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Quantitation of atorvastatin in human plasma using directly suspended acceptor droplet in liquid–liquid–liquid microextraction and high-performance liquid chromatography-ultraviolet detection

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ABSTRACT

A simple and sensitive methodology based on liquid–liquid–liquid microextraction (LLLME) followed by high-performance liquid chromatography-ultraviolet detection (HPLC-UV) has been successfully developed for the determination of atorvastatin (AT) in human plasma. AT was first extracted from 4.5 mL acidic aqueous sample (diluted plasma, donor phase, pH 1) at temperature 45 °C through 400 μL 1-octanol for 4.5 min, while being agitated by a stirring bar at 1250 rpm. Then, a 5.5 μL free suspended basic aqueous droplet (acceptor phase, pH 10) was delivered to the top-center position of the organic membrane. The mixture was stirred at 650 rpm for 7.5 min and the analyte was back-extracted into the droplet. Finally, the acceptor phase was taken into a microsyringe and injected directly into the HPLC. An enrichment factor of 187 along with substantial sample clean up was obtained under the optimized conditions. The calibration curve showed linearity in the range of 1–500 ng mL^{-1} with regression coefficient corresponding to 0.996. Limits of detection (S/N = 3) and quantification (S/N = 10) were 0.4 and 1 ng mL^{-1}, respectively. A reasonable relative recovery (91%) and satisfactory intra-assay (4.4–7.0%, $n = 6$) and inter-assay (4.9–7.7%, $n = 8$) precision illustrated good performance of the analytical procedure. This technique was eventually applied for the determination of AT in human plasma after oral administration of 40 mg single dose of drug. The protocol proved to be highly cost-effective and reliable for the screening purpose.

1. Introduction

Sample preparation is a tedious and yet unavoidable procedure in analytical chemistry [1]. The objective of this challenging and critical step is to transfer the analyte into a form that is pre-purified, concentrated and compatible with the analytical system [2,3]. The extracted and enriched analytes of interest from the sample matrix are often accomplished by procedures such as liquid–liquid extraction (LLE) [4,5] and solid-phase extraction (SPE) [6,7]. The invention of solid-phase microextraction (SPME) by Pawliszyn and co-worker [8] basically initiated the interest for microextraction techniques in analytical chemistry. SPME satisfies most of the requirements of a good sample preparation technique, including simplicity of use, automation, and low consumption of materials [9]. Thus, it has been applied to determine a broad range of organic compounds in numerous types of samples [10].

An alternative solvent-minimized sample preparation approach to complement SPME appeared in the middle-to-late 1990s [11–13]; liquid-phase microextraction (LPME) utilizes only a small amount of solvent (low microliter range) for concentrating analytes from aqueous samples. It is simply a miniaturized format of LLE and overcomes many of its disadvantages as well as some of those of SPME (e.g. non-dependence on a commercial supplier and sample carryover). LPME is simple to implement and use, generally fast, and is characterized by its affordability and reliance on widely available apparatus or materials [14]. The applications of LPME in environmental and biological analysis have been described in several papers [15–17].

LPME can be classified as two- and three-phase categories. In two-phase LPME, the target analytes are extracted from the aqueous sample matrix into the organic receiving phase [13,18,19]. Three-phase LPME (liquid–liquid–liquid microextraction, LLLME) is

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performed as hollow fiber or droplet based mode [20–24]. In hollow fiber LLLME, the analytes are first extracted from an aqueous sample matrix into the thin layer of the organic phase inside the wall pores of a hollow fiber, and then back-extracted into the acceptor phase located inside the hollow fiber [25]. In the other mode, the receiving phase is a microdrop of aqueous phase suspended from the tip of a microsyringe and located inside the organic phase [20,21,26]. Today, LLLME has been successfully utilized for the clean up and pre-concentration of ionizable analytes from a variety of matrixes [27–29], owing to its advantages.

Recently, Sarafraz-Yazdi et al. have developed a simple, efficient and novel LLLME that initially applied for the determination of diclofenac in environmental water samples [30]. The method was simplified droplet based LLLME using larger volumes of organic solvent to eliminate the use of microsyringe which is employed for suspending the acceptor drop at the tip of the needle. This quantitative LLLME is an efficient and satisfactory analytical procedure, because excellent accuracy and precision are demonstrated.

