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# A monolithic-phase based on-line extraction approach for determination of pharmaceutical components in human plasma by HPLC–MS/MS and a comparison with liquid–liquid extraction

Raymond Naxing Xu *, Leimin Fan, Grace E. Kim, Tawakol A. El-Shourbagy

*Abbott Laboratories, Department of Drug Analysis, 100 Abbott Park Road, Abbott Park, IL 60064, USA*

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**Abstract**

An automated procedure using monolithic-phase based on-line extraction is described for pharmaceutical component analysis in plasma by LC–MS/MS. In this approach, a short monolithic $C_{18}$ 4.6 mm × 10 mm cartridge is used for high flow extraction at 4 mL/min. Plasma samples were subjected to protein precipitation first with acetonitrile, and the supernatant was diluted and loaded onto a monolithic cartridge. Sample elution was accomplished with narrow-bore LC–MS/MS system. A method for determination of Amprenavir (APV) and Atazanavir (AZV) in human plasma was developed with this approach. After 0.1 mL of plasma was transferred into each well of a 96-well plate by a liquid handler, the rest of sample preparation time typically only takes about 20 min. A Phenomenex Luna C18(2) 2.0 mm × 150 mm analytical column was used for the separation at a flow rate of 0.3 mL/min. The run time for each sample was 4 min. The standard curve range was 2.77–1520 ng/mL for Atazanavir, and 4.50–2560 ng/mL for Amprenavir. The accuracy (%bias) at the lower limit of quantitation (LLOQ) for Atazanavir was 2.7% and the precision (%CV) at the LLOQ was 7.9%, while the accuracy at LLOQ for Amprenavir was −1.3% and the precision at LLOQ was 7.8%. The inter-day %bias and %CV of the quality control samples of Atazanavir were ≤4.5% and ≤6.5%, respectively. The inter-day %bias and %CV of the quality control samples of Amprenavir were ≤1.1% and ≤7.2%, respectively. Coefficients of determination, a measure of linearity, ranged from 0.993 to 0.999. Very low carry-over (0.006%) even after high standard sample was demonstrated in the monolithic-phase based method. Other characteristics of such method include high recovery and good tolerance to matrix effect, which was demonstrated by 12 lots of plasma. The back pressure of the monolithic extraction cartridge remained the same after 450 samples injected. The performance of the monolithic-phased on-line extraction method was compared with that done by an automated 96-well liquid–liquid extraction procedure, which was carried out using hexane:ethyl acetate as the extraction solvent. The results showed that similar precision and accuracy were achieved by both methods.


*Keywords:* High-throughput analysis; Monolithic column; On-line extraction; Liquid–liquid extraction; Liquid chromatography; Tandem mass spectrometry; Automation

## 1. Introduction

Liquid chromatography tandem mass spectrometry (LC–MS/MS) has become a widely used technique for the fast and sensitive determination of analytes, especially pharmaceutical compounds, in complex matrices such as biological fluids. The overall LC–MS/MS process for pharmaceutical analysis includes sample preparation, analytical separation and data acquisition. With the introduction of highly sensitive and multifunctional instruments such as newer types of mass spectrometers and autosamplers designed for easy operation, sample preparation remains as the time-limiting step when compared to analytical separation and data acquisition. To increase sample throughput, traditional procedures such as protein precipitation, liquid–liquid extraction (LLE), and solid-phase extraction (SPE) have been automated with robotic liquid handling systems in 96-well format [1–4]. Although not yet widely adopted in the industry, 384-well format has been shown able to increase analytical run sizes and sample throughput for LC/MS/MS determination of small drug molecules in biological samples [5,6].

On-line sample purification techniques have gained great interests in the recent years. One sample purification approach

* Corresponding author. Tel.: +1 847 9388158; fax: +1 847 9358785.
*E-mail address:* Raymond.Xu@abbott.com (R. Naxing Xu).

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is based on the use of small (typically 1 mm × 50 mm) HPLC columns packed with large particles (typically 30–50 μm), with a very high linear speed of the mobile phase. This combination provides the so-called turbulent flow chromatography for the rapid passage of the large biomolecules of the biological sample while the small-molecule analyte of interest is retained. Among commercially available columns available, Oasis HLB and Cohesive HTLC columns are most commonly used for direct plasma sample analysis [7,8]. Restricted access media and Prospekt system for solid-phase extraction have also been used for high-throughput bioanalysis [9,10].

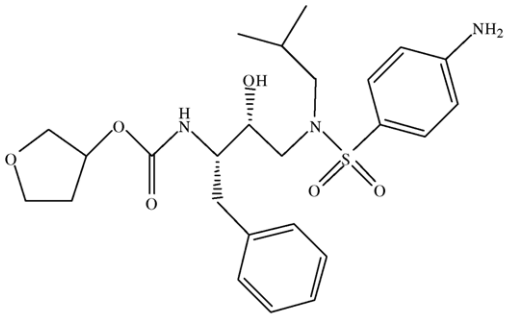
Recently introduced to achieve fast chromatographic separation, monolithic phases are also suitable for the direct injection of biological fluids [11]. Because of their high permeability, the extraction of biological samples can also be performed with a high flow rate without generating high back pressure. The flow rate can be 5–10 times higher than generally used conventional supports. The separation efficiency is less dependent on the flow rate of monolithic columns, which leads to short run time while maintaining separation efficiency. Monolithic phases produced by the sol–gel polymerization technology do not require frits at column extremities, which often remain the main source of endogenous material adsorption. Plumb et al. have demonstrated that monolithic supports can tolerate several millilitres of plasma without significant performance degradation [12].

