in plasma reached (C_{max}) was 0.087 μg/ml at 3 h after oral administration of 7.2 mg/kg/rat. The AUC_{0-12} was 0.4812 µg/ml*h and the apparent plasma half-life ($t_{1/2}$) was 7.5 h. This method was found to be suitable for examining atazanavir concentration in rats, after oral administration of atazanavir in a single dose.

Keywords: Atazanavir; High-performance liquid chromatography; Pharmacokinetic study

Introduction

Atazanavir (ATV) belongs to the HIV protease inhibitor (PI) class of the antiretrovirals (ARVs), which have played an important role in lowering the morbidity and mortality of HIV/AIDS [1]. The compound selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells, thus preventing formation of mature virions. Atazanavir is distinguished from other protease inhibitors in that it can be given once-daily and has lesser effects on the patient's lipid profile [2]. Moreover, several cytochrome P450 isoenzymes can be inhibited by atazanavir [3]. The pharmacokinetics of ATV allows for once daily dosing [4], which may improve patient compliance.

There are several methods described in the literature for the quantitative analysis of atazanavir in plasma, either alone [5-7] or in combination with other ARVs [7-10]. Some authors reported the use of mass spectrometry for detection [10], which is not routinely available in all laboratories. Furthermore, all the reported methods had run times exceeding 15 min, even those for the analysis of atazanavir alone.

HPLC-UV detection method was developed for atazanavir with LOQ of 0.044 μg/ml [11]. A validated HPLC method for the estimation of atazanavir was not appropriate for detection of low atazanavir concentration. In this method, the LOQ value was found to be 0.090 μg/ml and retention time was more than 8.3 min [12]. The purpose of the present study was to develop and validate a simple and time-saving RP-HPLC method with UV detection for the investigation of atazanavir after oral administration to Wistar rats. The method was validated according to Food and Drug Administration (FDA) and International Conference on Harmonization (ICH) guidelines with respect to linearity, precision, accuracy, and specificity and stability studies [13,14]. Indinavir (0.250 μg/ml) was used as an internal standard (IS).

Experimental

Chemicals and reagents

Atazanavir and Indinavir (99.8% w/w and 98.7% w/w, respectively, High performance liquid chromatography, HPLC) were provided ex-gratis by M/s Hetero Labs, Hyderabad, India. HPLC grade acetonitrile was purchased from SD fine-chem limited (Mumbai, India). Deionized water used in all the experiments was passed through a Milli-Q water purification system (18.2 MΩ/cm) Millipore (Bangalore, Karnataka, India).

Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with binary pump and SPD- 20AVPUV detector. Sample injection was done by Rheodyne injector with a 50 μL loop and a computer running Varian workstation version 6.42 software for data acquisition and processing. The chromatographic analysis was carried out on Phenomenex C18 column (250 mm × 4.6 mm I.D., 5 μm).

Chromatographic conditions

The mobile phase was composed of potassium dihydrogen phosphate (pH 3.5) and acetonitrile in a ratio of (58: 42, v/v) run under isocratic elution and pumped at a flow rate of 1 ml/min. The column was thermostated at 30°C. Under these conditions the run time was less than 8 min.

Preparation of Calibration Curve (CC) and Quality Control Samples (QC)

Eight-point calibration curve (CC) was prepared by serial dilution

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of atazanavir stock solution (100 μg/ml) in the range of 0.050, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/ml that were obtained by measuring the required amount of 100 μg/ml working standard solution, mixed with a sufficient quantity of mobile phase and making up to 10 ml. Similarly, six standard solutions were prepared by serial dilution of atazanavir stock solution (10 μg/ml) in the range of 0.005, 0.010, 0.020, 0.030, 0.040 and 0.050 μg/ml. Six standard solutions were obtained from the 10 μg/ml working standard solution, in order to determine the LOD and LOQ of the method. Calibration standards were prepared daily by spiking 100 μL of blank plasma with 10μl of the appropriate working solution resulting in concentrations of 0.050, 0.1, 0.5, 1.0, 1.5, and 2.0μg/ml and 0.005, 0.010, 0.020, 0.030, 0.040, and 0.050 μg/ml, respectively, of atazanavir. Stock solution (0.250 μg/ml) of IS was prepared in methanol and stored at −20°C. The solutions were stable for one day when stored at room temperature (20–25°C).The stock and standard solutions were prepared on a daily basis and stored in the dark at about 5°C. All solutions were used on the day they were prepared.