Atorvastatin ([R-(R*,R*)]-2-(4-fluorophenyl)-b, d-dihydroxy-5-(1-methylethyl)-3-phenyl-4 [(phenylamino) carbonyl] 1H-pyrrole-1-heptanoic acid, AT, $pK_a = 4.46$, $\log P_{ow} = 6.36$) is a potent inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis and has been demonstrated to be effective in reducing both cholesterol and triglyceride [31–33]. Also, AT is indicated to diminish the risk of myocardial infarction [34,35], stroke [36] which decreases the risk for revascularization procedures [37] and angina [38]. AT is rapidly absorbed after oral administration, however, due to pre-systematic clearance in the gastro-intestinal mucosa and metabolism in the liver, its absolute bioavailability is approximately 12%, and low plasma concentration is followed after taking the drug [39,40].

According to literature survey there have been several reports for the determination of AT in biological samples. Existing analytical methods for AT included an enzyme immunoassay [41], gas chromatography–mass spectrometry (GC/MS) [42] and also high-performance liquid chromatography equipped with mass spectrometry detection (HPLC-MS) [43,44]. GC–MS and HPLC–MS methods are sensitive and specific, but these devices may not be available in many pharmaceutical laboratories and need highly trained persons. In addition extraction and pre-concentration techniques, which are commonly applied to monitor the drug, are liquid–liquid extraction (LLE) [45,46] and solid-phase extraction (SPE) [47,48]. However, these sample pretreatment methods are considered expensive, time-consuming and labor-intensive, which often result in high blank values [49].

The aim of the present study is to assess the LLLME technique suitability for the determination of AT in human plasma. The factors affecting the microextraction efficiency were studied in detail and the optimal conditions were established. The resulting method was validated for quantitative purposes and applied to volunteer's plasma for sample analysis after oral administration of the drug in combination with high-performance liquid chromatography-ultraviolet detection (HPLC-UV).

2. Experimental

2.1. Materials and reagents

AT (purity 99%) and diclofenac sodium (DS, purity 99%), as internal standard (I.S.), were kindly supplied from Darou-Pakhsh Pharmaceutical Company (Tehran, Iran). All of organic solvents, sodium chloride, sodium hydrogen phosphate and hydrochloride acid were of analytical grade and purchased from Merck (Darmstadt, Germany). Trichloroacetic acid (TCAA) was obtained from Fluka (Buchs, Switzerland).

2.2. Instrument and apparatus

Separation, identification and quantification were performed with a Younglin HPLC system (ACME 9000, Korea), consisted of a Rheodyne 7725 injector (Cotati, CA, USA) equipped with a 5 μL sample loop. The separation was accomplished using a 150 mm × 4.6 mm i.d. × 5 μm particle size ODS-3 C_{18} analytical column (MZ, Germany) at room temperature (25 ± 0.5 °C). Isocratic mobile phase consisted of acetonitrile:0.1% acetic acid (70:30) was run through the column at flow rate of 1 mL min^{-1}. UV detection was performed at 246 nm and data were collected and processed with Autochrom 2000 software (Younglin, Korea). The temperature-controlling centrifuge (model: 2-16KC, Sigma, Germany) was used for centrifugation. The used water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). A 10 μL HPLC microsyringe (Bondaduz, Switzerland) was applied to introduce the acceptor phase and act as an injection syringe.

2.3. Preparation of standard solutions and plasma samples

Frozen, drug-free human plasma was obtained from the research center of Darou-Pakhsh pharmaceutical company (Tehran, Iran) and thawed at room temperature (25 ± 0.5 °C) before use. The plasma samples were obtained from healthy male volunteers. Stock solutions of AT (1 mg mL^{-1}) and I.S. (100 mg mL^{-1}) were prepared by dissolving proper amounts of pure substances in methanol, stored at 4 °C. Working solutions were prepared by diluting the stock solution with water. Spiked plasma samples were generated by adding 20 μL working solutions with different concentrations to 1 mL drug-free matrixes to yield final desired concentrations.