In this article, we explored the usage of a short monolithic $C_{18}$ 4.6 mm × 10 mm cartridge for LC–MS/MS bioanalysis with high flow on-line extraction at 4 mL/min. A major advantage of this method over off-line solid-phase extraction using liquid handlers is the short sample preparation time and the lower cost. This approach was tested for the analysis of Amprenavir (APV) and Atazanavir (AZV) in human plasma. Both Amprenavir and Atazanavir are synthetic peptide-like antiretroviral agents that inhibit the activity of the human immunodeficiency virus type-1 (HIV-1) protease. The performance of the monolithic-phase on-line extraction method was compared with that done by an automated 96-well liquid–liquid extraction procedure, one that has been well established in our laboratory [13,14].

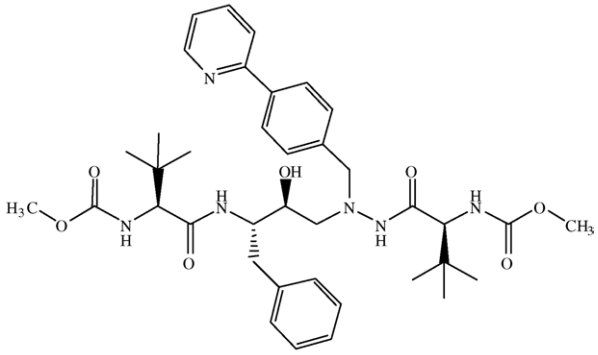
## 2. Experimental

### 2.1. Chemicals

Acetonitrile, hexane, and formic acid (FA) were purchased from EM Science (Gibbstown, NJ, USA). Ammonium acetate and sodium carbonate, both in ACS grade, were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was produced by a Millipore (Bedford, MA, USA) Milli-Q unit. Atazanavir, Amprenavir, and internal standard, ABT-093, were obtained from Abbott Laboratories (Abbott Park, IL, USA). Chemical structure of Amprenavir and Atazanavir is illustrated in Fig. 1. ABT-093 is a structurally similar molecule and the exact structure is not shown for proprietary reason. Normal human plasma with sodium heparin as anticoagulant was purchased from Biological Specialties Corporation (Colmar, PA, USA).




Amprenavir




Atazanavir

Fig. 1. Chemical structures of Amprenavir (APV) and Atazanavir (AZV).

### 2.2. Standard and quality control (QC) solutions

Stock solution was made in 50% acetonitrile in $H_2O$. Working solutions were prepared by diluting the stock solution of the analyte with 50% acetonitrile in $H_2O$. For standard preparation, three sets of weighing were used to prepare primary stock solutions of Amprenavir (APV) and Atazanavir (AZV) at the following levels: (1) 128 μg/mL APV, 75.9 μg/mL AZV; (2) 50.7 μg/mL APV, 31.2 μg/mL AZV; (3) 40.2 μg/mL APV, 23.4 μg/mL AZV. Human plasma standard levels 1–10, at concentrations of 4.50, 14.8, 19.3, 37.5, 123, 161, 313, 805, 1120, 2560 ng/mL for APV and concentrations of 2.77, 8.74, 11.3, 23.1, 72.8, 93.7, 192, 469, 687, 1520 ng/mL for AZV, were prepared by adding the appropriate volume of primary stock solution, or higher level standard solution into a 25 mL class A volumetric flask and diluting to the mark with normal human plasma with sodium heparin. Standards were then aliquoted into 4 mL polypropylene tubes and stored in a freezer maintained at approximately −20 °C. Only one weighing was used to prepare QC primary stock solution at 55.0 μg/mL APV, and 32.2 μg/mL AZV. Otherwise, quality control solutions were prepared in essentially the same manner at concentrations of 11.0, 550, 2200 ng/mL for APV and 6.43, 322, 1290 ng/mL for AZV.

### 2.3. Sample preparation for on-line extraction

Samples were thawed at room temperature, sonicated and mixed to ensure homogeneity. All steps of sample preparation

were handled in automated fashion. Sample transfer steps were accomplished by liquid handler with positive displacement capability (Hamilton Lab AT 2 Plus, Reno, Nevada, USA). Each plasma sample (0.100 mL) was loaded into the appropriate well of a clean 2.0 mL polypropylene 96-well plate.

After 0.050 mL of working IS solution at 1.06 μg/mL in 85% acetonitrile was transferred to each well, 0.3 mL of acetonitrile was added. The plate was covered and sonicated for 2–3 min. The plate was vortexed on a multitube vortexer at low speed (VWR, Model VX-2500) for 5 min. After being centrifuged at approximately 3000 rpm for 5 min at approximately 10 °C, 0.1 mL of supernatant was transferred from each well to a clean 96-well plate. Then 0.30 mL of 0.1% formic acid in 2 mM $NH_4OAc$ was added. After mixing, 0.1 mL of the solution was injected into LC–MS/MS system equipped with on-line extraction setup.

