



Comparison of extraction procedures for assessment of matrix effect for selective and reliable determination of atazanavir in human plasma by LC–ESI–MS/MS

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ABSTRACT

A comparative study with three conventional extraction techniques namely protein precipitation (PP), liquid–liquid extraction (LLE) and solid phase extraction (SPE) has been demonstrated to assess the magnitude of matrix interference by post-column analyte infusion and post extraction analyte spiking for the determination of atazanavir from human plasma. Severe ion suppression observed in PP and to a lesser extent in LLE was circumvented by SPE on LiChrosep Sequence extraction cartridge. Based on these observations a selective, rugged and high throughput SPE–LC–MS/MS method has been developed for reliable determination of atazanavir in human plasma. The chromatographic separation was achieved on a Hypersil Gold C18 (50 mm × 4.6 mm, 5 μm) analytical column using 5 mM ammonium formate in water:methanol (10:90, v/v) as the mobile phase under isocratic conditions. The method was validated over a wide dynamic concentration range of 10–6000 ng/mL. The mean relative recovery and absolute matrix effect across quality controls were 84.9 and 93.2%, respectively. The precision value for relative matrix effect between eight different lots of plasma, expressed as %CV of the slopes of the calibration lines was 2.41. The stability of atazanavir under different storage conditions varied from –8.4 to 5.4%. The method was successfully applied to a bioequivalence study of 300 mg atazanavir capsule formulation in 24 healthy Indian males under fasting condition.

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1. Introduction

The study of matrix interference in LC–ESI–MS/MS methods has become crucial as it can seriously compromise the integrity of a bioanalytical method. Matrix effect is a term used to designate suppression or enhancement in the measurement of analyte signal due to endogenous or exogenous components present in biological matrices like plasma, serum, blood, urine, tissues, cerebrospinal fluid and others. Matrix effect can directly impact the accuracy, precision, ruggedness and the overall reliability of a validated method. Assessment of matrix effect in quantitative bioanalysis for the determination of drugs has been a subject of several reports in the past decade [1–11]. The prominent causes associated with matrix interference include type of ionization source (ESI, APCI, APPI), ionization polarity, extraction procedure, and choice of internal standard, phospholipids, proteins, salts, chromatographic

conditions, mobile phase additives, buffers, ion-pairing agents, injection volume and many others. Amongst these, the use of inefficient extraction procedure to extract the drug and/or its metabolite from a biological matrix is one of the major contributors towards matrix effect. There are two common approaches to evaluate matrix interference, one is based on post-column analyte infusion [12], which gives a qualitative indication as evident from the chromatograms (suppression or enhancement) and the other way is by post extraction spiking [3], which gives a quantitative assessment by comparing the response of the analyte spiked into extracted blank matrix to the response of the analyte in neat solution. The concept of relative matrix effect, which holds high significance as proposed by Matuszewski et al. [3] gives a comparison of matrix effect values between different lots of biofluids. In another report Matuszewski [6] suggested the use of precision (%CV) of calibration line slopes obtained from different plasma sources as an indicator of relative matrix effect. He recommended that for a method to be practically free from relative matrix effect, the %CV values must not be greater than 3–4%.

Sensitive, selective and reliable determination of anti-HIV drugs in plasma is essential for studying drug–drug interaction, pharmacokinetic/pharmacodynamic properties, and therapeutic drug

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monitoring [13]. Atazanavir (ATV) is an azapeptide human immunodeficiency virus (HIV) type 1 protease inhibitor, which has played a significant role in lowering the morbidity and mortality of HIV. Its unique HIV resistance profile and favourable pharmacokinetics allows once-daily dosing. ATV is metabolized hepatically by CYP3A4 and is a strong inhibitor of this enzyme [14–16]. It is 86% bound to human serum proteins and protein binding is independent of concentration. The oral bioavailability of ATV is significantly enhanced in the presence of food. It is rapidly absorbed with a T_{\max} of ~2.5 h. It can be used alone as first line protease inhibitor or in combination therapy. ATV is available commercially under the brand name Reyataz[®] (Bristol-Myers Squibb) capsules in dose strength of 100, 150, 200 and 300 mg atazanavir sulphate [17].

Several methods are reported to quantify ATV in different biological matrices, either alone [18–24] or in combination [25–51] with several other protease inhibitors (PI), nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI), integrase inhibitor, raltegravir and an entry inhibitor, maraviroc. ATV is determined in peripheral blood mononuclear cells (PBMC) [28,31,47,48,51], cerebrospinal fluid and seminal plasma [31], serum [41], dried blood spots [42] and human plasma [24–27,29,30,32–40,43–46,49,50]. As a single analyte, ATV has been determined in PBMC [18] and human plasma [19–25]. Jemal et al. [18] have estimated ATV in PBMC by LC–MS/MS and discussed practical approaches to PBMC preparation and assay design for high-throughput analysis. Other methods based on HPLC–UV [19–23] in human plasma have a limit of quantitation ≥ 40 ng/mL. Schuster et al. [24] have developed an automated SPE–LC–MS/MS method for the determination of ATV with a sensitivity of 1.0 ng/mL, employing 0.25 mL plasma sample for processing. In these methods [19–24], plasma samples were prepared by either LLE or SPE with little emphasis on matrix interference and its assessment. ATV along with tipranavir has been estimated in human plasma by LC–MS/MS using a mixture of acetonitrile and methanol for protein precipitation [25]. A monolithic-phase based on-line extraction approach for the simultaneous determination of ATV and amprenavir in human plasma has been proposed by Xu et al. [34]. They compared the efficiency of automated 96-wells PP (with acetonitrile) and LLE (with hexane:ethyl acetate), however, similar precision and accuracy were achieved with both the methods. All other methods deal with simultaneous determination of ATV along with six or more antiretrovirals in different biological matrices by HPLC–UV [26,27,29,31–33,35,37,39,43], UPLC–diode array detection [45] and LC–MS/MS [28,30,36,38,40–42,44,46–51]. Amongst all LC–MS/MS based methods for the determination of ATV in human plasma, few studies have reported a detailed investigation of matrix effect [38,40,46,49,50]. Thus, in the present study a systematic evaluation of matrix interference was investigated by using the three conventional extraction techniques namely PP, LLE and SPE for reliable determination of atazanavir in human plasma. Based on the outcome a reliable and rugged method has been proposed for the analysis of ATV in human plasma with desired sensitivity. Ion suppression/enhancement was studied by post column infusion of analyte and post extraction spiking technique. The present method is highly selective for ATV in presence of endogenous plasma matrix components and nine other antiretroviral drugs (amprenavir, darunavir, ritonavir, lopinavir, tipranavir, saquinavir, nelfinavir, nevirapine and etravirine).

2. Experimental

2.1. Chemicals and materials

Reference standards of atazanavir (99.6%) and indinavir (IS, 99.1%) were obtained from Cadila Pharmaceuticals Ltd.

(Ahmedabad, India) and Vivan Life Sciences Pvt. Ltd. (Mumbai, India), respectively. LC–MS grade methanol, acetonitrile and ethyl acetate were procured from Mallinckrodt Baker, S.A. de C.V. (Estado de Mexico, Mexico). Ammonium formate, sodium hydroxide, acetic acid and formic acid of purity $\geq 99\%$ were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC grade dichloromethane and *n*-hexane were purchased from Merck Specialties Pvt. Ltd. (Mumbai, India). Solid phase extraction cartridges LiChrosep Sequence (30 mg, 1 cm³) were obtained from Merck (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma with K₂EDTA as anticoagulant was obtained from in house clinical laboratory of Cadila Pharmaceutical Ltd. (Ahmedabad, India) and was stored at -20°C until use.

2.2. Liquid chromatography and mass spectrometric conditions

A Waters Acquity UPLC system (Milford, MA, USA) was used for setting the reverse-phase liquid chromatographic conditions. The analysis of ATV and IS was performed on a Hypersil Gold C 18 [50 mm \times 4.6 mm (length \times inner diameter), with 5 μm particle size] from Thermo Scientific (USA) and was maintained at 35°C in column oven. The mobile phase consisted of 5 mM ammonium formate, pH 3.0 adjusted with formic acid in water:methanol (10:90, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 0.7 mL/min with 50% flow splitting. The total chromatographic run time was 2.5 min.

