Determination of Atazanavir in Human Plasma by High-Performance Liquid Chromatography With UV Detection

Dario Cattaneo[1,2,*], Franco Maggiolo[3], Diego Ripamonti[3], and **Norberto Perico[1,2]**

[1]Department of Medicine and Transplantation, Ospedali Riuniti di Bergamo – Mario Negri Institute for Pharmacological Research, Bergamo, Italy; [2]Center for Research on Organ Transplantation, "Chiara Cucchi De Alessandri & Gilberto Crespi"; and [3]Division of Infectious Diseases, Ospedali Riuniti di Bergamo, Bergamo, Italy

Abstract

A high-performance liquid chromatographic method for the determination of atazanavir (ATV) in human plasma is developed and validated. The method involves a rapid and simple solid-phase extraction (SPE) of ATV using Bond-elut C18 3 mL cartridge. The separation of ATV from internal standard and endogenous components is achieved using an isocratic elution on an octyl column and an UV detector set at 260 nm. The method is linear from 20 to 10,000 ng/mL (mean r^2 = 0.9991, n = 10). The observed intra- and inter-day assay precision ranged from 2.2% to 14.7% [at the lower limit of quantitation (LOQ)], whereas accuracy varies between 1.0% and 14% (at LOQ). Mean drug recovery is 80.5% for ATV and 78.4% for IS. The method is found to be precise and accurate, practical enough for therapeutic drug monitoring in routine clinical practice and is applied for the assessment of 24-h ATV plasma concentration-time profiles in HIV-infected pregnant women.

Introduction

New antiretroviral drugs effective against the human immunodeficiency virus (HIV) are continuing to be developed. Recently, an HIV-1 protease inhibitor (PI), namely atazanavir (ATV), was marketed. Preliminary observations showed that, unlike other PIs, ATV administration was associated with low incidence of severe liver injury (1). Moreover, ATV may have an additional advantage over other PIs because of its favourable effect on lipid profile (2), eventually lowering the risk of cardiovascular events (3). Some concerns, however, have been raised on its pharmacokinetic characteristics. Indeed, this azapeptide is primarily metabolized by P450 cytochromes, a condition which increases the risk of drug-to-drug interactions (4). As an additional drawback, evidence is now available that a fixed daily dose of ATV, either given alone or boosted with ritonavir, is associated with considerable inter-patient variability of the drug trough concentration (4,5). All together, these conditions underline the importance of ATV therapeutic drug monitoring as a guide to optimize daily drug exposure for each patient.

So far, several high-performance liquid chromatographic (HPLC) methods have been described for the assessment of ATV concentration in human plasma (6–11). Although these methods meet the criteria for validated analysis of ATV, they require gradient elution mode (6,7), liquid–liquid extraction (7,8,9), or need expensive detectors (i.e., mass spectrometer, diode-array, or fluorimeter) not commonly available in clinical laboratories (10,11). Here we propose a new technique that uses a solid-phase extraction, an isocratic elution on a reverse-phase column, and an UV detection.

Experimental

Reagents

ATV sulphate (Figure 1) was kindly provided by Bristol-Myers Squibb Company (Princeton, NJ), whereas diazepam, used as the internal standard (IS), was purchased from Sigma (Milan, Italy). Different in-house substances have been tested as potential IS, such as imipramine, desipramine, trimetoprim, azathioprine, xantines, and bupivacaine, together with some benzodiazepines. Diazepam was the one associated with highest recovery and optimal retention time, being eluted earlier than ATV in a position of the chromatogram not affected by interfering peaks. Therefore, we selected it as the IS. Stock solutions containing 1 mg/mL of ATV and IS were appropriately prepared in methanol and stored at –20°C. Working solutions of ATV (1 and 20 µg/mL)

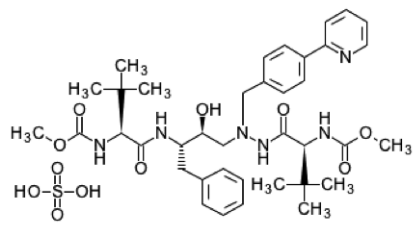

Figure 1. Chemical structure of atazanavir sulphate.