### 2.4. Sample preparation for liquid–liquid extraction

Samples were treated with the same manner as the on-line extraction procedure until the step of adding IS solution to each well of the 96-well plate. Then 0.050 mL of 500 mM sodium carbonate, and 1.20 mL of extracting solvent (hexane:ethyl acetate 1:1) were added. The plate was heat-sealed with Pierceable Sealing foil (Abgene House, Epsom, Surrey, UK) and mixed and vortexed on a multitube vortexer (VWR, Model VX-2500) for 5 min. After the plate was centrifuged at 3000 rpm for 5 min at approximately 10 °C to separate layers, the seal was punctured and 0.9 mL of the organic layer was transferred from each well to a clean 96-well plate. The organic extract was evaporated to dryness under a stream of nitrogen at approximately 35 °C. Then 0.30 mL of 50:50 acetonitrile:0.1% formic acid in 2 mM $NH_4OAc$ was added to the each well of the clean plate. After mixing, 20 μL of the solution was injected into LC–MS/MS.

### 2.5. LC–MS/MS instrumentation

An Agilent 1100 pump (Hewlett-Packard, Waldbronn, Germany) with a two-way solvent selector (Parker Instrumentation, Fairfield, NJ) was used to deliver a high flow through the extraction column to load and wash the sample and subsequently to flush and equilibrate the extraction column. A Shimadzu LC-$10AD_{vp}$ pump (Shimadzu, Columbia, MD, USA) was used to deliver the flow to elute the analytes from the extraction column and to perform separation on the analytical column. A Shimadzu SIL-$HT_C$ autosampler/controller was used to inject samples. The mobile phase of the analytical column was used as the wash solvent for both the syringe and the injector. A Chromolith RP-18e 10 mm × 4.6 mm cartridge (Merck KGaA, Darmstadt, Germany, ordered through VWR International) was used as the extraction column. A Valco 10-port valve (Valco Instruments, Houston, TX, USA) was used to control on-line extraction and liquid flow to mass spectrometer as shown in Fig. 2.

A Luna 5 μm, C18(2), 150 mm × 2.0 mm column from Phenomenex (Torrance, CA, USA) was used as the analytical column. An isocratic HPLC method was employed for separation. Mobile phase consisted approximately 70:30 (v/v) acetonitrile:2 mM ammonium acetate in 0.1% formic acid. The flow rate

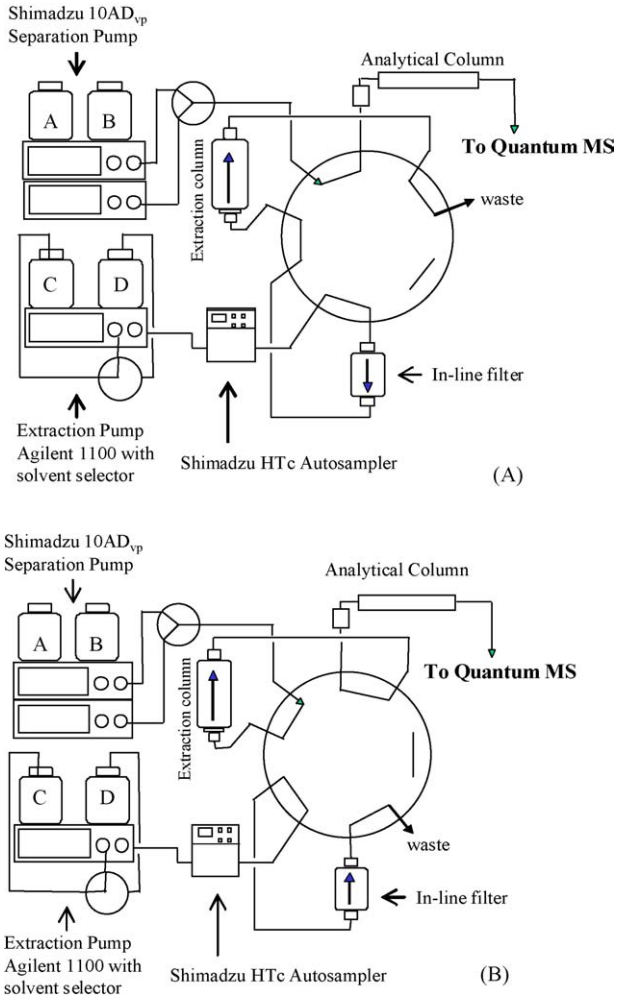



Fig. 2. Schematic diagrams of the instrumental setup for the on-line extraction with Chromolith 4.6 mm × 10 mm cartridge as extraction column: (A) sample loading and extraction mode and (B) elution and separation mode. For both on-line extraction method and liquid–liquid extraction method, Shimadzu pump used solvent A (mobile phase) for isocratic separation. Solvent B, typically optioned for gradient elution, was not used for either method.

for this program was set to 0.3 mL/min. The analytical column was maintained at room temperature of approximately 22 °C.

For liquid–liquid extraction method, a Intersil ODS2 2.0 mm × 10 mm guard column (Thermo-Hypersil-Keystone, Bellefonte, PA) was used before the Luna 5 μm, C18(2) analytical column. The Shimadzu LC-$10AD_{vp}$ pump was used to deliver the flow for separation and the Shimadzu SIL-$HT_C$ autosampler/controller was used to inject samples. Mobile phase and separation conditions were kept the same as the on-line extraction method.

LC–MS/MS detection was performed using a Thermo Finnigan (San Jose, CA, USA) Quantum triple quadrupole mass spectrometer with an electrospray ionization source operated in the positive ion mode. The computer control system was Xcalibur™ Version 1.3. The spray voltage was 5000 V. The source temperature was 350 °C. The sheath gas setting was 35 and auxiliary valve flow setting was 20. Following are some other parameters of the mass spectrometer acquisition file. The

selected reaction monitoring (SRM) detection channel for APV was *m*/*z* 506.2–245.2 with collision energy setting at 10 V and tube lens setting at 187. The SRM detection channel for AZV was *m*/*z* 705.3–168.2 with collision energy setting at 46 V and tube lens setting at 202. The SRM detection channel for IS was *m*/*z* 747.3–322.2 with collision energy setting at 19 V and tube lens setting at 193.