Ionization and detection of ATV and IS was carried out on a triple quadrupole mass spectrometer, Waters Quattro Premier XE (Milford, MA, USA), equipped with turbo ion spray interface and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor \rightarrow product ion transitions for ATV m/z 705.2 \rightarrow 167.9 and m/z 614.2 \rightarrow 421.2 for IS. The source dependent parameters maintained for ATV and IS were source temperature: 120°C ; desolvation temperature: 350°C ; cone gas flow: 50 ± 10 L/h; desolvation gas flow: 900 ± 10 L/h. The optimum values for compound dependent parameters (MRM file parameters) like collision energy and cone voltage set were 45 eV and 30 V for ATV and 42 eV and 29 V for IS, respectively. The dwell time was set at 200 ms for the analyte and IS. Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

2.3. Standard stock, calibration standards and quality control sample preparation

The standard stock solution of ATV (1 mg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with stock solution. Calibration curve standards were made at 10.0, 20.0, 50.0, 140, 400, 1000, 3000, 4800, 6000 ng/mL concentrations, respectively, while quality control samples were prepared at five concentration levels, viz. 4400 ng/mL (HQC, high quality control), 2200/300 ng/mL (MQC1/2, medium quality control), 30.0 ng/mL (LQC, low quality control) and 10.0 ng/mL (LLOQ QC, lower limit of quantification quality control). Stock solution (1 mg/mL) of the internal standard was prepared by dissolving 5.0 mg of indinavir in 5.0 mL of methanol. Its working solution (5 $\mu\text{g/mL}$) was prepared by appropriate dilution of the stock solution in methanol:water (50:50, v/v). All the solutions (standard stock, calibration standards and quality control samples) were stored at $2-8^{\circ}\text{C}$ until use.

2.4. Sample extraction protocols

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature.

2.4.1. Protein precipitation

To an aliquot of 100 μL of spiked plasma sample, 25 μL of IS (5.0 $\mu\text{g}/\text{mL}$) was added and vortexed for 10 s. Protein precipitation was carried out with 500 μL of acetonitrile/methanol/0.1% acetic acid or 0.2 M NaOH in water:acetonitrile (15:85, v/v)/0.1% acetic acid or 0.2 M NaOH in water:methanol (15:85, v/v) in polypropylene micro centrifuge vials. The vials were capped and vortexed vigorously for 1 min, followed by centrifugation at $17,949 \times g$ for 5 min at 25 °C. After centrifugation, 200 μL of the supernatant was transferred in pre-labelled autosampler vials, diluted with 200 μL Milli-Q water and briefly vortexed; 5 μL was used for injection in the chromatographic system.

2.4.2. Liquid–liquid extraction

To an aliquot of 100 μL of spiked plasma sample, 25 μL of IS (5.0 $\mu\text{g}/\text{mL}$), 50 μL , 0.1% formic acid/0.2 M NaOH was added and vortexed for 10 s. Extraction of analyte and IS was done in 2.0 mL of dichloromethane/ethylacetate:*n*-hexane (50:50, v/v) solvent mixture on a rotary mixer (rotospin) for 5 min at $32 \times g$. Centrifugation of the samples was done at $3204 \times g$ for 5 min at 10 °C. The organic layer (1.5 mL) was separated and evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under a gentle stream of nitrogen. The dried samples were reconstituted with 1000 μL of mobile phase, vortexed to mix for 10 s and 5 μL was used for injection in the chromatographic system.

2.4.3. Solid phase extraction

To an aliquot of 50 μL of spiked plasma sample, 25 μL of IS (5.0 $\mu\text{g}/\text{mL}$) was added and vortexed for 10 s. Further, 300 μL of Milli-Q water was added and vortex mixed for another 10 s. Centrifugation of the samples was done at $3200 \times g$ for 2 min at 10 °C. The samples were loaded on LiChrosep Sequence (1 cm^3 , 30 mg) extraction cartridges which were preconditioned with 1 mL of methanol followed by 1 mL of water. Washing of samples was done by 1 mL with Milli Q water twice. After the washing step, the cartridges were dried for 1 min by applying nitrogen (1.72×10^5 Pa) at 2.4 L/min flow rate. Elution of analyte and IS was done using 1.0 mL of mobile phase into pre-labelled vials. The contents were vortexed to mix for 15 s and 5 μL was used for injection in the chromatographic system.

2.5. Method validation procedures

The bioanalytical method was fully validated following the USFDA guidelines [52]. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of ATV (2200 ng/mL) and IS (5.0 $\mu\text{g}/\text{mL}$) at the start of each batch during method validation. System performance was studied by injecting one extracted blank (without drug and IS) and one ULOQ and LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. Carry over effect of auto sampler was checked to verify any carryover of analyte at the start and at the end of each batch. The design of the experiment comprised of the following sequence of injections viz. extracted blank plasma \rightarrow ULOQ sample \rightarrow extracted blank plasma \rightarrow LLOQ sample \rightarrow extracted blank plasma.

The selectivity of the method towards endogenous plasma matrix components was assessed in ten different batches of plasma (seven normal K_2EDTA plasma and one each of

lipidemic, haemolyzed and heparinized plasma). The selectivity of the method towards commonly used medications in human volunteers was done for acetaminophene, cetirizine, domperidone, ranitidine, diclofenac and ibuprofen in six different batches of plasma having K_2EDTA as anticoagulant. The effect of potential concomitant antiretroviral drugs namely amprenavir (APV, 506.2/156.1), darunavir (DRV, 548.3/392.0), ritonavir (RTV, 721.3/296.2), lopinavir (LPV, 629.3/447.4), tipranavir (TPV, 603.0/172.2), saquinavir (SQV, 671.2/225.1), nelfinavir (NFV, 568.1/330.2), nevirapine (NVP, 267.1/225.9) and etravirine (ETV, 435.0/303.9) was studied for ionization (ion suppression/enhancement), analytical recovery (precision and accuracy) and chromatographic interference (interference with MRM of ATV and IS). Working solutions (200 ng/mL) of each drug were prepared in methanol and water (95:5, v/v) and analyzed at LQC and HQC levels in triplicate. These sets were processed along with freshly processed calibration curve standards (CS) and two sets (8 samples) of qualifying QC samples (HQC, MQC-1, MQC-2 and LQC). As per the acceptance criteria, the % accuracy should be within 85–115%.

The linearity of the method was determined by analysis of five linearity curves containing nine non-zero concentrations. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. A correlation coefficient (r^2) value > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least ten times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) not greater than 20% and accuracy within 80–120%. The deviation of standards other than LLOQ from the nominal concentration should not be more than $\pm 15\%$.

For determining the intra-batch accuracy and precision, replicate analysis of plasma samples of atazanavir was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC2, MQC1 and HQC samples. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The precision (%CV) at each concentration level from the nominal concentration should not be greater than 15%. Similarly, the mean accuracy should be within 85–115%, except for the LLOQ where it can be from 80 to 20% of the nominal concentration. Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment [4,12]. A standard solution containing ATV (at ULOQ level) and IS was infused post column via a ‘T’ connector into the mobile phase at 10 $\mu\text{L}/\text{min}$ employing infusion pump. Aliquots of 5 μL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS/MS chromatograms were acquired for ATV and IS. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of ATV or IS indicates ion enhancement.

The relative recovery, absolute matrix effect and process efficiency were assessed as recommended by Matuszewski et al. [3]. All three parameters were evaluated at HQC, MQC1, MQC2 and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. The overall ‘process efficiency’ (%PE) was calculated as $(\text{ME} \times \text{RE})/100$. Further, the effect of plasma matrix (relative matrix effect) on analyte quantification was also checked in eight different plasma lots. To investigate this matrix effect, calibration curves consisting of nine non-zero

concentrations were constructed and the precision (%CV) values for slopes were calculated. For a method to be practically free from relative matrix effect the %CV should not exceed 3–4% [6]. Further, the deviation of the back calculated concentrations should be within 85–115% of the nominal concentrations.