* Author to whom correspondence should be addressed: Mario Negri Institute for Pharmacological Research, Via Gavazzeni 3–24020 Ranica (Bergamo), Italy; email: dcattaneo@marionegri.it.

and IS (4.5 µg/mL) were prepared by dilution with methanol and stored at 4°C. According to previous observations (11), stock solutions were stable for at least 10 months, whereas working solutions were prepared every month. Acetonitrile, methanol (BDH, Milan Italy), and distilled water (MilliQ system, Millipore, Milan, Italy) were all HPLC grade.

Instrumentation and chromatographic conditions

The HPLC system consisted of an analytical column (Ultrasphere octyl, with 4 µm beads, 4.6 mm × 15 cm) (Beckman Coulter, Fullerton, CA) heated at 30°C by a Croco-cil oven equipped with a 125 Solvent Module pump, an autosampler (model 508), set at 15°C, and a UV detector (model 166) set at 260 nm. The mobile phase was a mixture of 45% water, 20% methanol, and 35% acetonitrile. The mobile phase was filtered under vacuum using a polycarbonate 0.4-µm membrane. Flow rate at isocratic elution was 1 mL/min. Data were collected and processed using a 32 Karact software. All equipment was purchased from Beckman (Fullerton, CA).

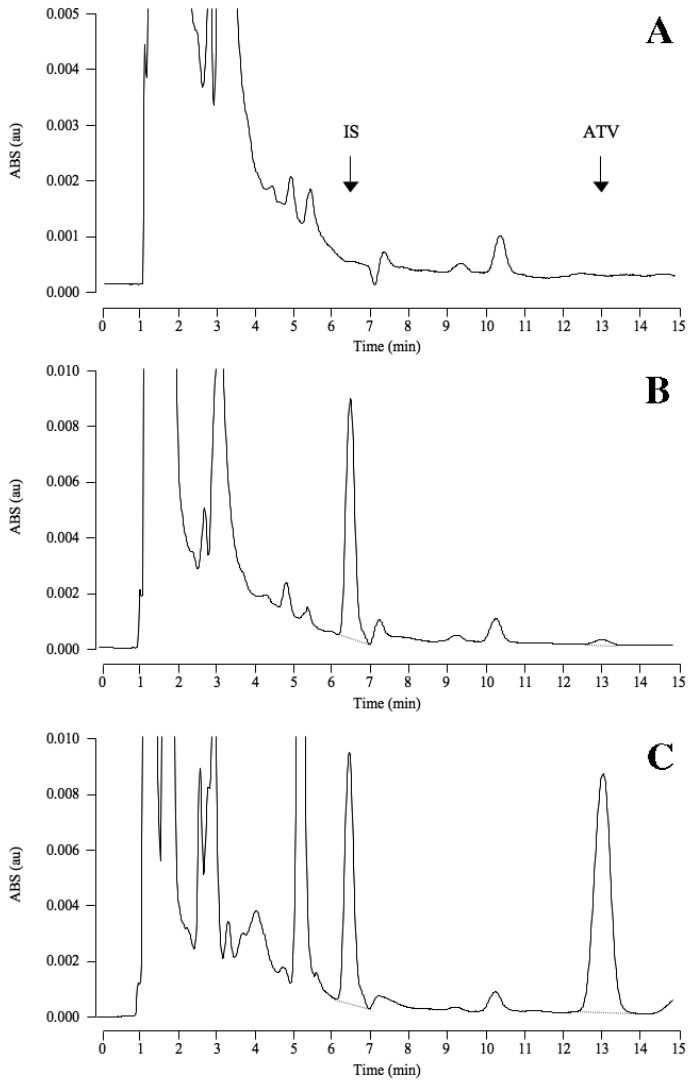

Figure 2. HPLC chromatograms of extracts from drug-free plasma from a healthy volunteer (A), drug-free plasma spiked with 50 ng/mL ATV (atazanavir) (B), and plasma extracted from a patient receiving ATV (C) as part of her HAART therapy (ATV concentration: 1912 ng/mL). ABS: absorbance.

Sample preparation

Blood samples from healthy volunteers (for calibrators and QCs) as well as from HIV-positive patients were drawn into vacutainer tubes containing K_3EDTA as anticoagulant and transported on ice to the laboratory, then immediately centrifuged for 10 min at 3000 g at 4°C. Plasma was decanted and stored at –80°C until analysis. According to recent findings (8,11), we decided not to perform heat viral inactivation of human plasma, due to the fact that ATV is heat-sensitive.