### 2.6. System operation for on-line extraction procedure

After the diluted supernatant from the plasma precipitation was injected by the HTc autosampler, the sample was loaded onto the extraction column by solvent C, a solution containing 25:75 acetonitrile:2 mM $NH_4OAc$ in 0.1% formic acid (v/v), at a flow rate of 4 mL/min. At 0.7 min after injection, the valve switched to the elution position, which put the extraction and separation columns in tandem in the flow path of the separation pump (Shimadzu). The separation pump running an isocratic flow of mobile phase (A) at 0.3 mL/min elutes analytes to mass spectrometer without the need of diverting. An elution step of 1.3 min was given before the switching valve was switched back to the original position (configuration A in Fig. 2). The extraction pump then started to deliver solvent D, 95% acetonitrile with 0.01% formic acid, at a flow rate of 4 mL/min for 0.5 min to flush the extraction column. For the rest of the run cycle, the extraction pump delivered solvent C at 4 mL/min to recondition the Chromolith cartridge for the next sample. The run time for the assay of one sample is 4 min. The time program of the Agilent 1100 pump is listed in Table 1.

HTc autosampler/controller sent the signal to inject the sample and to start the program on the Agilent 1100 pump. It also sent out a signal to the mass spectrometer to start the data acquisition. For overnight operation, a contact-closure signal was sent from the HTc autosampler/controller to Agilent 1100 pump to shut down the pump at the end of sample analysis.

### 2.7. Calibration curves and quantitation of samples

Xcalibur™ Version 1.3 was used for the data acquisition, peak area integration, regression, and quantitation. For each analytical batch, a calibration curve was derived from the peak area ratios (analyte/internal standard) using weighted linear least-squares regression of the area ratio versus the concentration of the standards. A weighting of $1/x^2$ (where $x$ is the concentration of a given standard) was used for curve fitting. The regression equation for the calibration curve was used to back-calculate the measured concentration at each standard level and the results were compared with the theoretical concentration to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard level measured.

## 3. Results and discussion

The on-line system was found to be very rugged for the analysis of plasma samples after a simple protein-precipitation treatment. Because of its highly porous nature, the back pressure generated on the extraction column was very low (175 psi) at the flow rate of 4 mL/min. The back pressure on the extraction column and the analytical separation column remained the same after over 450 injections of samples. In our experience, the extraction column can usually last for at least four 96-well batches without any performance concerns.

Precision and accuracy of the on-line extraction method was demonstrated by six consecutive analytical batches. Each batch contained a single set of calibration standards, six replicates of QCs at three concentration levels, six replicates of LLOQ (lower limit of quantitation) evaluation samples, and six replicates of ULOQ (upper limit of quantitation) evaluation samples. Each batch also contained other test samples such as system suitability sample.

Statistical data of calibration curve parameters computed from six consecutive analytical curves are listed in Table 2. The correlation coefficients of six calibration curves were all $\geq$0.993. The standards showed a linear range of 2.11–1520 ng/mL for Atazanavir, and 4.50–2560 ng/mL for Amprenavir, respectively, using weighted (1/concentration$^2$) least-square linear regression. The precision and accuracy data for LLOQ, ULOQ, and QC samples are summarized in Table 3. The data show that this method is consistent and reliable with low %CV and %bias values. The accuracy (%bias) at the lower limit of quantitation (LLOQ) for Atazanavir was 2.7% and the precision (%CV) at the LLOQ was 7.9%, while the accuracy at LLOQ for Amprenavir was −1.3% and the precision at LLOQ was 7.8%. The inter-day %bias and %CV of the quality control samples of Atazanavir were $\leq$3.0% and $\leq$6.5%, respectively. The inter-day %bias and %CV of the quality control samples of Amprenavir were $\leq$1.1% and $\leq$7.2%, respectively. Coefficients of determination, a measure of linearity, ranged from 0.993 to 0.999 in all standard curves for either Amprenavir or Atazanavir.

Fig. 3 showed the chromatograms from a blank sample. Representative chromatograms of a LLOQ sample and a quality control sample (mid QC) are shown in Fig. 4(a and b), respectively.

Table 1
Time program on Agilent 1100 pump

| Time (min) | Event |
|---|---|
| 0 | Sample loaded onto extraction column with loading solvent |
| 0.7 | The flow-control valve switched, extraction column in tandem with analytical column |
| 0.72 | Agilent 1100 solvent selector switched to deliver washing solvent |
| 2.0 | The flow-control valve switched again to wash extraction column by washing solvent |
| 2.5 | Agilent solvent selector switched to deliver loading solvent to extraction column for conditioning |
| 3.5 | Program end, flow rate stays at 4 mL/min through the program |