All stability results were evaluated by measuring the area ratio response (ATV/IS) of stability samples against freshly prepared comparison standards with identical concentration. Stock solutions of ATV and IS were checked for short term stability at room temperature and long term stability at 5 °C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler stability (wet extract), bench top (at room temperature) and freeze–thaw stability were performed at LQC and HQC using six replicates at each level. Freeze–thaw stability was evaluated by successive cycles of freezing (at –20 °C and –70 °C) and thawing (without warming) at room temperature. Long term stability of spiked plasma samples stored at –20 °C and –70 °C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

To authenticate the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed by different analyst while the second batch was studied on two different columns. Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at 10,000 ng/mL ATV concentration in the screened plasma. The precision and accuracy for dilution integrity standards at 1/2 (5000 ng/mL) and 1/10th (1000 ng/mL) dilution were determined by analyzing the samples against calibration curve standards.

2.6. Bioequivalence study design and incurred sample reanalysis

The design of the study comprised of “A randomized, open-label, balanced, two-treatment, two-period, two-sequence, single dose, two-way crossover bioequivalence study of test (300 mg from an Indian Pharmaceuticals Company, India) and a reference (REYATAZ[®], 300 mg capsule from Bristol Myers Squibb, Princeton, NJ, USA) formulation of atazanavir sulphate in 24 healthy, adult (18–45 years) Indian subjects under fasting condition”. Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [53]. The subjects were fasted 10 h before administration of the drug formulation. They were orally administered a single dose of test and reference formulations after recommended wash out period of 10 days with 200 mL of water. Blood samples were collected at pre-dose (0.00), 0.33, 0.67, 1.0, 1.33, 1.67, 2.0, 2.25, 2.50, 3.0, 3.25, 3.5, 4.0, 4.25, 4.5, 5.0, 6.0, 8.0, 10, 12, 16, 24, 36 h after oral administration of the dose for test and reference formulation in labelled K₂EDTA–vacutainers. Plasma was separated by centrifugation and kept frozen at –20 °C till the completion of period and then at –70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. An incurred sample re-analysis (assay reproducibility test) was also conducted by computerized random selection of subject samples, 10% of total samples analyzed. The selection criteria included samples, which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the

same sample using the same procedure. The percent change in the value should not be more than ±20% [54].

$$\% \text{Change} = \frac{\text{repeat value} - \text{initial value}}{\text{mean of repeat and initial values}} \times 100$$

2.7. Statistical analysis

The pharmacokinetic parameters of ATV were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA). The C_{max} values and the time to reach maximum plasma concentration (T_{max}) were estimated directly from the observed plasma concentration vs. time data. The area under the plasma concentration–time curve from time 0 to 36 h (AUC_{0–36}) was calculated using the linear trapezoidal rule. The AUC_{0–inf} was calculated as: AUC_{0–inf} = AUC_{0–36} + C_t/K_{el}, where C_t is the last plasma concentration measured and K_{el} is the elimination rate constant; K_{el} was determined using linear regression analysis of the logarithm linear part of the plasma concentration–time curve. The t_{1/2} of ATV was calculated as: t_{1/2} = ln 2/K_{el}. To determine whether the test and reference formulations were pharmacokinetically equivalent, C_{max}, AUC_{0–36}, and AUC_{0–inf} and their ratios (test/reference) using log transformed data were assessed; their means and 90% CIs were analyzed by using SAS[®] software version 9.1.3 (SAS Institute Inc., Cary, NC, USA). The formulations were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant (P ≥ 0.05) and the 90% confidence intervals (CI) for these parameters fell within 80–125%.

3. Results and discussion

3.1. Method development

To develop a selective, rugged and a reliable method for the estimation of ATV in human plasma, the three commonly used extraction procedures were systematically investigated. The chromatographic and mass spectrometric conditions were suitably optimized to get the desired sensitivity, selectively and a good linearity in regression curves.

3.2. Mass spectrometry

The present study was conducted using positive ion electrospray ionization (ESI) mode for MRM LC–MS/MS analyses as ATV and indinavir both have secondary amino groups. Q1 MS full scan spectra for ATV and IS predominantly contained protonated precursor [M+H]⁺ ions at m/z 705.2 and 614.2, respectively. The most abundant and consistent product ions in Q3 MS spectra for ATV and IS were observed at m/z 167.9 and 421.2 by applying collision energy of 45 and 42 eV, respectively (Fig. 1). The product ion fragment of ATV at m/z 167.9 corresponded to the elimination of 4-(pyridin-2-yl)phenylmethyl group from the parent moiety. For indinavir the stable product ion fragment at m/z 421.2 was obtained by elimination of pyridine-3-yl methyl and tert-butyl carboxamide groups from the precursor ion. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response for the analyte. A dwell time of 200 ms for ATV and IS was adequate and no interference was observed between their MRMs.

3.3. Optimization of extraction technique

Reported procedures for the estimation of ATV (as single analyte) in human plasma have used either LLE [21,23] or SPE

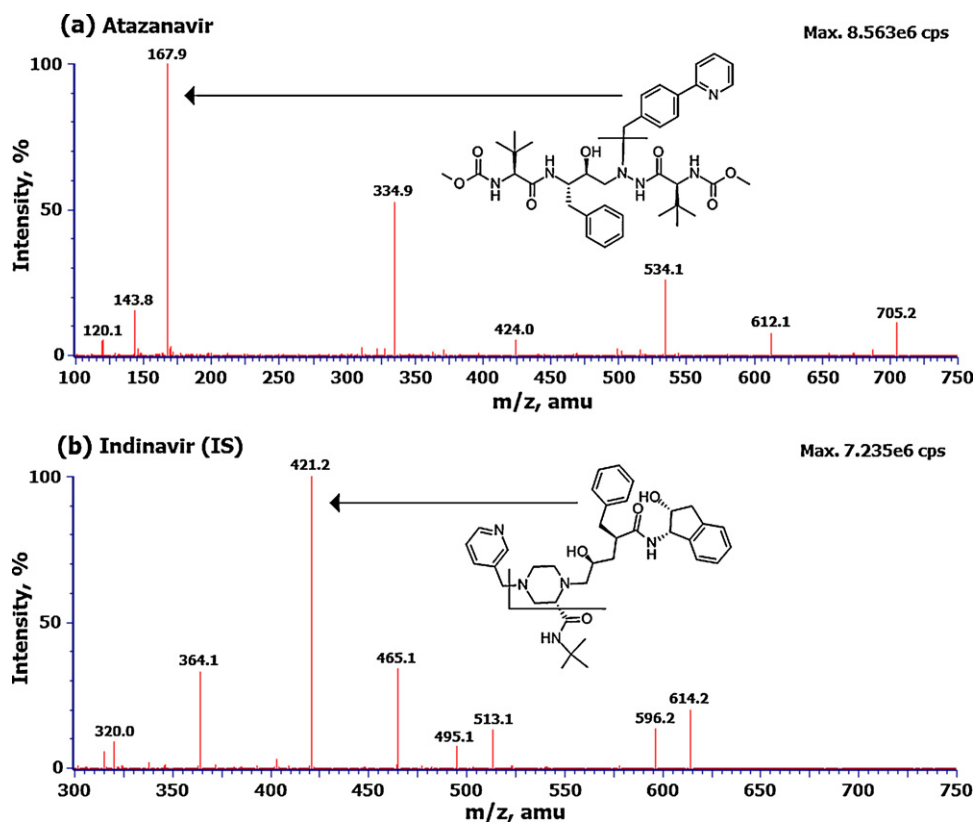


Fig. 1. Product ion mass spectra of (a) atazanavir (m/z 705.2 \rightarrow 167.9, scan range 100–750 amu) and (b) internal standard, indinavir (m/z 614.2 \rightarrow 421.2, scan range 300–750 amu) in positive ionization mode.