Five hundred microliters of each plasma sample were mixed with 50 µL of IS working solution and 100 µL of methanol in eppendorff vials. Subsequently, the solution was vortex-mixed for 30 s and then centrifuged for 10 min (13.000 × *g*) at room temperature. After centrifugation, 600 µL of the clean supernatant were poured in a polypropylene tube, and after addition of 1 mL of water, sample was mixed and loaded onto a preconditioned (1 mL of methanol followed by 1 mL of water) Bond-elut cartridge (C18, 200 mg, 3-mL, Varian, Leinì, Italy) on a Vac Elut 20 Manifold (Varian, Leinì, Italy). The cartridges were washed with a solution of methanol–water (5:95), and ATV and IS were eluted with 2 mL of methanol. The eluate was taken to dryness (either using nitrogen stream or a centrifugal evaporator both at 37°C) and reconstituted in 150 µL of methanol. Each sample was then transferred to polypropylene vials. Thirty microliters of each sample were injected into the HPLC system.

Ethical and safety concerns

The study was approved by the bio-ethical Committee of our Institution. Healthy volunteers, as well as HIV-infected pregnant women and controls (HIV-infected patients not given ATV as part of their maintenance therapy) gave their informed consent prior to starting the study. Moreover, the laboratory personnel was informed about the nature of the study and the potential risk associated with the use of such procedure not involving sterilization of plasma by heat. Samples were treated as per good laboratory practice, using standard safety procedures (eye protection, use of disposable materials, etc.).

Calibration and method validation

The linearity of the method was tested by constructing standard curves (n = 10) from 20 to 10000 ng/mL of ATV, plotting the peak height ratios of the drug to IS versus the nominal drug concentration, and applying a linear least squares regression analysis without weighing. The method was considered linear if the mean coefficient of regression (r^2) was equal or better than 0.99. For this purpose, appropriate volumes of ATV from stocked working solutions were added to 500 µL of plasma from healthy volunteers to achieve 7 different concentrations (20, 100, 300, 1000, 3000, 6000, and 10000 ng/mL).

Method validation was conducted in agree-

ment with FDA procedures (12). The specificity was evaluated as lack of matrix interference by analysis in triplicate of human drug-free plasma from different volunteers (n = 10). In addition, to test potential concomitant medication or xenobiotic interference, plasma from different HIV-positive patients (n = 30) given a different combination of therapies was analyzed. Quality control (QC) samples were prepared spiking concomitantly known volumes of ATV from stocked working solutions to drug-free human plasma in order to obtain three concentrations within the linear range at low, medium, and high levels (50, 500, and 5000 ng/mL of ATV, respectively). The within- and between-day coefficient of variation (CV) and the accuracy of the method were assessed by calculating daily and overall CVs and bias values for QC samples (five replicates at each concentration per analytical run) that were assayed in five separate analytical runs. The assay was considered acceptable if precision at each concentration was less than 15% for both within- and between-day variability (12) and the accuracy was within ± 15%. The lowest identifiable discrete and reproducible concentration that showed a precision of 20% and accuracy of 80–120% was accepted as lower limit of quantitation (LOQ). To determine the extraction efficiency, the peak height ratios of spiked plasma samples were compared to those obtained from direct injections (n = 5) of the same amount of ATV (at 50 and 5000 ng/mL).

The assay was then assessed in vivo to estimate ATV 24-h plasma concentration-time profiles in 9 HIV-positive pregnant women. During each analytical run, a number of QC samples were prepared separately (usually one at start and one a the end the analytical run). If QCs fall out of the 15% of their nominal values, the run was rejected.

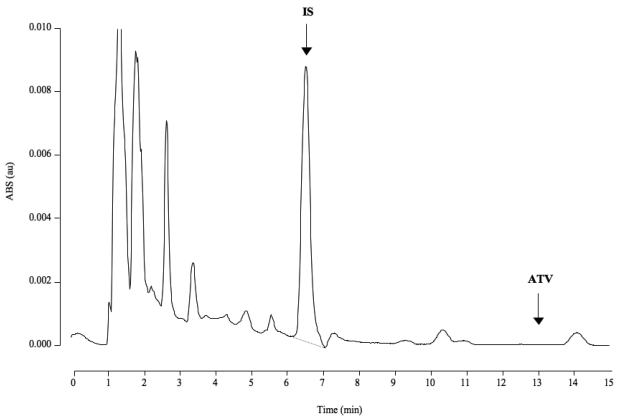

Figure 3. Typical HPLC chromatogram of extract from the plasma of a HIV-positive patient on multiple drug therapy that did not include ATV. ABS: absorbance.