Table 2
Summary of calibration curves obtained for the analysis of Amprenavir (APV) and Atazanavir (AZV) by monolithic-phase on-line extraction method

| | Concentration (ng/mL) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | STD 1, 4.50 | STD 2, 14.8 | STD 3, 19.3 | STD 4, 37.5 | STD 5, 123 | STD 6, 161 | STD 7, 313 | STD 8, 805 | STD 9, 1120 | STD 10, 2560 | Coefficient of determination ($r^2$) |
| (I) APV | | | | | | | | | | | |
| Mean | 4.45 | 15.2 | 20.2 | 35.7 | 119 | 152 | 319 | 830 | 1170 | 2490 | 0.9964 |
| S.D. | 0.10 | 0.70 | 0.52 | 1.97 | 2.37 | 7.57 | 11.6 | 15.5 | 56.1 | 104 | 0.0020 |
| %CV | 2.2 | 4.6 | 2.6 | 5.5 | 2.0 | 5.0 | 3.6 | 1.9 | 4.8 | 4.2 | 0.2 |
| %Bias | −1.2 | 3.2 | 4.4 | −4.7 | −3.5 | −5.7 | 2.0 | 3.2 | 5.1 | −2.7 | – |
| *n* | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| | Concentration (ng/mL) | | | | | | | | | | |
| | STD 1, 2.77 | STD 2, 8.74 | STD 3, 11.3 | STD 4, 23.1 | STD 5, 72.8 | STD 6, 93.7 | STD 7, 192 | STD 8, 469 | STD 9, 687 | STD 10, 1520 | Coefficient of determination ($r^2$) |
| (II) AZV | | | | | | | | | | | |
| Mean | 2.78 | 8.67 | 11.3 | 22.8 | 73.0 | 95.3 | 198 | 471 | 703 | 1420 | 0.9983 |
| S.D. | 0.05 | 0.30 | 0.31 | 0.61 | 1.77 | 1.14 | 6.49 | 12.0 | 24.2 | 60.5 | 0.0007 |
| %CV | 1.7 | 3.4 | 2.7 | 2.7 | 2.4 | 1.2 | 3.3 | 2.5 | 3.4 | 4.3 | 0.1 |
| %Bias | 0.2 | −0.8 | 0.4 | −1.3 | 0.2 | 1.6 | 3.2 | 0.5 | 2.3 | −6.3 | – |
| *n* | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

Mean values in the table are average of the back-calculated concentrations from the standard curve.

Table 3
Inter-day accuracy and precision of the LLOQ, QC, ULOQ evaluation samples for Amprenavir (APV) and Atazanavir (AZA) by monolithic-phase on-line extraction method

| | Concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | LLOQ 4.50 | Low QC 11.0 | Mid QC 550 | High QC 2200 | ULOQ 2560 |
| (I) APV | | | | | |
| Mean | 4.44 | 10.9 | 553 | 2220 | 2560 |
| Inter-run S.D. | 0.35 | 0.70 | 39.9 | 107 | 135 |
| Inter-run %CV | 7.8 | 6.4 | 7.2 | 4.8 | 5.2 |
| Inter-run %bias | −1.3 | −0.5 | 0.6 | 1.1 | 0.1 |
| *n* | 36 | 36 | 36 | 36 | 36 |
| | Concentration (ng/mL) | | | | |
| | LLOQ 2.77 | Low QC 6.43 | Mid QC 322 | High QC 1290 | ULOQ 1520 |
| (II) AZV | | | | | |
| Mean | 2.85 | 6.62 | 323 | 1250 | 1450 |
| Inter-run S.D. | 0.23 | 0.43 | 14.0 | 54.5 | 84.1 |
| Inter-run %CV | 7.9 | 6.5 | 4.3 | 4.4 | 5.8 |
| Inter-run %bias | 2.7 | 3.0 | 0.5 | −2.7 | −4.5 |
| *n* | 36 | 36 | 36 | 36 | 36 |

Mean values in the table are average of the back-calculated concentrations from the standard curve.

Estimated signal to noise ratio at LLOQ was greater than 200:1 for both analytes, which demonstrated that sufficient sensitivity was achieved. For the current on-line extraction method, 100 μL out of approximate 450 μL of protein-precipitation supernatant was transferred to a clean well of another plate, which was then diluted with 300 μL of reconstitution solution and only 100 μL of the mixture was loaded onto on-line extraction column for LC–MS/MS analysis. The overall sample usage percentage was 5.6%. With the loading speed at 4 mL/min, it is projected that sensitivity can be easily improved with higher injection volume if needed.

Extraction recovery was determined by comparing the response factors (area/on-column amount) of the appropriate peaks of extracted QC samples with those of post-extraction spiked plasma blanks at similar concentrations. For both analytes, almost 100% extraction recovery was determined. Matrix effects from co-eluting endogenous components in biological fluids have been well documented in the literature to compromise the reproducibility and accuracy of the analysis [15,16]. To demonstrate that the assay performance is independent from the sample matrix, low QC was prepared in 12 different matrix lots and the accuracy and precision of these low QC evaluation