[19,20,22,24] for sample preparation with little or no information on ion suppression or matrix interference. D'Avolio et al. [38] have used a mixture of methanol:acetonitrile (50:50, v/v) as precipitating agent for the simultaneous determination of ATV along with 11 other antiretroviral agents. Similarly, a combination of methanol and zinc sulphate was used for protein precipitation followed by on-line solid phase extraction for the simultaneous quantification of ATV and ten other antiretrovirals [46]. They investigated the matrix effect by the post column infusion method and by the measurement of precision of standard line slopes. Based on the selectivity, matrix effect, analytical recovery and reproducibility requirements, all three conventional extraction procedures were studied. PP was with acetonitrile/methanol along with 0.1% acetic acid/0.2 M NaOH, LLE with dichloromethane and

ethyl acetate:*n*-hexane mixture along with 0.1% formic acid/0.2 M NaOH, and SPE with LiChrosep Sequence extraction cartridges were initiated during method development. In protein precipitation, severe perturbations in the response were seen with all the precipitating agents as evident by post column analyte infusion experiment. A massive ion suppression was observed at the retention time of ATV and IS and also between 0.5 and 0.8 min using acetonitrile as precipitating agent as shown in Fig. 2a. Additionally, significant enhancement was also observed in the region of 0.7–0.8 min. Replacing acetonitrile with methanol resulted in considerable improvement in the response (Fig. 2b). Addition of 0.1% acetic acid with both the solvents resulted in a marginal decrease in ion suppression. Thus, LLE was tried with solvents reported previously for estimation of ATV in human plasma [21,23].

Table 1
Mean relative recovery and absolute matrix effect of atazanavir using protein precipitation and liquid–liquid extraction under different extraction conditions at LQC level.

Protein precipitation			Liquid–liquid extraction		
Extraction conditions	Relative recovery (%)	Absolute matrix effect (%)	Extraction conditions	Relative recovery (%)	Absolute matrix effect (%)
Acetonitrile	24.3	33.5	Dichloromethane	48.7	63.6
Acetonitrile:0.1% acetic acid in water (85:15, v/v)	25.2	35.7	Dichloromethane in presence of 0.1% formic acid	47.3	62.9
Acetonitrile:0.2 M NaOH in water (85:15, v/v)	28.2	38.3	Dichloromethane in presence of 0.2 M NaOH	52.4	68.7
Methanol	28.7	39.5	Ethyl acetate: <i>n</i> -hexane (50:50, v/v)	50.1	61.3
Methanol:0.1% acetic acid in water (85:15, v/v)	29.7	39.3	Ethyl acetate: <i>n</i> -hexane (50:50, v/v) in presence of 0.1% formic acid	51.3	62.5
Methanol: 0.2 M NaOH in water (85:15, v/v)	32.5	41.6	Ethyl acetate: <i>n</i> -hexane (50:50, v/v) in presence of 0.2 M NaOH	54.5	70.6

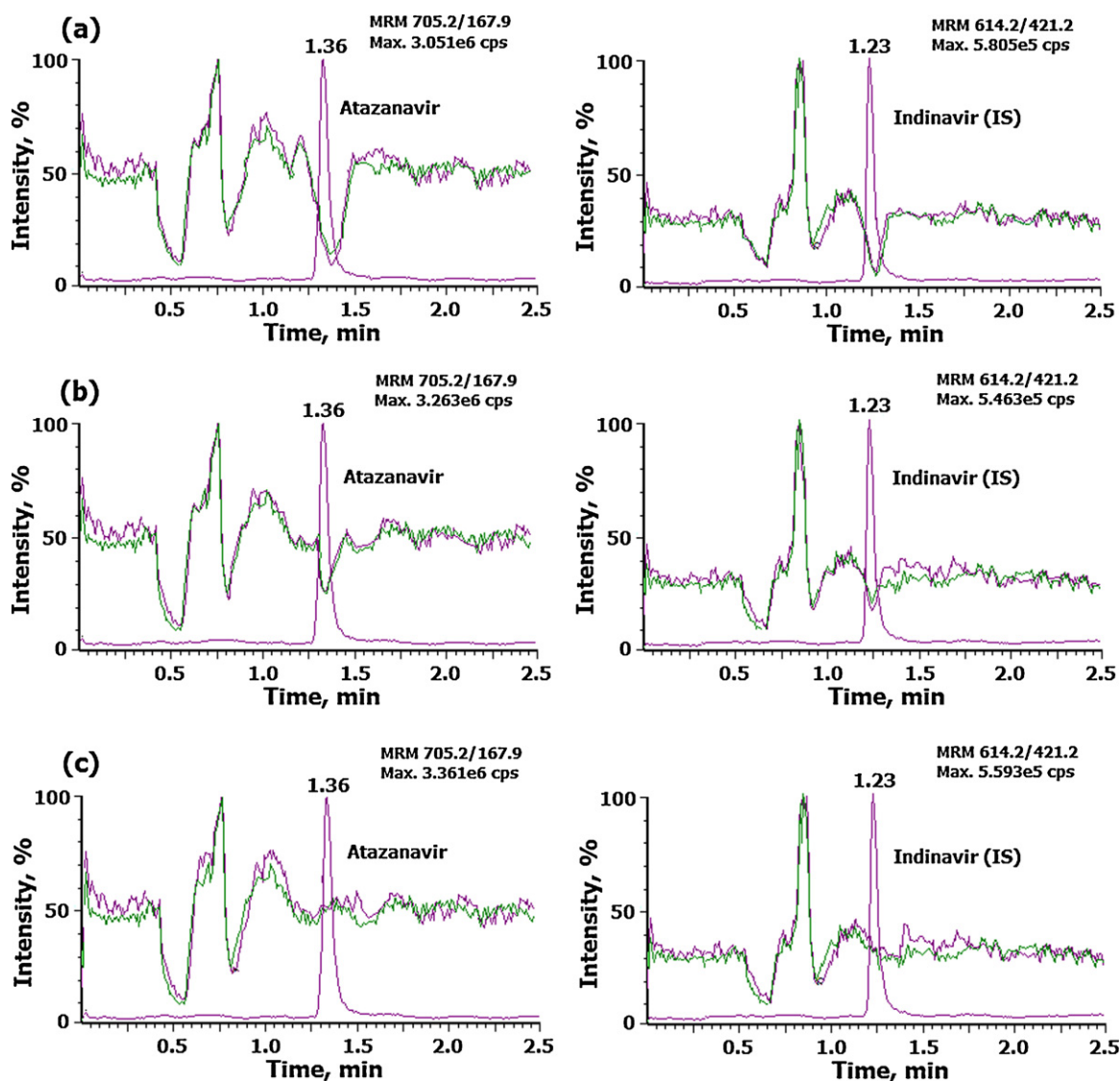


Fig. 2. Post column infusion LC–MS/MS chromatograms of two blank plasma extracts from (a) PP with acetonitrile, (b) PP with methanol and (c) LLE with dichloromethane with overlaid chromatograms of atazanavir at ULOQ level and indinavir (IS).

Though considerable improvement was observed at the retention time of ATV and IS, nevertheless, severe ion suppression and enhancement was still present between 0.5 and 0.8 min for both the solvents systems. Fig. 2c shows post-column infusion chromatograms for LLE with dichloromethane. Similar consequences were seen in the chromatograms for ethyl acetate:*n*-hexane mixture and also under acidic and alkaline conditions. Quantitative assessment of matrix effect was also done by post extraction spiking as recommended by Matuszewski et al. [3] and the results are shown in Table 1 for all relevant experiments. This can be related to the amount of non-volatile substances present with the analyte of interest in different extraction procedures. King et al. [4] have calculated the amount of non-volatile materials left after solvent evaporation in these three extraction procedures. The most severe ion-suppression was observed in protein precipitation with acetonitrile (3.35 mg), followed by LLE and SPE (0.2–0.3 mg). The results obtained in the present investigation are in good agreement with their findings. Thus SPE was initiated with LiChrosep Sequence extraction cartridges using 50 μ L plasma volume for sample preparation. Quantitative and precise recoveries were obtained at all QC levels, with practically negligible matrix interference. Results of post-column infusion experiment in Fig. 3 indicate no ion

suppression or enhancement at the retention time of ATV and IS. Though there was some ion enhancement around 0.5 min, but it did not interfere in the quantitation of ATV.