Results

Chromatography and detection

Under the given conditions, IS and ATV eluted at retention times of 6.5 and 13 min, respectively. The total time of the chromatographic run was 15 min. The selectivity of ATV measurement was good, without interference from endogenous compounds. To test possible matrix effects, we analyzed in triplicate different sources of matrix (from 10 healthy volunteers), and no detectable interfering peaks were found. Representative chromatograms of extract from a drug free plasma sample (A), a drug-free plasma sample spiked with known amount of ATV to produce a final concentration of 50 ng/mL (B), and the extract from a patient given ATV as part of highly active antiretroviral therapy, (C, ATV concentration: 1912 ng/mL) are shown in Figure 2.

HIV-positive patients are usually treated with different drugs beside atazanavir. Therefore, to test potential interferences from concomitant medication and relative metabolites, we also analyzed plasma samples from 30 HIV-positive patients given different combinations of antiviral drugs (a list of medications tested for interference is given: Abacavir, Zidovudine, Lamivudine, Nevirapine, Indinavir, Ritonavir, Lopinavir, Tipranavir, Cyclosporine, Mycophenolate mofetil, Sirolimus, Statins, ACE inhibitors, Angiotensin II receptor blockers, Beta-blockers, Diuretics, Steroids, Fluconazole, and Aciclovir). A typical chromatogram from a patient on multiple drug therapy that did not include ATV is given in Figure 3. It contains additional peaks that are not present in the spiked samples. However, none of them eluted at the retention times of ATV or IS.

Table I. Performance of the HPLC Method for the Determination of ATV Plasma Concentrations (n = 5)

	Spiked ATV concentration (ng/mL)			
	20 (LOQ)	50 (low QC)	500 (medium QC)	5000 (high QC)
Within-day assay				
Mean ± SD (ng/mL)	20.4 ± 2.6	52.4 ± 2.3	513.4 ± 34.7	5047.8 ± 112.5
Precision (CV%)	11.6	4.4	6.8	2.2
Accuracy (%)	12.0	4.8	2.7	1.0
Between-day assay				
Mean ± SD (ng/mL)	22.8 ± 3.4	52.6 ± 5.0	517.4 ± 43.4	5071.8 ± 153.5
Precision (CV%)	14.7	9.6	8.4	3.0
Accuracy (%)	14.0	5.2	3.5	1.4

* ATV: atazanavir; CV: coefficient of variation.

Validation

The analysis showed satisfactory precision with intra- and inter-assay CV less than 15%. Similarly, the accuracy was, in every instance, within the acceptance limit (Table I). The LOQ was set at 20 ng/mL. The method was linear in the range of drug concentrations of 20–10000 ng/mL (n = 10), with an acceptable coefficient of regression (mean r^2 = 0.9991), $y = 0.00246x - 0.01102$. Mean drug recovery, calculated by comparing the peak-height ratios of extracted plasma

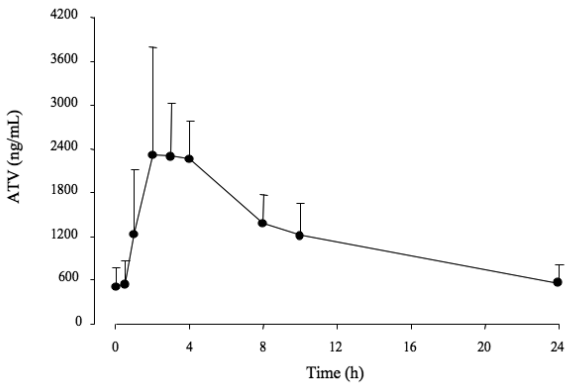

Figure 4. Mean ATV plasma concentration-time profile from HIV-infected pregnant patients (n = 9). The concentrations are expressed as mean ± standard deviation.

samples with those obtained from un-extracted calibrators with the same amount of drug was 80.5% for ATV and 78.4% for IS. Recovery was comparable at both ATV concentrations (50 and 5000 ng/mL, data not shown).