Quality control (QC) samples (low quality control (LQC), 0.1 μg/ml; medium quality control (MQC), 1.0 μg/ml; high quality control (HQC), 2.0 μg/ml; limit of quantification (LOQ), 0.009 μg/ml were prepared by spiking 0.1ml aliquot of blank plasma with 10 μl of spiking solution of drug as well as the IS. All solutions were stored in the refrigerator at 4.0 ± 2.0°C. The bulk spiked CC and QC samples were stored at −20°C and brought to room temperature before use.

Sample preparation

To a 50 μl of rat plasma, 10 μl of IS and 100 μl of atazanavir were added and the mixture was incubated at 37°C for 1 h. Atazanavir was then extracted using 30 μl of acetonitrile followed by vortexing for 2 min. After vortexing, the samples were subjected to centrifuge at 12,000×g for 10 min. The supernatant was decanted into a china dish and evaporated to dryness at room temperature. This was further reconstituted with 100 μl of mobile phase and vortexed for 30 s and 20 μl was injected into an HPLC system. Atazanavir was detected at a wavelength of 249 nm.

Method validation study

The developed method was validated as per ICH guidelines using atazanavir with respect to the following parameters: accuracy, precision, LOD, LOQ, specificity, stability, and system suitability.

Linearity

For testing linearity, five calibration standards were prepared in the concentration range of 0.05–2.0 μg/ml (0.050, 0.1, 0.5, 1.0, 1.5 and 2.0 μg/ml). Standard curve was achieved by plotting peak area against concentration, and the evaluation of linearity was completed by linear regression analysis using least square method.

Limit of detection and limit of quantitation

Normally, limit of detection (LOD) and limit of quantitation (LOQ) are estimated at a signal to noise ratio of 3:1 and 10:1, respectively [13]. LOD and LOQ were determined based on the response and slope of a specific calibration curve obtained from six standard solutions (0.005, 0.010, 0.020, 0.030, 0.040, and 0.050 μg/ml) that were in proximity of these limit concentration values.

Selectivity, specificity and linearity

Selectivity was verified by analyzing the blank plasma from rats to test interference at the analyte retention times. By employing the proposed extraction procedure each blank plasma sample was tested and then compared with the results of plasma samples spiked with atazanavir (n=6) in calibration standard to ensure no interference of atazanavir from plasma. Spiked plasma samples that contained increasing concentrations of atazanavir from 0.050 to 2.0 μg/ml were analyzed according to the procedure described above. The linearity was detected by calculating the correlation coefficient (r) of the curves by means of least-squared linear regression method. All calibration curves of atazanavir were constructed prior to the experiments with correlation coefficient of ($r^2 > 0.9989$).

Accuracy

The accuracy of the assay method was evaluated in triplicate at three different concentration levels (0.1, 1.0, and 2.0 μg/ml), and the percentage recoveries were calculated.

Precision: The precision is usually reported as the percent relative standard deviation (%RSD) of a set of responses. Precision was represented into two categories, namely, repeatability (intraday precision) and intermediate precision (interday precision).

Repeatability or intraday precision: Repeatability was tested by analyzing six determinations at three different concentrations, namely, low, medium, and high within the linearity range.

Intermediate or interday precision: The inter-day variability of this method was assessed over three days at three low, medium, and high concentrations of atazanavir standard in replicates of six.

Pharmacokinetic study in Rats: The pharmacokinetic studies were carried out in healthy male Wistar rats (200– 250 g), and the animals were fasted overnight before dosing with free access to water. The animals were acclimatized to laboratory conditions over the week before experiments and fed with standard rat diet, under controlled conditions of a 12:12 h light : dark cycle, with a temperature of 22 ± 3°C and a relative humidity of 50 ± 5% RH. The experimental protocol was approved by the Institutional Animal Ethical Committee (AACP/IAEC/Jun-2012-02).

Twelve rats were randomly separated into two groups (six animals each group). The grouping of animals was as follows:

Group I: Control normal rats (received saline solution)

Group II: Administered with pure drug (as solution) (7.2 mg/kg/rat) [15]

At regular time intervals 0, 0.5, 1, 2, 3, 4, 6, 8, 10 and 12h samples of blood were withdrawn (100 μl) from the retro-orbital plexus by microcapillary technique under light ether anesthesia into heparinized microcentrifuge tubes (50 units heparin/ml of blood). Plasma was separated by centrifugation at 12,000×g for 15 min and analyzed by the following method. Plasma samples were deproteinated with 1ml of acetonitrile, vortexed for 2 min, and centrifuged at 12,000×g for 10 min. The supernatant was decanted into a China dish and evaporated to dryness at room temperature. This was further reconstituted with 100 μl of mobile phase and vortexed for 30 s and 20 μl was injected into an HPLC system. Atazanavir was detected at a wavelength of 249 nm.