3.4. Optimization of chromatographic conditions

To have a rugged and efficient chromatography, efforts were made to minimize matrix interference, achieve short run time in order to ensure high throughput and attain high sensitivity with good peak shapes. The analytical potential of four different reversed-phase columns was evaluated namely Zorbax Eclipse XDB C18 (100 mm \times 4.6 mm, 5 μ m, pore size 80), Hypurity C18 (50 mm \times 2.1 mm, 5 μ m), Luna C18 (100 mm \times 4.6 mm, 5 μ m) and Hypersil Gold C18 (50 mm \times 4.6 mm, 5 μ m) for extracted samples. Separation was tried using various combinations of methanol/acetonitrile in acidic buffer (2–20 mM ammonium formate) and additives like formic acid (0.01–0.1%) on these columns to find the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. There was adequate retention on Luna C18 column, however the peaks were very broad probably due to very high carbon loading (19%), while noticeable tailing was observed on Zorbax Eclipse C18 column. Hypurity

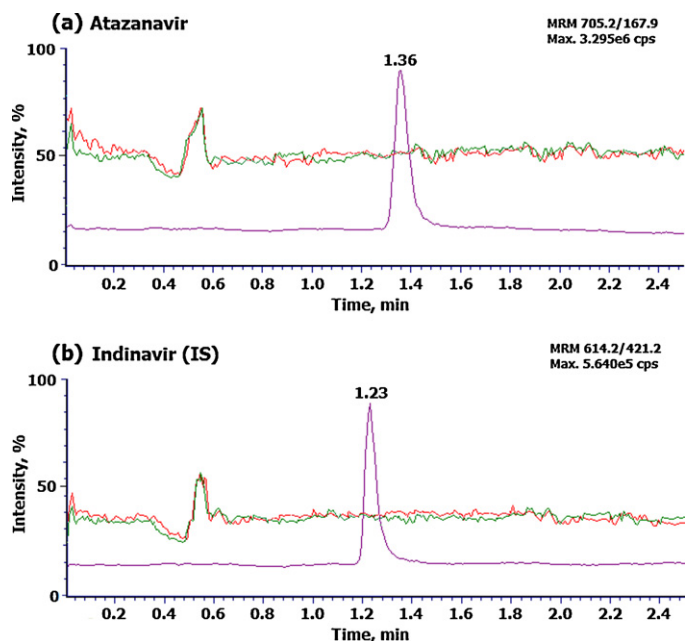


Fig. 3. Post column infusion LC-MS/MS chromatograms of two blank plasma extracts from SPE with overlaid chromatograms of atazanavir and indinavir (IS).

C18 offered better peak shape but the drug was not adequately retained on the column and was eluted at 0.62 min as shown in Fig. 4a–c. Nevertheless in the present work, the best chromatographic conditions as a function of analyte peak intensity, peak shape, adequate retention and analysis time was achieved with Hypersil Gold C18 (50 mm × 4.6 mm, 5 μm) as evident from Fig. 4d, using 5 mM ammonium formate, pH 3.0 adjusted with formic acid in water:methanol (10:90, v/v) as the mobile phase under isocratic conditions. The total chromatographic run time was 2.5 min with a retention time of 1.36 min for ATV, was the shortest compared to previous assays [18–24]. Further, the reproducibility of retention time for extracted ATV samples, expressed as %CV was ≤1.2% for 100 injections on the same column. The sensitivity achieved for ATV (as single analyte) in the present work was 10.0 ng/mL, which is greater compared to other methods reported in human plasma [19–23], except the work of Schuster et al. [24]. Based on the selectivity (unperturbed and stable base line) and signal to noise ratio ($S/N \geq 40$), it was possible to further lower the LLOQ by about four folds, however, it was not required based on the results of subject samples. Representative MRM ion chromatograms in Fig. 5 of extracted blank human plasma (double blank), blank plasma fortified with IS (m/z 614.2 → 421.2), ATV at LLOQ (m/z 705.2 → 167.9) and an actual subject sample at 2 h demonstrates the selectivity of the method. The chromatograms showed acceptable peak shape for the analyte and IS. None of the concomitant antiretroviral drugs studied or the commonly used medications by human volunteers interfered in the determination of ATV. The retention times obtained for the antiretroviral drugs

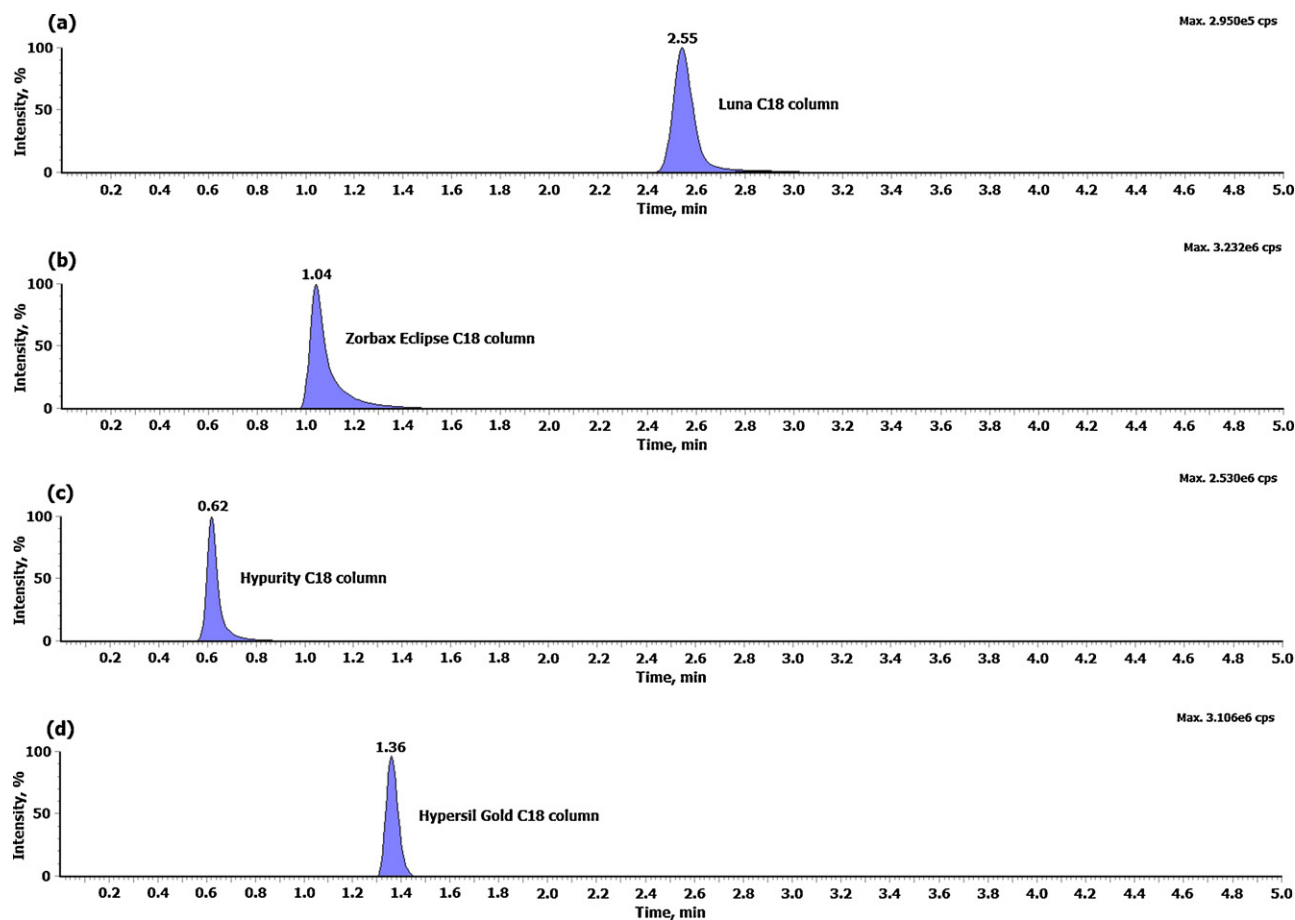


Fig. 4. Chromatograms of atazanavir (m/z 705.2 → 167.9) at ULOQ level obtained on (a) Luna C18 (100 mm × 4.6 mm, 5 μm), (b) Zorbax Eclipse XDB C18 (100 mm × 4.6 mm, 5 μm, pore size 80) and (c) Hypurity C18 (50 mm × 2.1 mm, 5 μm) (d) Hypersil Gold C18 (50 mm × 4.6 mm, 5 μm) analytical columns. Mobile phase: 5 mM ammonium formate, pH 3.0 adjusted with formic acid in water: methanol (10:90, v/v); flow rate: 0.7 mL/min.