The present method has been used to measure ATV 24-h plasma concentration-time profiles in 9 HIV-infected women at 30–36 weeks of gestation given the drug at 300 mg/day boosted with 100 mg of ritonavir. Despite the fixed daily drug dose used, ATV concentration showed marked interpatient variability in the 24-h mean pharmacokinetic profile (Figure 2). A detailed description of this study has been provided elsewhere (13).

Discussion

We described a new HPLC method with UV detection for assaying ATV concentration in human plasma samples. The major advantage of this method as compared to others previously published (6–11,14) rests on the simple extraction procedure, the use of an UV detector, which is common in most laboratories, and the relatively shorter chromatographic run time in contrast to other assays (6,14). Of note, the use of solid-phase extraction (SPE) over the more conventional liquid–liquid extraction (LLE) (8,9) may result in a faster preparation step with a lower requirement of toxic organic solvents. Moreover, according to our previous experience with other analytes, SPE gives cleaner chromatograms compared with LLE and prevents fast degradation of the analytical column. As an additional improvement compared to previous publications, the proposed method does not necessitate the use of phosphate salts (either in the mobile phase or during the extraction procedures) that might precipitate in the HPLC system, ultimately resulting in unacceptable high pressure and failure of the analysis. The performance of the proposed method complies in the spirit of FDA guidelines (12) for the validation of a bioanalytical method in terms of linearity, precision, accuracy, and recovery. In particular, the accuracy, expressed as % bias from the nominal concentrations of the QCs, was between 1.0% and 5.2%, whereas the precision, expressed as %CV, ranged from 2.2% to 9.6%. All these values were similar to those previously reported in other HPLC–UV methods developed for the assessment of ATV concentration in human plasma (6–10,14). The LOQ for ATV was 20 ng/mL, which is greatly below the minimum drug concentration reported in different ATV-based therapies (4,5,9) and observed by us during pharmacokinetic evaluations performed in HIV-positive pregnant women (13).

The present work certainly has some shortcomings. The stability of the solutions or the frozen plasma was not assessed because these data have been already published (11). Moreover, the extraction phase required a high volume of plasma and the use of solid-phase cartridges, which can be expensive. As an additional drawback, this method has been developed for the assessment of only one agent, whereas several assays are available to date for the simultaneous quantitation of different anti-HIV drugs (7,9,10,15). It must be pointed out, however, that these methods require gradient procedures that result in chromatographic time largely exceeding 30 min for each analytical run. The present method has a run time of 15 min, allowing the extraction and analysis of ATV in at least 60 samples (including calibration points and controls) in less than 24 h.

The instability of atazanavir during heating HIV-inactivation may represent a further limitation to the safety application of therapeutic drug monitoring in the routine clinical practice. Indeed, 15–50% decreases in atazanavir concentrations in plasma samples treated at 60°C for 30–60 min have been reported (8,11). Actually, the structural features in the molecules making it unstable to heat are poorly understood. To our knowledge, only two papers have partially faced this topic. Seshachalam and co-workers (16) have shown that atazanavir was stable under thermal, photolytic, and oxidative conditions in a neutral pH ambient. However, when the substance was heated under acidic or alkaline conditions, significant degradation was observed. Others (17) have documented that atazanavir has a low thermal stability in water solution. From this point of view, the development of alternative methods based on derivatizations to stabilize the molecule might represent a step forward, avoiding the potential exposure of laboratory personnel to the HIV virus.

In conclusion, the reported method is a simple HPLC procedure for the determination of ATV in human plasma and provides a suitable alternative to mass spectroscopy. Our method may have applications for pharmacokinetic studies, drug-to-drug interaction evaluations, and implementing ATV therapeutic drug monitoring.

Acknowledgments

The authors thank the Association for Research on Transplantation (ART) for continuous support.

References

1. J.A. Pineda, R. Palacios, A. Rivero, L. Abdel-Kadel, M. Marquz, P. Cano, J.A. Mira, J. Torre-Cisneros, F. Lozano, and J. Santos. Low incidence of severe liver toxicity in patients receiving antiretroviral combinations including atazanavir. *J. Antimicrob. Chemother.* **57:** 1016–17 (2006).