2.4. Directly suspended droplet LLLME procedure and deproteinization of plasma

The experimental microextraction setup is illustrated in Fig. 1. A water bath was placed on a magnetic stirring plate (IKA-Werke, Staufen, Germany) and the temperature (in the range of 25–55 °C) was controlled. 4.5 mL of the acidic aqueous sample (diluted plasma) was transferred in a 5 mL commercially available glass vial. A stirring bar (7 mm × 3 mm) was used to facilitate the mass transfer process. 400 μL of an organic phase was added to the sample solution by a 500 μL syringe (Hamilton, Reno, NV, USA) and stirred at 1250 rpm. After extraction for a prescribed time, the acceptor phase (5.5 μL, adjusted to pH 11 with 0.1 M Na_2HPO_4) was delivered to the top-center position of the organic membrane. The mixture was agitated at 600 rpm for a specified time and back-extraction was performed. Subsequently, the 5 μL of microdrop was taken into a microsyringe and injected into the HPLC system.

AT is extensively bounded to plasma proteins (98%) [50], and should be librated prior to extraction. Plasma sample (1 mL) was spiked with particular level of the drug and vortexed for 3 min. The mixture was acidified with 75 μL hydrochloric acid (37%) to disturb the drug protein binding. Then, 100 μL TCAA (100%, w/v) was added to denature the proteins. These processes eventually led to the precipitation of proteins. Subsequently, the sample was centrifuged at 10,000 rpm for 5 min. A volume of 0.75 mL of the supernatant was transferred to the sample vial, mixed with proper amounts of I.S (45 μL of the stock solution added to sample vial to achieve final concentration of 1 mg mL^{-1}) and then, diluted with water to 4.5 mL. The pH was adjusted to 1 with 0.1 mol L^{-1} HCl. To adjust the pH to desired value, a micropipette was used for the addition of appropriate amount of concentrated HCl.

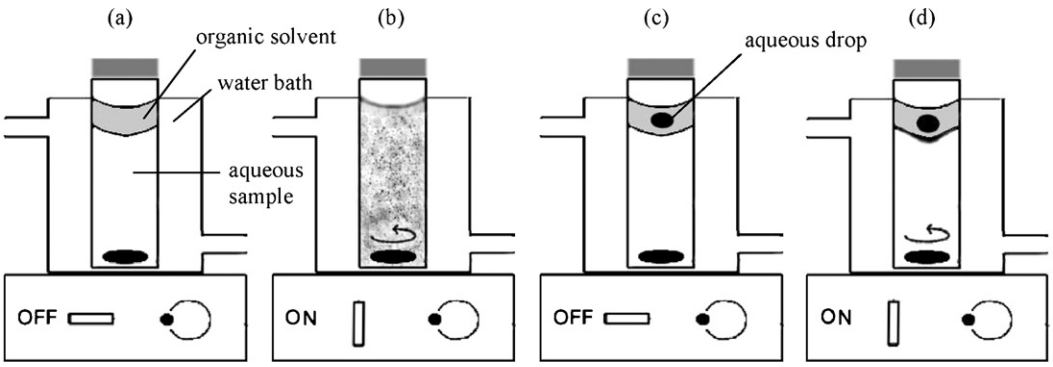

Fig. 1. Directly suspended droplet LLLME setup.

3. Results and discussion

The important aims of LLLME are to separate and clean up the analytes from the complex matrixes and to provide an efficient pre-concentration prior to HPLC or capillary electrophoresis (CE) determination. It involves the extraction of ionizable compounds from the aqueous sample (donor phase) at suitable pH into the organic layer (as membrane), followed by back-extraction into the receiving aqueous phase (acceptor phase) by adjusting the pH on the desired value. This back-extraction step introduces extra selectivity since neutral compounds will preferably stay in the organic phase. Extraction was induced by the pH difference inside and outside an organic membrane located at the interface [25,51,52].