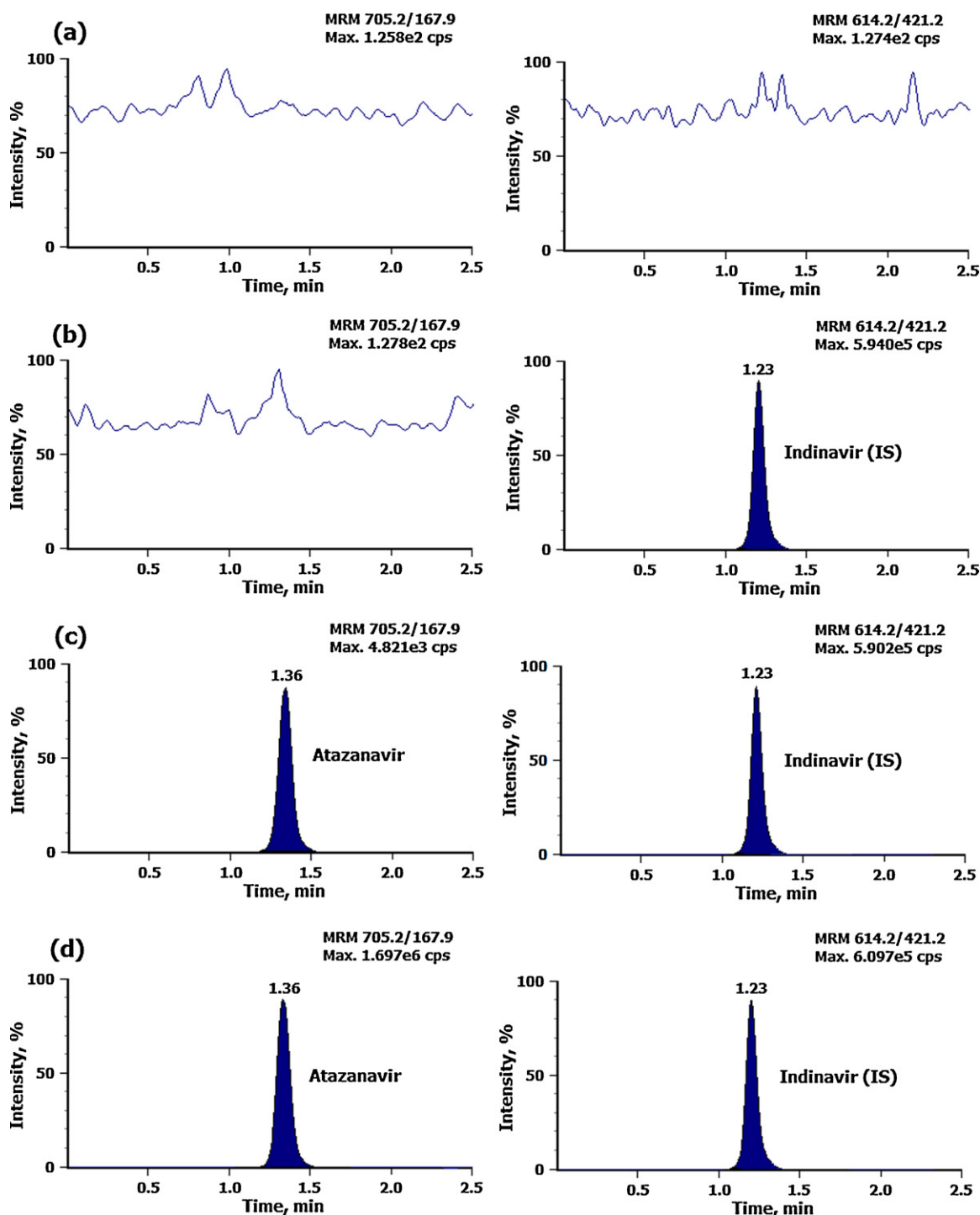


Fig. 5. MRM ion-chromatograms of (a) double blank plasma (without IS), (b) blank plasma with indinavir (IS), (c) atazanavir at LLOQ (m/z 705.2 \rightarrow 167.9) and IS (d) real subject sample at 2.0 h after administration of 300 mg atazanavir sulphate capsule.

under the optimized experimental conditions were APV (1.41 min), DRV (1.42 min), RTV (1.93 min), LPV (1.97 min), TPV (2.12 min), SQV (1.29), NFV (1.27 min), NVP (1.12 min) and ETV (2.35 min). However, due to their different MRM transitions there was no interference in the quantification of ATV. The % accuracy results were within 95.3–104.6% at both the QC levels. The average matrix factor value calculated as the response of post spiked sample/response of neat solution in mobile phase at the LLOQ level was 0.97, which indicates a minor suppression of 3%.

Ideally, a deuterated analogue should be the first-choice internal standard, but due to its unavailability, a general IS was used to minimize analytical variation due to solvent evaporation, integrity of the column and ionization efficiency. Indinavir, which belongs to the same class of protease inhibitors but not co-formulated with ATV was selected as an internal standard in the present work. It had similar chromatographic behaviour and was easily separated and eluted along with the analyte. There was no effect of IS on analyte recovery, sensitivity or ion suppression.

Table 2
Intra-batch and inter-batch accuracy and precision for atazanavir using solid phase extraction.

QC ID	Nominal conc. (ng/mL)	Intra-batch				Inter-batch			
		n	Mean conc. found (ng/mL) ^a	Accuracy (%)	CV (%)	n	Mean Conc. found (ng/mL) ^b	Accuracy (%)	CV (%)
HQC	4400	6	4545	103.3	4.8	30	4518	102.7	3.9
MQC1	2200	6	2228	101.3	5.9	30	2251	102.3	5.6
MQC2	300	6	309	103.0	3.6	30	313	104.3	4.2
LQC	30.0	6	30.8	102.6	5.7	30	31.1	103.6	6.0
LLOQ QC	10.0	6	9.45	94.5	5.1	30	9.76	97.6	5.5

n, total number of observations; CV, coefficient of variation.

^a Mean of six replicates at each concentration.

^b Mean of six replicates for five precision and accuracy batches.

Table 3
Absolute matrix effect, relative recovery and process efficiency for atazanavir using solid phase extraction.

A (%CV)	B (%CV)	C (%CV)	Absolute matrix effect, % ME (B/A) × 100	Relative recovery, % RE (C/B) × 100	Process efficiency, % PE (C/A) × 100
HQC 423, 146(2.2)	400,334 (5.7)	335,080(5.2)	94.6 (96.3) ^a	83.7 (82.1) ^a	79.2 (79.1) ^a
MQC1 218, 225(2.3)	200,464 (5.8)	169,943(3.3)	91.8 (94.6) ^a	84.8 (85.2) ^a	77.8 (80.6) ^a
MQC2 29, 507(1.1)	27,589 (5.5)	23,862(4.5)	93.5 (92.2) ^a	86.5 (83.6) ^a	80.8 (77.1) ^a
LQC 2975(3.0)	2766 (5.7)	2339(4.7)	92.8 (93.5) ^a	84.6 (83.8) ^a	78.7 (78.4) ^a

A, mean area response of six replicate samples for atazanavir prepared in mobile phase (neat samples); B, mean area response of six replicate samples for atazanavir prepared by spiking in extracted blank plasma; C, mean area response of six replicate samples for atazanavir prepared by spiking before extraction; and CV, coefficient of variation.

