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Short communication

Liquid chromatographic–tandem mass spectrometric determination of amprenavir (agenerase) in serum/plasma of human immunodeficiency virus type-1 infected patients receiving combination antiretroviral therapy

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Abstract

A selective assay method for quantitation of amprenavir (agenerase) in human immunodeficiency virus type-1 infected patient serum or plasma using liquid chromatography–tandem mass spectrometry (LC–MS–MS) is described. Amprenavir and an internal standard (reserpine) are extracted by liquid–liquid extraction and chromatographically separated by a reversed-phase C_{18}-analytical column. The triple quadrupole LC–MS–MS system is operated in the positive-ion mode and multiple reaction monitoring is used for drug quantitation. The method has been validated over the range of 0.05–10.0 μg/ml. The RSDs for the intra-day and inter-day determinations ranged from 5.3 to 6.1% and from 4.7 to 6.2%, respectively. The average assay accuracy at two different concentrations ranged from 96.0 to 103.0% and the extraction recovery of amprenavir was 90.8%. The lower limit of quantitation was 0.05 μg/ml. Using a short microbore column, the analysis was completed in less than 5 min.

Keywords: Human immunodeficiency virus; Validation; Amprenavir; Agenerase; Enzyme inhibitors; Reserpine

1. Introduction

Amprenavir is a synthetic peptide-like antiretroviral agent that inhibits the activity of the human immunodeficiency virus type-1 (HIV-1) protease [1]. This protease inhibitor prevents maturation of the virus, and causes the formation of immature, non-infectious virions. Recently, data have emerged showing a possible link between antiretroviral drug concentrations and antiviral effect [2–5]. This relationship has been reported with the use of protease inhibitors, whose activity does not involve intracellular conversion unlike nucleoside reverse transcriptase inhibitors (NRTIs), and whose half-lives are relatively short as compared to non-nucleoside reverse transcriptase inhibitors (NNRTIs). Consequently, there is a need to develop new, validated methodologies for monitoring of antiretroviral agents, specifically HIV-1 protease inhibitors. Recently, several high-performance liquid chromatography (HPLC) methods have been reported for the quantitation of amprenavir or amprenavir together with other antiretroviral agents in biological fluids using fluorescence or ultraviolet detector [6–8]. Some of these methods are very sensitive and the analysis can be completed in a relatively short time. However, when other commonly used antiretroviral agents are present in combination, the analysis might be prolonged due to

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necessary chromatographic separation to avoid possible interference. We report here a rapid and selective assay for quantitation of amprenavir (agenerase) in patient serum or plasma using liquid chromatography–tandem mass spectrometry (LC–MS–MS). We developed and validated this method in order to study the pharmacokinetics of amprenavir in humans and to monitor the amprenavir serum or plasma level in HIV-1 infected patients receiving combination antiretroviral therapy.

2. Experimental

2.1. Materials

Amprenavir was kindly supplied by Glaxo Wellcome (Research Triangle Park, NC, USA). Reserpine, formic acid and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Methanol, acetonitrile and diethyl ether were purchased from J.T. Baker (Phillipsburg, NJ, USA). Drug free serum (lyophilized) was obtained from Bio-Rad (Irvine, CA, USA) and drug free plasma was from Interstate Blood Bank (Memphis, TN, USA). HPLC-grade water was obtained using the nanopure reagent-grade water system from Barnstead (Boston, MA, USA).

2.2. Preparation of standard solution

Stock solutions of amprenavir (1 mg/ml) and reserpine (1 mg/ml) were prepared by dissolving the appropriate amount of authentic amprenavir and reserpine, accurately weighed, in methanol and stored at 4°C for up to 6 months. During assay validation and patient specimen quantitation several stock solutions of amprenavir and reserpine were prepared and used for the analysis. The final working concentration of amprenavir was 10 μg/ml, and reserpine was 500 μg/ml.