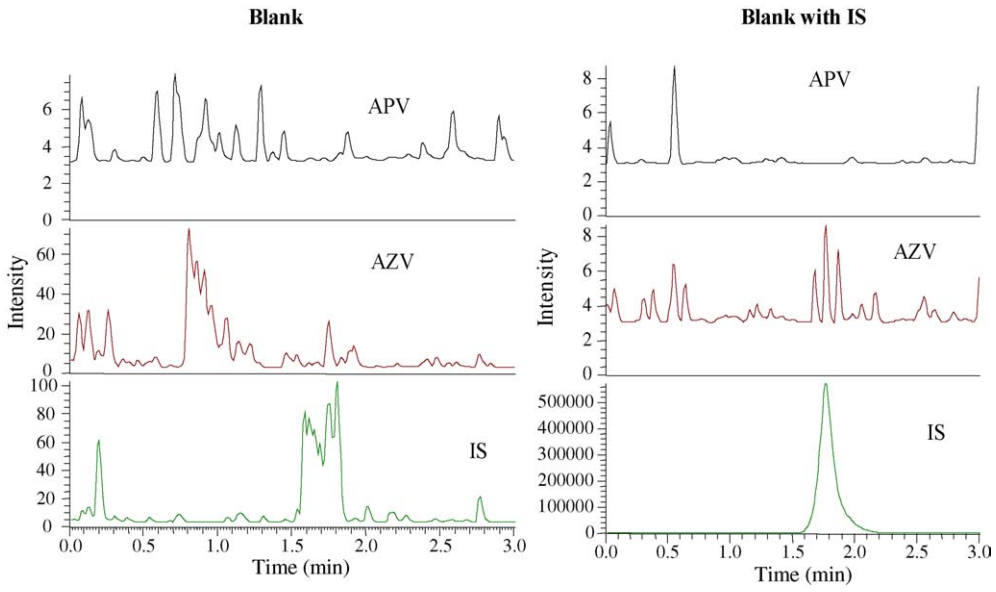



Fig. 3. Representative ion chromatograms of an extracted blank sample and those of an extracted blank spiked with internal standard (IS) by the on-line extraction method.

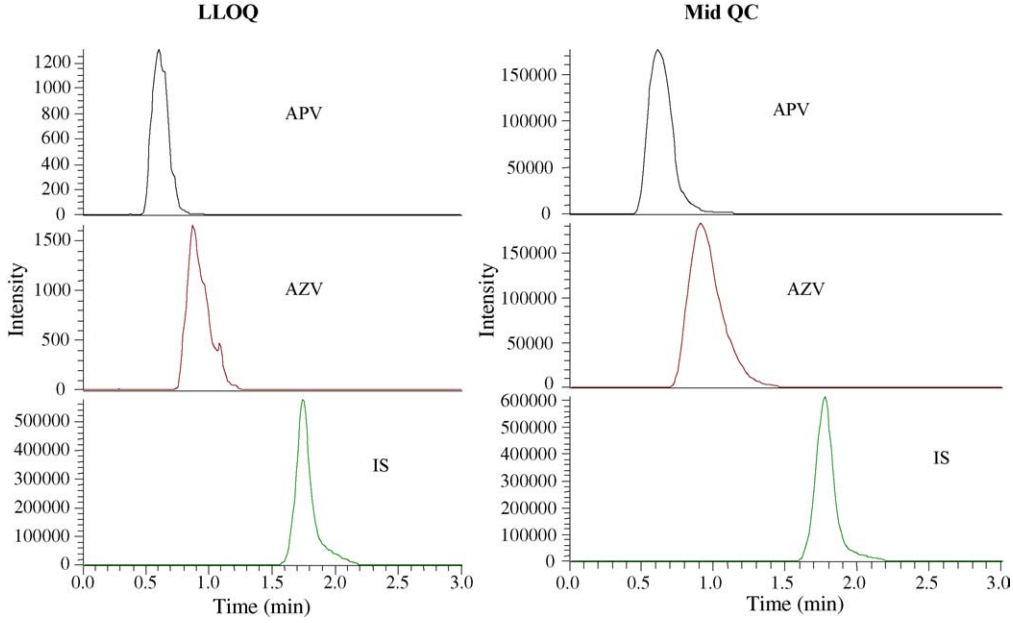



Fig. 4. Representative ion chromatograms of an LLOQ sample (4.50 ng/mL for Amprenavir and 2.77 ng/mL for Atazanavir) and a mid QC sample (550 ng/mL for Amprenavir and 322 ng/mL for Atazanavir) by on-line extraction method.

sample in six replicates were determined with a same calibration curve. The accuracy (%bias) of these low QC evaluation samples for Amprenavir was ≤9.4% and the precision (%CV) was ≤7.7%, while the accuracy of the evaluation samples for Atazanavir was ≤9.5% and the precision was ≤9.3%. The results as summarized in Table 4 suggested that matrix effect for the assay was well within the measurement errors.

To compare the on-line extraction method to 96-well liquid–liquid extraction approach, three analytical batches were prepared by liquid–liquid extraction method described in Section 2. Representative chromatograms of a LLOQ sample and a quality control sample (mid QC) are shown in Fig. 5(a and b), respectively. Each batch contained the same amount of evaluation samples as those done in on-line extraction method. Statistical data of calibration curve parameters computed from three consecutive analytical curves are listed in Table 5. Coefficients of determination ranged from 0.990 to 0.999 in all standard curves for either Amprenavir or Atazanavir. The precision and accuracy data for LLOQ, QC, and ULOQ samples are summarized in Table 6. The accuracy (%bias) at the lower limit of quantitation (LLOQ) for Atazanavir was −2.4% and the precision (%CV) for samples at the LLOQ was 10.2%, while the accuracy at LLOQ for Amprenavir was −5.8% and the precision for the samples at LLOQ was 18.0%. The inter-day %bias and %CV