^a Values for internal standard, indinavir.

3.5. Assay performance and validation

Throughout the method validation, the precision (%CV) of system suitability test was observed in the range of 0.01–0.26% for the retention time and 0.97–1.20% for the area response of ATV and IS, while the signal to noise ratio for system performance was ≥ 40 for analyte and IS. Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was practically negligible carry-over ($\leq 0.02\%$) during auto-sampler carryover experiment as shown in Fig. 6. No enhancement in the response was observed in double blank (without analyte and IS) after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of ATV and IS, respectively.

All five calibration curves were linear over the concentration range of 10–6000 ng/mL for ATV. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. The mean linear equation was $y = (0.0007 \pm 0.00001)x - (0.0007 \pm 0.00003)$, where y is the peak area ratio of the analyte/IS and x the concentration of the analyte. The mean and standard deviation value for correlation coefficient (r^2) observed were 0.9982 and 0.0006, respectively. The accuracy and precision (%CV) for the calibration curve standards ranged from 94.7 to 105.6% and 0.2 to 3.5%, respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 10.0 ng/mL in plasma at a signal-to-noise ratio (S/N) of ≥ 40 .

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC-2, MQC-1, LQC and LLOQ QC levels (Table 2). The intra-batch precision (%CV) ranged from 3.6 to 5.9% and the accuracy was within 94.5 to 103.3%. For the inter-batch experiments, the precision varied from 3.9 to 6.0% and the accuracy was within 97.6 to 104.3%.

The relative recovery, absolute matrix effect and process efficiency data for ATV and IS is presented in Table 3. The relative recovery of the analyte is the 'true recovery', which is unaffected by

the matrix as it is calculated by comparing the area ratio response (analyte/IS) of extracted (spiked before extraction) and unextracted (spiked after extraction) samples. The relative recovery and process efficiency obtained for ATV and IS was $\geq 83.7\%$ and $>79\%$, respectively, and was consistent at all QC levels. The coefficient of variation (%CV) of the slopes of the calibration lines for relative matrix effect in eight different plasma lots did not exceed 2.41% (Table 4).

The stability of ATV in human plasma and stock solutions was examined under different storage conditions, while the stability of IS as checked in stock and working solutions. Samples for short-term stability remained stable upto 28 h, while the stock solutions of ATV and IS were stable for minimum of 72 days at refrigerated temperature of 5 °C. ATV in control human plasma (bench top) at room temperature was stable for at least 8 h at 25 °C and for minimum of five freeze and thaw cycles at –20 °C and –70 °C. Spiked plasma samples stored at –20 °C and –70 °C for long term stability experiment were found stable for a minimum period of 72 days. Autosampler stability (wet extract) of the spiked quality control samples maintained at 5 °C was determined up to 69 h without

Table 4
Relative matrix effect in eight different lots of human plasma for atazanavir using solid phase extraction.

Plasma lot	Slope of calibration curve
Lot-1	0.00072
Lot-2	0.00068
Lot-3	0.00070
Lot-4	0.00071
Lot-5	0.00069
Lot-6 (heparinized)	0.00067
Lot-7 (haemolyzed)	0.00068
Lot-8 (lipemic)	0.00069
Mean	0.00069
±SD	0.000017
%CV	2.41

CV: coefficient of variation

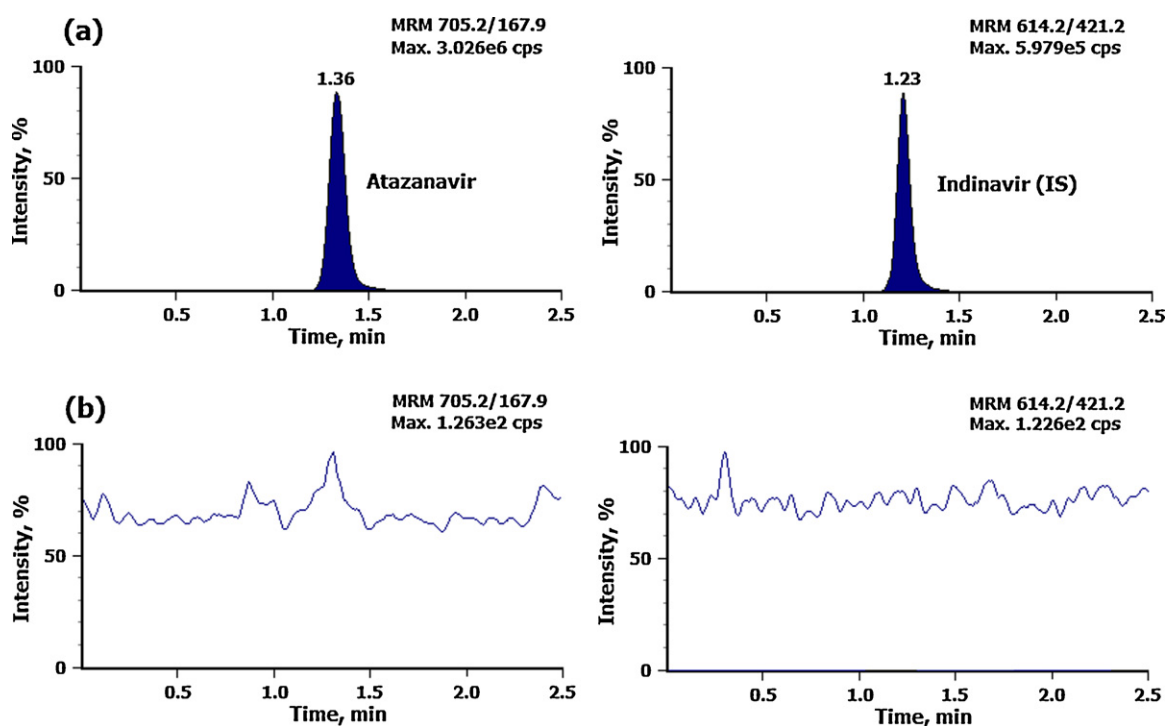


Fig. 6. MRM ion-chromatograms for carry over test of atazanavir (m/z 705.2 \rightarrow 167.9) and indinavir (IS, m/z 614.2 \rightarrow 421.2). (a) atazanavir at ULOQ and IS (b) double blank plasma (without analyte and IS).

significant drug loss. Different stability experiments in plasma at two QC levels; with the values for percent change is shown in Table 5.

Method ruggedness was evaluated using re-injection of analyzed samples on two different Hypersil Gold C18 (50 mm \times 4.6 mm, 5 μ m) columns having batch no. 0607310A and 0383075U, respectively, and also with different analysts. The precision (%CV) and accuracy values for two different columns ranged from 2.9 to 6.3% and 97.1 to 103.0%, respectively, at all four quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 2.7–5.3% and 101.1–104.3%, respectively, at these levels. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision for dilution integrity of 1/2 and 1/10th dilution were 2.4 and 0.7%, while the

accuracy results were 96.4% (4820 ng/mL) and 105.4% (1054 ng/mL), respectively, which is well within the acceptance limit of 15% for precision (%CV) and 85–115% for accuracy.

3.6. Application to a bioequivalence study and incurred sample reanalysis

The validated method has been successfully used to quantify ATV concentration in human plasma samples after administration of a single 300 mg dose of test and reference formulation of atazanavir sulphate. Fig. 7 shows the plasma concentration vs. time profile of atazanavir in human subjects under fasting condition. The method was sensitive enough to monitor the atazanavir plasma concentration up to 36 h. In all approximately 2000 samples including the calibration, QC, volunteer samples and ISR samples were run and analyzed during a period of 5 days and the precision and accuracy were well within the acceptable limits. The mean

Table 5

Stability results for atazanavir under different conditions ($n=6$).