2.3. Mass spectrometric and chromatographic conditions

MS analyses were performed on a PE-Sciex (Foster City, CA, USA) Model API-2000 triple quadrupole mass spectrometer equipped with a turboionspray source. The LC–MS–MS system is operated in the positive-ion mode and multiple reaction monitoring (MRM) is used for drug measurement. The transition of the protonated parent/daughter-ion pairs at m/z 506–418 and m/z 609–195 are used for recording the selected-ion mass chromatograms for amprenavir and reserpine, respectively. A Shimadzu (Columbia, MD, USA) HPLC system which consists of two Model LC-10-AD pumps, a Model SCL-10-A controller, and a Model DGU-14-A degasser, was used. The separation was carried out in a reversed-phase system with a Phenomenex (Torrance, CA, USA) C_{18} Luna column as the stationary phase (50×2.0 mm I.D., particle size 3 μm) and acetonitrile–water (1:1, v/v) containing 0.1% formic acid as the mobile phase. The flow-rate of the mobile phase was 150 μl/min, and the turboionspray source temperature was held constant at 300°C. For sample introduction into the system, a Perkin-Elmer (Norwalk, CT, USA) Model 200 autoinjector was used.

2.4. Sample preparation and standard curve

Serum or plasma samples were randomly obtained from HIV-1 infected patients receiving amprenavir and other antiretroviral agents. This may include co-medication with other protease inhibitors as well as with nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. The time from dosage of amprenavir to specimen collection was not known. The specimens were not treated for virus inactivation prior to extraction. A total of 15 μg (30 μl) of reserpine as the internal standard, 0.25 ml of 0.5 *M* sodium hydroxide, and 3.0 ml of diethyl ether were added to 0.5 ml of patient serum or plasma in a 15-ml screw top culture tube. The mixtures were vortexed for 20 s, rapidly inverted by hand 60 times and centrifuged at 2500 rpm (1000 *g*) for 8 min at 20°C. The organic phase (upper layer) was transferred into a new glass tube for further processing. A second 3.0-ml aliquot of diethyl ether was added to the aqueous residue, and extracted using the same procedure described above. The two organic phases were combined and evaporated to dryness under a gentle stream of nitrogen. The dried residue was redissolved in 250 μl of

mobile phase, and 10 μl was injected into the LC–MS–MS system.

A standard curve for amprenavir, obtained by unweighted least-squares linear regression analysis, was determined in a similar manner using 0.5 ml of drug-free serum or plasma to which known amounts of amprenavir (0.025, 0.05, 0.1, 0.5, 1.0 and 5.0 μg) were added. For the two controls, 0.5 ml of drug-free serum or plasma were spiked with 0.25 and 2.5 μg of amprenavir. Concentrations of amprenavir were determined by the ratio of the peak areas of amprenavir to the peak area of the internal standard plotted against the known concentrations of the standards.

3. Results and discussion

Fig. 1 shows the total ion-chromatogram (selective multiple reaction monitoring of two parent/daughter-ion pairs for amprenavir and reserpine) of an extracted patient serum sample receiving amprenavir therapy. A total of 15 μg of reserpine was added as an internal standard to the specimens prior to extraction. As can be seen from the chromatogram, amprenavir and the internal standard are chromatographically separated from each other. Potential interference from endogenous compounds or other commonly used antiretroviral agents could be excluded since only the transition of the parent/daughter ion-pairs of amprenavir and reserpine have been selectively monitored by the MS–MS detector. By using a short microbore analytical column, the analysis was completed in less than 5 min. The standard curves ($n=20$) for amprenavir in human serum exhibited good linearity (regression coefficient: $r^2 \geq 0.996$) over the concentration range of 0.05–10.0 μg/ml. Table 1 shows the RSDs (based on two different concentrations) for the intra-day and inter-day determinations which ranged from 5.3 to 6.1% ($n=20$) and from 4.7 to 6.2% ($n=20$), respectively. The average assay accuracy of amprenavir, determined at two different concentrations, ranged from 96.0 to 103.0% ($n=40$) as shown in Table 1. Using liquid–liquid extraction technique, the extraction recovery of amprenavir was 90.8%. The extractability of amprenavir was determined by comparing the peak area ratios of amprenavir to the internal standard measured in the extracted (serum spiked with amprenavir, followed by extraction) versus those in the unextracted (pure amprenavir in methanol solution, no extraction performed) samples supplemented with the same amount of amprenavir. In both cases, the internal standard (not subject to extraction) was added to the extracted as well as unextracted samples just prior to injection into the LC–MS–MS system. The lower limit of quantitation, defined as the lowest amprenavir level used for generating the standard curve, was 0.05 μg/ml.