Table 4
A summary of accuracy and precision results of low QC prepared in 12 different lots of plasma matrix by monolithic-phase on-line extraction method

| | Lot # | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | Overall |
| (I) APV | | | | | | | | | | | | | |
| Mean | 11.2 | 11.6 | 11.8 | 10.9 | 11.5 | 10.9 | 11.7 | 12.0 | 11.4 | 11.8 | 11.1 | 11.3 | 11.4 |
| %CV | 5.7 | 7.6 | 6.0 | 3.6 | 7.6 | 7.2 | 3.7 | 7.7 | 4.0 | 2.5 | 2.5 | 2.8 | 5.1 |
| %Bias | 2.1 | 5.3 | 6.9 | −1.1 | 4.2 | −0.6 | 6.4 | 9.4 | 3.9 | 7.5 | 1.2 | 2.4 | 4.0 |
| *n* | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 72 |
| (II) AZV | | | | | | | | | | | | | |
| Mean | 6.50 | 6.00 | 6.48 | 6.41 | 6.32 | 6.35 | 6.49 | 7.04 | 6.37 | 6.82 | 6.34 | 6.40 | 6.46 |
| %CV | 8.2 | 9.3 | 8.4 | 6.5 | 4.0 | 6.3 | 5.7 | 7.8 | 4.7 | 4.2 | 5.6 | 4.4 | 6.3 |
| %Bias | 1.9 | −6.7 | 0.8 | −0.4 | −1.7 | −1.3 | 0.9 | 9.5 | −1.0 | 6.0 | −1.5 | −0.5 | 0.5 |
| *n* | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 72 |

Mean values in the table are average of the back-calculated concentrations from the standard curve. Theoretical concentration of low QC was 11.0 and 6.43 ng/mL for Amprenavir and Atazanavir, respectively.

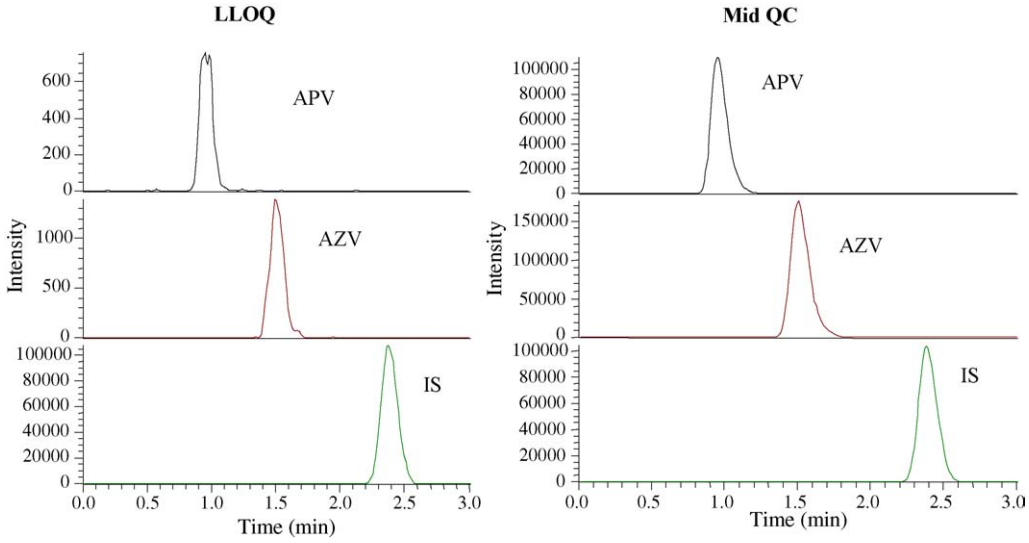



Fig. 5. Representative ion chromatograms of an LLOQ sample (4.50 ng/mL for Amprenavir and 2.77 ng/mL for Atazanavir) and a mid QC sample (550 ng/mL for Amprenavir and 322 ng/mL for Atazanavir) by liquid–liquid extraction method.

of the quality control samples of Atazanavir were ≤2.0% and ≤6.7%, respectively. The inter-day %bias and %CV of the quality control samples of Amprenavir were ≤8.5% and ≤12.0%, respectively.

The results demonstrated that reliable and reproducible data can be generated by either on-line extraction method or liquid–liquid extraction method. However, 96-well liquid–liquid extraction method requires good heat sealing of the plate to avoid contamination during nixing step. This is mainly because that organic solvent typically used for liquid–liquid extraction is not miscible with aqueous solution and intense mixing and/or inverting the sealed plated are required to achieve expected assay performance. A liquid–liquid extraction method usually needs a drying step that adds to sample preparation time. Both 96-well liquid–liquid extraction method and monolithic-phase on-line extraction approach share a common feature of being cost-effective. One noteworthy aspect of on-line extraction approach is the lack of use of volatile organic solvents such as hexane, heptane, ethyl acetate, or methyl *tert*-butyl ether, which are required in almost daily life of analytical chemists routinely performing liquid–liquid extraction methods.

Protein precipitation was typically considered a quick sample preparation method. But it is also widely considered a "crude" way for sample treatment when compared to solid-phase extraction and liquid–liquid extraction. This is because that endogenous materials and certain percentage of proteins remain after protein precipitation. Its low selectivity can induce analyte co-precipitation or mass spectrometry signal suppression [15,16]. The on-line extraction approach described here is a hybrid sample preparation technique that differentiates itself from either protein precipitation or solid-phase extraction. Besides protein precipitation, it adds another dimension of sample purification without adding much of sample preparation time because of the high speed loading, extraction, and washing on the monolithic cartridge.