Storage condition	Level	Mean stability sample (ng/mL) \pm SD	% Change
Bench top stability; 8 h	HQC	4282 \pm 318.5	-2.7
	LQC	31.4 \pm 3.3	4.8
Wet extract stability; 69 h	HQC	4410 \pm 182.3	0.2
	LQC	31.6 \pm 3.6	5.4
Freeze and thaw stability; 5 cycles, -20 $^{\circ}$ C	HQC	4212 \pm 160.3	-4.3
	LQC	29.1 \pm 1.3	-2.8
Freeze and thaw stability; 5 Cycles; -70 $^{\circ}$ C	HQC	4077 \pm 118.4	-7.3
	LQC	28.3 \pm 0.8	-5.8
Long term stability in plasma; 72 days, -20 $^{\circ}$ C	HQC	4030 \pm 68.2	-8.4
	LQC	27.7 \pm 1.6	-7.7
Long term stability in plasma; 72 days, -70 $^{\circ}$ C	HQC	4120 \pm 263.0	-6.4
	LQC	28.5 \pm 1.2	-5.0

SD, standard deviation.

n , number of replicates at each level, %Change = $\frac{\text{mean stability samples} - \text{mean comparison samples}}{\text{mean comparison samples}} \times 100$

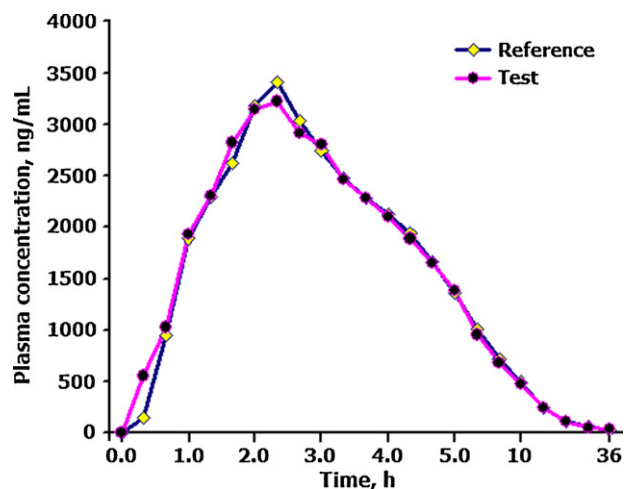


Fig. 7. Mean plasma concentration–time profile of atazanavir after oral administration of test (300 mg atazanavir sulphate capsules of an Indian Company) and a reference (REYATAZ[®], 300 mg atazanavir sulphate capsules from Bristol-Myers Squibb, Princeton, NJ, USA) formulation to 24 healthy Indian subjects fasting conditions.

pharmacokinetic parameters obtained for the test and reference formulation are presented in Table 6. These results can be compared with previous work on atazanavir (400 mg) monotherapy in healthy volunteers [23,55]. The C_{max} and AUC_{0-t} values obtained in the present work were lower compared to both these reports. This variation can be attributed to the dose strength, genetic difference, gender type (body size and muscle mass), type of food etc. However, T_{max} and $t_{1/2}$ values were comparable with the work of Martin et al. [55]. The 90% confidence interval of individual ratio geometric mean for test/reference was within 99–104% for AUC_{0-t} , AUC_{0-inf} and C_{max} under fasting conditions. Further, there was no adverse event during the course of the study.

Incurred sample reanalysis (ISR) study has now become an essential part of the bioanalytical process to assess the quality of bioanalytical assays. It reaffirms the reproducibility and reliability of a validated bioanalytical method. This was done by random selection of subject samples (10% of total samples analyzed). Out of 105 incurred samples studied, 62 samples showed %change for assay reproducibility within $\pm 5\%$, while the remaining 43 samples were within $\pm 15\%$ as shown in Fig. 8. This authenticates the reproducibility of the proposed method.

Table 6

Mean pharmacokinetic parameters following oral administration of 300 mg test and reference tablet formulation of atazanavir in 24 healthy Indian subjects under fasting condition.

Parameter	Test Mean \pm SD	Ref. Mean \pm SD
C_{max} (ng/mL)	3525 \pm 259	3526 \pm 219
T_{max} (h)	2.115 \pm 0.228	2.174 \pm 0.222
$t_{1/2}$ (h)	9.194 \pm 3.028	9.267 \pm 3.382
AUC_{0-36h} (h ng/mL)	21,346 \pm 5930	20,814 \pm 6287
AUC_{0-inf} (h ng/mL)	21,869 \pm 6175	21,289 \pm 6504
K_{el} (1/h)	0.075 \pm 0.002	0.074 \pm 0.003

C_{max} , maximum plasma concentration.

T_{max} , time point of maximum plasma concentration.

$t_{1/2}$, half life of drug elimination during the terminal phase.

AUC_{0-t} , area under the plasma concentration–time curve from zero hour to 36 h.

AUC_{0-inf} , area under the plasma concentration–time curve from zero hour to infinity.

K_{el} , elimination rate constant.

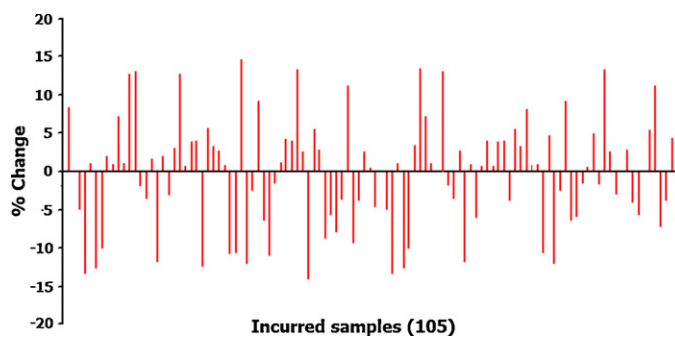


Fig. 8. Graphical representation of results for 105 incurred samples of atazanavir.

3.7. Comparison with reported methods

Although previous methods [38,40,46,49,50] have reported studies on matrix effect, however, there are no reports on systematic evaluation of magnitude of matrix interference under different extraction conditions for ATV. In the present work all three conventional extraction procedures are discussed extensively and an optimized SPE method has been proposed with minimum matrix interference for determination of ATV. The method presented has the highest sensitivity compared to majority of the methods developed for ATV alone [19–23] and in combination with other antiretroviral drugs [25,26,29,30,35,37–40,46,49] in human plasma, except few methods which have a sensitivity less than 10 ng/mL [24,32,34]. The plasma volume for samples preparation is only 50 μ L, which is considerably less than all other methods except the work of D'Avolio et al. [38], which uses a similar volume. Moreover, the total analysis time (extraction and chromatography) is the shortest compared to all other methods reported for ATV as a single analyte [18–24]. Also, the on-column loading of ATV at ULOQ was only 1.5 ng per sample injection volume, which is significantly lower compared to all other reported procedures.

4. Conclusions

Few methods based on LC–ESI–MS/MS determination of ATV have studied the effect of matrix in human plasma. In the present work, a systematic evaluation of matrix interference has been demonstrated using three conventional extraction procedures for the determination of ATV by LC–ESI–MS/MS. The results show significant ion-suppression for samples prepared by protein precipitation with methanol and acetonitrile, while to a much lesser extent using LLE with dichloromethane. However, the best results were achieved through SPE with LiChrosep Sequence extraction cartridges with minimum matrix interference. The proposed method is highly reliable and rugged for routine sample analysis. The method offers significant advantages over those previously reported, in terms of lower sample requirements, practically free from matrix interference, simplicity of extraction procedure and overall analysis time. The efficiency of SPE and a chromatographic run time of 2.5 min per sample make it an attractive procedure in high-throughput bioanalysis of ATV. The method is selective in presence commonly used medications by healthy volunteers and nine antiretroviral drugs studied. Overall the developed method has shown adequate sensitivity, excellent selectivity, no ion suppression and desired reproducibility for the quantification of ATV in human plasma in a clinical study. Incurred sample reanalysis, which has now become mandatory for clinical and non-clinical study has been demonstrated to prove the reproducibility of the proposed method in healthy subject samples.

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