Results and Discussion

Method development and optimization of HPLC-UV conditions

A liquid chromatographic method for the estimation of atazanavir in rat plasma has been developed and validated according to the principles of Good Laboratory Practices. An appropriate wavelength

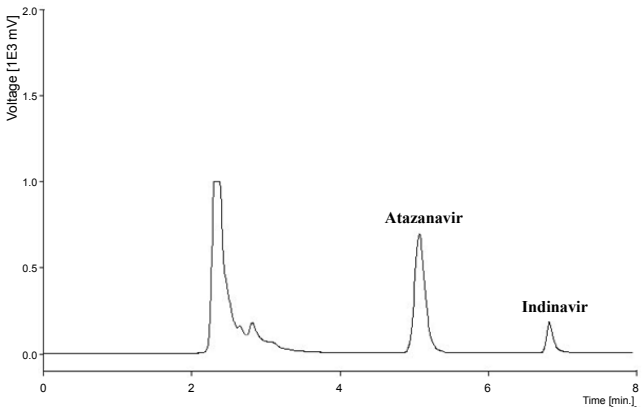

Figure 1: At optimized conditions: (a) chromatographic profile of the plasma spiked with atazanavir (2 μg/ml) in the presence of the I.S (0.250 μg/ml).

Concentration (µg/ml)	Observed concentration (µg/ml)	% Precision	% Accuracy
Intra-day			
0.1	0.098 ± 0.002	2.00	98.00
1.0	0.988 ± 0.016	1.61	98.80
2.0	2.105 ± 0.011	0.54	105.25
Inter-day			
0.1	0.094 ± 0.003	3.19	94.00
1.0	0.981 ± 0.027	2.75	98.10
2.0	1.996 ± 0.068	3.40	99.80

Table 1: Intra-day and inter-day precision and accuracy of atazanavir in rat plasma (n = 6).

Sample condition	Spiked concentration (µg/ml)	Mean determined concentration (µg/ml)	Accuracy (%)
Bench-top stability[a]	0.1	0.096	96.00
	1.0	0.97	97.00
	2.0	1.99	99.50
Freeze–thaw stability[b]	0.1	0.098	98.00
	1.0	0.99	99.00
	2.0	2.07	103.5
One-week stability[c]	0.1	0.092	92.00
	1.0	0.89	89.00
	2.0	2.38	95.20
[a]Exposed at ambient temperature (25 °C) for 4h.			
[b]After three freeze–thaw cycles			
[c]Stored at −16 °C.			

Table 2: Stability of atazanavir in rat plasma (n = 6)

was important for good sensitivity. Atazanavir has a special conjugation structure which leads to strong UV absorption at the wavelength of 249 nm. Therefore, the detection wavelength was set at 249 nm. It was necessary to use an IS in extraction techniques and HPLC method to compensate for extraction variation, efficiency, and analytical errors. Indinavir was adopted as the IS in this study for the reasons that it is structurally similar to atazanavir and its behavioural characteristics and properties conform to the chemical requirement for IS in HPLC. In addition, indinavir is commercially available in high purity, and it is stable and nonreactive with sample or mobile phase. Meanwhile, it also has good response at the detection wavelength of 249 nm.

The mixture of potassium dihydrogen phosphate (pH 3.5) and acetonitrile (58: 42, v/v) at a flow rate of 1ml/min could achieve the above purpose that was found to be optimum and provided adequate peak separation, with less tailing and resulted in good resolution among all the other combinations tested which was finally adopted as the mobile phase.

Limit of detection and limit of quantitation

Concentrations of LOD and LOQ were found to be 0.003 μg/ml and 0.012 μg/ml, respectively.

Specificity

Specificity is expressed as the capability of a method to distinguish the analyte from all potentially intrusive substance [16]. The specificity of the method was scrutinized by blank plasma detection, peak purity, and spiking blank plasma with pure standard compounds. Blank plasma had no interference, when atazanavir and the IS were eluted. At optimized conditions, the separation of atazanavir and indinavir was completed within 8 min (Figure 1).

Linearity

Each sample was analyzed in replicates of six to verify the reproducibility of detector response at each concentration level. The detector responses were found to be linear over the concentration range from 0.050 to 2.0 μg/ml. The regression equation for the graph is y = 0.7986x–0.6198, and the correlation coefficient R^2 is 0.9989 showing excellent correlation between the area and the concentration.