2. T. Swainston Harrison and L.J. Scott. Atazanavir: a review of its use in the management of HIV infection. *Drugs.* **65:** 2309–36 (2005).
3. (No authors listed). Some PIs have greater impact than others on heart disease. Patients on atazanavir had less heart problems. *AIDS Alert.* **21:** 17–18 (2006).
4. A. Le Tiec, C. Barrail, C. Goujard, and A.M. Taburet. Clinical pharmacokinetics and summary of efficacy and tolerability of atazanavir. *Clin. Pharmacokinet.* **44:** 1035–50 (2005).
5. S. Smith, D.E. Jeganathan, and J. Ray. Atazanavir plasma concentrations vary significantly between patients and correlate with increased serum bilirubin concentrations. *HIV Clin. Trials* **7:** 34–38 (2006).
6. S. Colombo, N. Guignard, C. Marzolini, A. Telenti, J. Biollaz, and L.A. Decosterd. Determination of the new HIV-protease inhibitor atazanavir by liquid chromatography after solid-phase extraction. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **810:** 25–34 (2004).
7. E. Dailly, F. Raffi, and P. Jolliet. Determination of atazanavir and other antiretroviral drugs plasma levels by high performance liquid chromatography with UV detection. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* **813:** 353-358 (2004).
8. R.W. Sparidans, F. Dost, K.M. Crommentuyn, A.D. Huitema, J.H. Schellens, and J.H. Beijnen. Liquid chromatographic assay for the protease inhibitor atazanavir in plasma. *Biomed. Chromatogr.* **20:** 72–76 (2006).
9. O. Tribut, M.C. Verdier, C. Arvieux, H. Allain, C. Michelet, and D. Bentue-Ferrer. Simultaneous quantitative assay of atazanavir and 6 other HIV protease inhibitors by isocratic reversed-phase liquid chromatography in human plasma. *Ther. Drug Monit.* **27:** 265–69 (2005).
10. R. Verbesselt, E. Van Wijngaerden, and J. de Hoon. Simultaneous determination of 8 HIV protease inhibitors in human plasma by isocratic high-performance liquid chromatography with combined use of UV and fluorescence detection: Amprenavir, indinavir, atazanavir, ritonavir, lopinavir, saquinavir, nelfinavir and M8-nelfinavir metabolite. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **845:** 51–60 (2007).
11. A. Schuster, S. Burzawa, M. Jemal, E. Loizillon, P. Couerbe, and D. Whigan. Quantitative determination of the HIV protease inhibitor atazanavir (BMS-232632) in human plasma by liquid chromatography-tandem mass spectrometry following automated solid-phase extraction. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **788:** 377–86 (2003).
12. FDA guidance for industry, *Center for Drug Evaluation and Research, US Department for Health and Human Services*, May (2001).
13. D. Ripamonti, D. Cattaneo, F. Maggiolo, M. Airoldi, L. Frigerio, P. Bertuletti, M. Ruggeri, and F. Suter. Atazanavir plus low-dose ritonavir in pregnancy: pharmacokinetics and placental transfer. *AIDS*, 21: 2409–2415 2007.
14. A. Loregian, S. Pagni, E. Ballarin, E. Sinigalia, S.G. Parisi, and G. Palù. 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 (2006).
15. J.M. Poirier, P. Robidou, and P. Jaillon. Simple and simultaneous determination of the hiv-protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir plus M8 nelfinavir metabolite and the nonnucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma by reversed-phase liquid chromatography. *Ther Drug Monit* **27:** 186–92 (2005).
16. U. Seshachalam, D.V.L. Narasimha, B. Haribabu, and K.B. Chandrasekhar. Determination of atazanavir in the presence of its degradation products by a stability-indicating LC method. *Chromatographia* **65:** 355–58 (2007).
17. K. Michalik, Z. Drzazga, A. Michnik, and M. Kaszuba. Thermal stability study of the protease inhibitors nelfinavir and atazanavir sulfate. *J. Therm. Anal. Cal.* **88:** 401–404 (2007).

Manuscript received April 17, 2007;
revision received July 12, 2007.