3.1. Optimization of the LLLME process

A univariate approach was employed to optimize the influential factors in this method. The optimization was executed using 1 mL plasma containing 250 ng AT. In order to improve the precision and accuracy of the procedure in all of the experiments, DS with the concentration of 100 ng mL^{-1} was used as the I.S. and was added into the donor phase. Quantifications were performed by calculating peak areas relative to the I.S. from the average of 3 replicate measurements.

3.1.1. Selection of the organic solvent

The selection of appropriate organic solvent is of great importance in LLLME, in order to achieve satisfactory analyte pre-concentration [53,54]. Several organic solvents with different polarities were used to study their effects on extraction efficiency. The solvents including isooctane, 2-octanone, 1-octanol and hexyl acetate were examined. Fig. 2 depicts that 1-octanol presented the maximum extraction efficiency and thus was selected as the most suitable solvent.

3.1.2. Phase volume ratios

In three-phase LPME higher enrichment factors can be resulted by decreasing the volume ratio of acceptor to donor phase. However, in many cases optimization of the enrichment factor is not the primary goal, but rather optimization of recovery (R), defined by IUPAC as the ratio of the moles extracted (nA) and the number of moles initially present in the sample (nD):

$$R = \frac{nA}{nD}$$

At equilibrium the maximum recovery will be limited by the distribution coefficient and the volume ratio between the sample and acceptor phases [25,52]. To enhance recovery the acceptor volume must therefore be increased.

The phase ratio was varied by changing the volume of the microdrop in the range 3.0–6.0 μL, while the donor solution volume was kept constant (4.5 mL). It was found that the signal intensity of the analyte increased with the amplification of drop volumes. However, from a practical point of view, the drop greater than 5.5 μL was unstable especially with a high stirring speed. Accordingly, the following investigations were carried out with the drop volume of 5.5 μL to get higher extraction efficiency.

Due to the design of our extraction device, the volume of the organic phase was also important and needed to be optimized. The best volume of the organic solvent was found to be 400 μL. A smaller volume of organic solvent tended to cause instability of the aqueous drop during agitation, whereas the extraction efficiency is reduced if a larger volume of organic phase is used. Hence, a 400 μL volume of organic solvent was selected as the optimum on the basis of these observations.

3.1.3. The donor phase and acceptor drop pHs

The pH difference between donor and acceptor phases can promote the transfer analyte from the former to the latter [55]. To get a high distribution coefficient result leading to a high preconcentration, the donor solution should be sufficiently acidic to keep the drug in molecular form, and consequently reduce their solubility within this phase. The acceptor phase should be basic in order to promote dissolution of the acidic analyte.

The experiments were conducted to optimize the composition (leading to variation of pH) of both donor and acceptor solutions. First, the effect of the donor phase's pH on extraction efficiency was investigated. Solutions with different acidic pHs (0.5–3.5) were prepared by addition of appropriate amounts of HCl, while pH of the

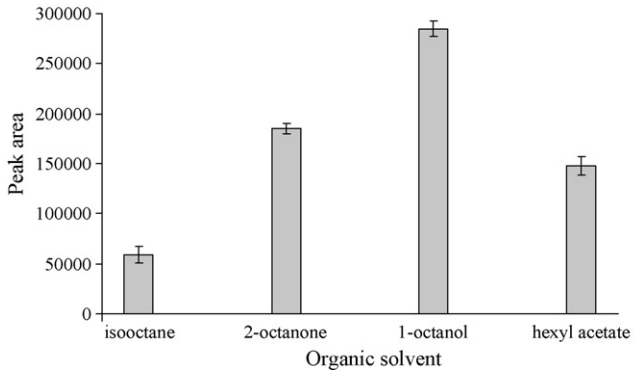

Fig. 2. Extraction efficiencies obtained for different organic solvents. Conditions: 4.5 mL aqueous sample (pH 1) at room temperature (25 ± 0.5 °C); organic phase volume: 400 μL; acceptor phase: 5.5 μL aqueous drop adjusted to pH 11 with 0.1 mol L^{-1} Na_2HPO_4; extraction and back-extraction time: 6.0 and 9.0 min, respectively; stirring rate for extraction and back-extraction: 1250 and 600 rpm, respectively.