Precision

The percentage relative standard deviation (%RSD) of the area of atazanavir during intraday study was found to be less than 5 and for interday study was found to be less than 6.5, which indicated a good precision of the method (Table 1). Intra-day and inter-day precision (% R.S.D) of the methods were lower than 4% and were within the acceptable limits to meet the guidelines for bioanalytical method validation which is considered to be ≤ 15% [17,18].

Accuracy

The quantitative recovery of atazanavir achieved ranged from 94.0 to 105.25% with a low %RSD value. The results of the recovery experiments done at three concentration levels and the %RSD values are given in Table 1.

Stability

Bench-top stability was investigated to ensure that atazanavir was not degraded in plasma samples at room temperature for a time period to cover the sample preparation. It was measured by divulging the QC samples to ambient laboratory conditions for 10 h. Freeze-thaw stability was measured over three cycles. Because of the need for occasional delayed injection of extraction samples, the stability of reconstituted samples was assessed at ambient temperature for 12 h. The freezer storage stability of atazanavir rat plasma at -20°C was evaluated by assaying QC samples at the beginning and one week later. All stability QC samples were analyzed in six replicates. The results indicated that atazanavir had an acceptable stability under those conditions (Table 2).

Application of the assay

The validated method was successfully applied to investigate the content of atazanavir in *in vivo*, after administered orally to rats. Oral administration of atazanavir in the present study resulted in a sharp C_{max} of 0.087 μg/ml at 3 h after which the plasma concentration declined rapidly, indicating a rapid absorption of atazanavir. The area under the

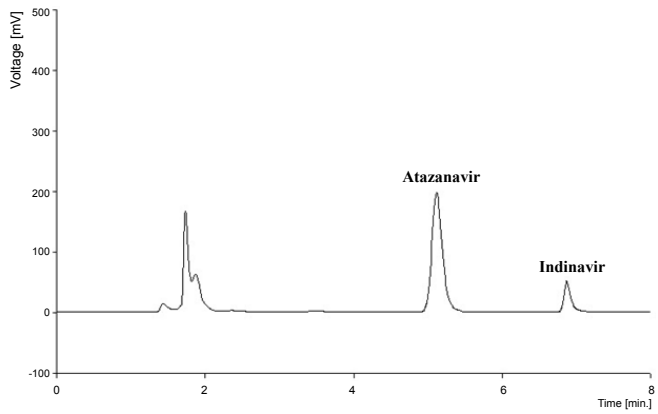

Figure 2: Chromatogram of plasma sample collected from rats 3 h after oral administration of atazanavir.

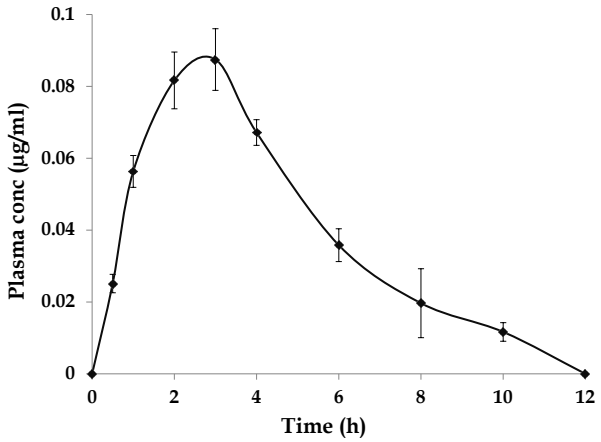

Figure 3: Plasma concentration-time curve of atazanavir after being orally administered male Wistar rats at dose of 7.2mg/kg/rat (*n* = 6, mean ± S.D).

Pharmacokinetic parameters	Atazanavir
C_{max} (µg/ml)	0.0874 ± 0.032
t_{max} (h)	3.2 ± 0.014
$t_{1/2}$ (h)	7.5 ± 0.190
AUC_{0-12} (µg/ml*h)	0.481 ± 0.023
K_a (1/h)	0.2921 ± 0.005
MRT	4.736 ± 0.189

Data presented as mean ± standard deviation (n=6)

Table 3: Pharmacokinetic parameters of atazanavir at a dose of 7.2 mg/kg/rat.

concentration versus time curve was 0.4812 μg/ml*h. The representative chromatogram of a plasma sample, which was collected from Wistar rats 3 h following oral administration of atazanavir as portrays in Figure 2. The plasma profile of atazanavir is shown in Figure 3. The results substantiate the suitability of the developed method for determining atazanavir concentration in plasma after oral administration. The pharmacokinetic data of atazanavir after oral administration in rats is shown in Table 3.