In the current method, a Hamilton Lab AT 2 Plus equipped with 12 tips was programmed for accurate liquid handling to aliquot samples from individual tubes to 96-well deep well plates

Table 5
Summary of calibration curves obtained for the analysis of Amprenavir (APV) and Atazanavir (AZV) by a 96-well liquid–liquid extraction method

| | Concentration (ng/mL) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | STD 1, 4.50 | STD 2, 14.8 | STD 3, 19.3 | STD 4, 37.5 | STD 5, 123 | STD 6, 161 | STD 7, 313 | STD 8, 805 | STD 9, 1120 | STD 10, 2560 | Coefficient of determination ($r^2$) |
| (I) APV | | | | | | | | | | | |
| Mean | 4.59 | 13.7 | 18.5 | 36.2 | 120 | 157 | 307 | 799 | 1170 | 2840 | 0.9962 |
| S.D. | 0.07 | 1.28 | 0.62 | 1.80 | 2.25 | 7.63 | 13.7 | 23.1 | 34.0 | 91.4 | 0.0023 |
| %CV | 1.6 | 9.4 | 3.4 | 5.0 | 1.9 | 4.9 | 4.5 | 2.9 | 2.9 | 3.2 | 0.2 |
| %Bias | 2.1 | −7.3 | −4.0 | −3.5 | −2.8 | −2.6 | −1.9 | −0.7 | 4.7 | 10.7 | – |
| *n* | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | Concentration (ng/mL) | | | | | | | | | | |
| | STD 1, 2.77 | STD 2, 8.74 | STD 3, 11.3 | STD 4, 23.1 | STD 5, 72.8 | STD 6, 93.7 | STD 7, 192 | STD 8, 469 | STD 9, 687 | STD 10, 1520 | Coefficient of determination ($r^2$) |
| (II) AZV | | | | | | | | | | | |
| Mean | 2.76 | 8.93 | 11.1 | 23.1 | 72.2 | 93.9 | 192 | 458 | 704 | 1520 | 0.9995 |
| S.D. | 0.03 | 0.22 | 0.08 | 0.86 | 1.28 | 1.03 | 4.29 | 9.31 | 17.1 | 18.3 | 0.0001 |
| %CV | 1.1 | 2.4 | 0.8 | 3.7 | 1.8 | 1.1 | 2.2 | 2.0 | 2.4 | 1.2 | 0.0 |
| %Bias | −0.2 | 2.1 | −1.6 | −0.2 | −0.8 | 0.1 | −0.1 | −2.2 | 2.5 | 0.4 | – |
| *n* | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |

Mean values in the table are average of the back-calculated concentrations from the standard curve.

Table 6
Inter-day accuracy and precision of the LLOQ, QC, ULOQ evaluation samples for Amprenavir (APV) and Atazanavir (AZA) by liquid–liquid extraction method

| | Concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | LLOQ 4.50 | Low QC 11.0 | Mid QC 550 | High QC 2200 | ULOQ 2560 |
| (I) APV | | | | | |
| Mean | 4.24 | 10.6 | 568 | 2390 | 2750 |
| Inter-run S.D. | 0.76 | 1.27 | 39.2 | 122 | 133 |
| Inter-run %CV | 18.0 | 12.0 | 6.9 | 5.1 | 4.8 |
| Inter-run %bias | −5.8 | −3.6 | 3.3 | 8.5 | 7.2 |
| *n* | 18 | 18 | 18 | 18 | 18 |
| | Concentration (ng/mL) | | | | |
| | LLOQ 2.77 | Low QC 6.43 | Mid QC 322 | High QC 1290 | ULOQ 1520 |
| (II) AZV | | | | | |
| Mean | 2.70 | 6.51 | 328 | 1300 | 1490 |
| Inter-run S.D. | 0.27 | 0.44 | 20.4 | 77.4 | 81.9 |
| Inter-run %CV | 10.2 | 6.7 | 6.2 | 5.9 | 5.5 |
| Inter-run %bias | −2.4 | 1.2 | 2.0 | 1.4 | −1.6 |
| *n* | 18 | 18 | 18 | 18 | 18 |

Mean values in the table are average of the back-calculated concentrations from the standard curve.

and add IS. The Hamilton was also used for adding acetonitrile and dilution reagent, although such function can be equally performed well by other liquid handlers such as Tomtec.

The carry-over of the on-line extraction system was found to be extremely low. When calculated from the carry-over signals after an upper limit of quantitation sample, the percentage of the carry-over was typically at 0.002% when calculated from the peak areas. The highest carry-over observed in six consecutive runs was less than 0.006%. This is quite surprising giving the fact the on-line extraction systems typically has high carry-overs because of additional switch valve(s) and connection tubing used. In comparison, the percentage of the carry-over from the liquid–liquid extraction method is in the range of 0.01–0.03%.

Although applications using monolithic materials have been on the rise in the recent years and research efforts have demonstrated the potential of such material for bioanalytical chemistry, currently the number of commercially available monolithic columns is still limited. Further development in monolithic supports to expand the column types and column dimensions are necessary to address application needs in clinical and pharmaceutical laboratories.

## 4. Conclusion

In conclusion, a new automated approach has been developed to perform high-throughput LC–MS/MS quantitation of compounds in plasma samples. The approach combines rugged on-line high-flow extraction method based on monolithic material with the narrow-bore analytical column for efficient separation and high sensitivity. A total extraction and separation cycle time of 4 minor less can be achieved for samples containing multiple analytes in plasma. A method for determination of Amprenavir and Atazanavir in human plasma was developed with this approach. In an evaluation of over 450 plasma injections, reproducible and reliable quantitative data were obtained for multiple analytes using same monolithic extraction cartridge. Precision and accuracy of the batches performed by this approach satisfied that required for GLP bioanalysis and the results are comparable with that obtained by liquid–liquid extraction procedure. Monolithic-phase on-line extraction approach demonstrated very low carry-over, high recovery, and was matrix-independent.

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