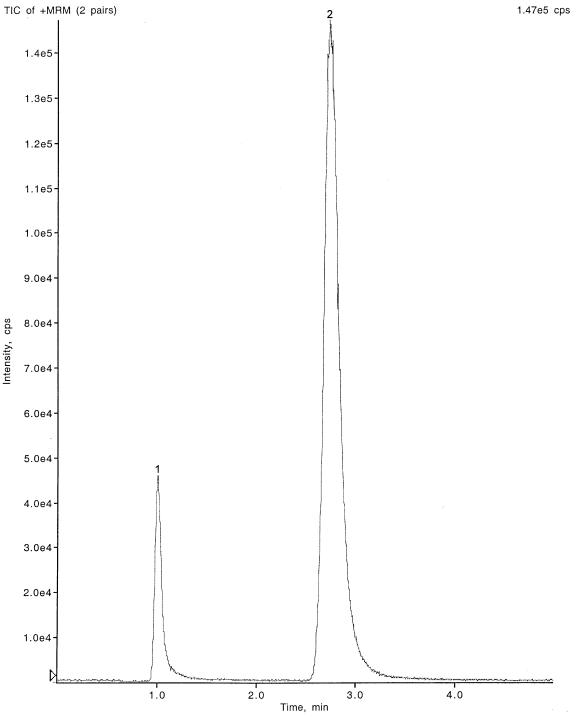

Fig. 1. Total ion-chromatogram (selective multiple reaction monitoring of two parent/daughter-ion pairs: m/z 506–418 for amprenavir and m/z 609–195 for reserpine) of an extracted patient serum sample containing 1.53 μg/ml of amprenavir. A total of 15 μg of reserpine was added as the internal standard prior to extraction. Peaks: 1, reserpine; 2, amprenavir.

The mean amprenavir serum levels in 58 (78%) patients receiving amprenavir therapy was 1.83 μg/ml (range from 0.05 to 8.50 μg/ml). In 16 samples (22%) the serum amprenavir levels were below the lower limit of quantitation (0.05 μg/ml). Even though these patients' serum amprenavir levels may have limited clinical information, since the exact

Table 1
Precision and accuracy of the assay for amprenavir in human serum

Nominal (μg/ml)	Mean (μg/ml)	SD (μg/ml)	RSD (%)	Accuracy (%)
Intra-day (n=20)				
0.50	0.49	0.03	6.1	98.0
5.00	5.13	0.27	5.3	102.6
Inter-day (n=20)				
0.50	0.48	0.03	6.2	96.0
5.00	5.15	0.24	4.7	103.0

n, number of replicates; RSD, relative standard deviation; SD, standard deviation.

specimen collection time after amprenavir intake was not known, the wide variation strongly suggests that amprenavir serum level monitoring in the treatment of HIV-1 infection should be considered.

The LC–MS–MS method described in this report has advantages over the published HPLC methods, i.e. the relatively short time of analysis and the assay specificity especially when other antiretroviral medication are present in the patient's specimen. These advantages can be achieved by using a liquid chromatography system in combination with a triple quadrupole MS–MS detector. The method described here is adequately sensitive to quantitate amprenavir concentrations for pharmacokinetic studies as well as for monitoring of amprenavir levels in the therapeutic range for HIV-1 infected patients. Measurement of protease inhibitor drug concentrations should be considered in the treatment of HIV-1 infection. Protease inhibitor drug monitoring may improve efficacy, help to establish compliance, and help to evaluate drug–drug interactions in HIV-1 infected patients.

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