Conclusion

A specific, linear, accurate, reliable, and reproducible new method of atazanavir in rat plasma was developed and fully validated over the range 0.050-2.0 μg/ml with LOQ of 0.012 µg/ml. The method was successfully applied to measure the drug concentration in plasma after oral administration to rats. Reproducible high recovery of atazanavir was achieved. Because of its highly satisfactory sensitivity, accuracy, linearity, and specificity, this HPLC methodology could thus be an appropriate tool for further determination of atazanavir in plasma samples in the pharmacokinetic studies.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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References

1. Harrison TS, Scott LJ (2005) Atazanavir-a review of its use in the management of HIV infection. Drugs 65: 2309-2336.
2. Chitturi SR, Somannavar YS, Peruri BG, Nallapati S, Sharma HK, et al. (2011) Gradient RP-HPLC method for the determination of potential impurities in atazanavir sulfate. J Pharm Biomed Anal 55: 31-47.
3. Cateau E, Tournier N, Dupuis A, Moalc GL, Venisse N (2005) Determination of atazanavir in human plasma using solid-phase extraction and high-performance liquid chromatography. J Pharm Biomed Anal 39: 791-795.
4. Singh G, Pai RS (2013) High-Performance Liquid Chromatographic Method for Analysis of Emtricitabine in Rat Plasma: Method Development, Validation and Application to a Pharmacokinetic Study. ISRN Chromatography Article ID 329072.
5. Cattaneo D, Maggiolo F, Ripamonti D, Perico N (2008) Determination of atazanavir in human plasma by high-performance liquid chromatography with UV detection. J Chromatogr Sci 46: 485-489.
6. Loregian A, Pagni S, Ballarin E, Sinigalia E, Parisi SG, et al. (2006) Simple determination of the HIV protease inhibitor atazanavir in human plasma by high-performance liquid chromatography with UV detection. J Pharm Biomed Anal 42: 500-505.
7. Elens L, Veriter S, Fazio VD, Vanbinst R, Boesmans D, et al. (2009) Quantification of 8 HIV-protease inhibitors and 2 nonnucleoside reverse transcriptase inhibitors by ultra-performance liquid chromatography with diode array detection. Clin Chem 55: 170-174.
8. Weller DR, Brundage RC, Balfour HH, Vezina HE (2007) An isocratic liquid chromatography method for determining HIV non-nucleoside reverse transcriptase inhibitor and protease inhibitor concentrations in human plasma. J Chromatogr B Biomed Sci Appl 848: 369-373.
9. Notari S, Bocedi A, Ippolito G, Narciso P, Pucillo LP, et al. (2006) Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 831: 258-266.
10. Avolio AD, Siccardi M, Sciandra M, Baietto L, Bonora S, et al. (2007) HPLC-MS method for the simultaneous quantification of the new HIV protease inhibitor darunavir, and 11 other antiretroviral agents in plasma of HIV-infected patients. J Chromatogr B Biomed Sci 859: 234-240.
11. Sparidans RW, Dost F, Crommentuyn KML, Huitema ADR, Schellens JHM, et al. (2006) Liquid chromatographic assay for the protease inhibitor atazanavir in plasma. Biomed Chromatogr 20: 72-76.
12. Muller AC, Kanfer I (2010) An efficient HPLC method for the quantitative determination of atazanavir in human plasma suitable for bioequivalence and pharmacokinetic studies in healthy human subjects. J Pharm Biomed Anal 53: 113-118.
13. Food and Drug Administration (2001) Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Rockville,Md, USA.
14. ICH Harmonised Tripartite Guideline (2005) Validation of Analytical Procedures: Methodology, Q2 (R1), International Conference on Harmonisation of Technical Requirements for Registrations of Pharmaceuticals for Human Use, ICH, Geneva, Switzerland.

15. Fukushima K, Terasaka S, Haraya K, Kodera S, Seki Y, et al. (2007) Pharmaceutical approach to HIV protease inhibitor atazanavir for bioavailability enhancement based on solid dispersion system. Biol Pharm Bull 30: 733-738.

16. Singh G, Pai RS, Pandit V (2012) Development and validation of a HPLC method for the determination of trans-resveratrol in spiked human plasma. J Adv Pharm Tech Res 3: 130-135.

17. Singh G, Pai RS, Pandit V (2014) *In vivo* pharmacokinetic applicability of a simple and validated HPLC method for orally administered trans-resveratrol loaded polymeric nanoparticles to rats. J Pharm Investig 44: 69-78.

18. Singh G, Pai RS (2014) Optimization (central composite design) and validation of HPLC method for investigation of emtricitabine loaded poly(lactic-co-glycolic acid) nanoparticles: *in vitro* drug release and in *vivo* pharmacokinetic studies. The Scientific World Journal.