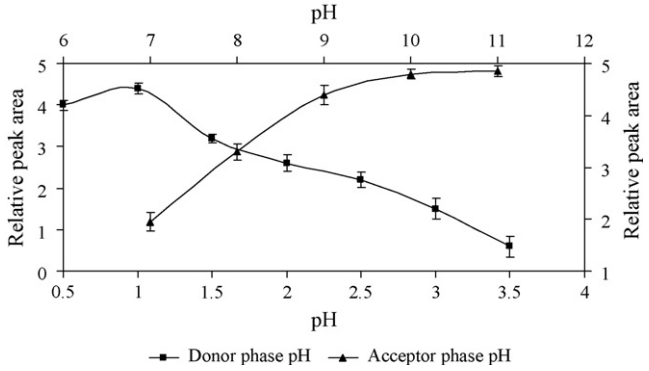

Fig. 3. Effect of the donor and acceptor phases' pH on the extraction of AT. Conditions: as with Fig. 2, organic solvent: 1-octanol.

acceptor phase was adjusted to 11.0 using 0.1 mol L^{-1} Na_2HPO_4. Since pH 1 provided the highest extraction efficiency (Fig. 3), it thus was selected for subsequent experiments. Secondly, the acceptor phase's pH, which was adjusted by 0.1 mol L^{-1} Na_2HPO_4 in the range of 7–11, was examined. The results, also shown in Fig. 3, indicate that the receiving phase with pH 10 could ionize the analyte more conveniently to prevent re-entering into the organic phase. Also, in order to investigate the effect of concentration of phosphate buffer on the extraction efficiency, aqueous solutions with various concentrations of Na_2HPO_4 (0.01, 0.05, 0.1 and 0.5 mol L^{-1}) were prepared and the extraction procedure was conducted. The results show that the best extraction efficiency was obtained at: 0.05 mol L^{-1} of Na_2HPO_4. Therefore, further experiments were done using the aqueous acceptor solutions containing 0.05 mol L^{-1} Na_2HPO_4 with pH 10.

3.1.4. Effect of the temperature

The temperature of extraction can influence the mass transfer rate and the partition coefficient of the analyte. So, it affects extraction kinetics and the time required to reach equilibrium diminishes [53,55]. The effect of sample solution temperature was studied in the range of 25–55 °C. An increase in analytical signal was observed up to 45 °C during this study (Fig. 4). After this point, some practical difficulties such as instability of the microdrop and reduction of its volume due to its higher solubility in the organic phase could occur. Thus, the sample temperature was held at 45 °C for the rest of the experiments as it gave a higher efficiency.

3.1.5. Effect of the ionic strength

The addition of salt to an analytical sample can potentially cause the formation of hydration spheres, which reduce the amount of water available to dissolve the analyte in water. This effect resulted in an increase in analyte recovery in microextraction procedures [56–58]. The NaCl concentration effect (0–20%, w/v) was investigated and the extraction efficiency was monitored. Based on the obtained results (Fig. 4), addition of salt did not improve the extraction efficiency. While the extraction efficiency was highest without the addition of sodium chloride, it subsequently decreased as more salt was added. This may be related to the formation of a physical barrier across donor phase and organic solvent interface, which could prevent the mass transfer of analyte into the organic phase. Hence, no salt was added in the subsequent experiments.

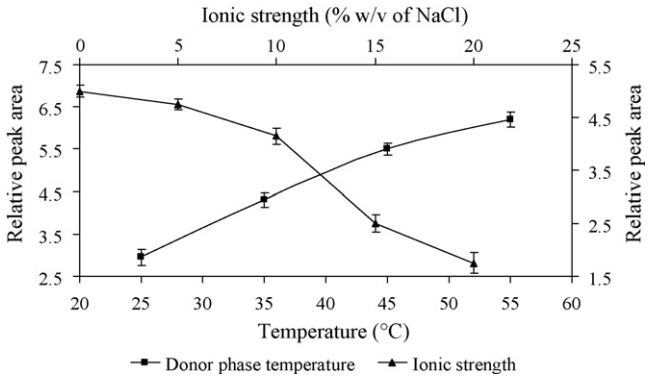

Fig. 4. Influence of the temperature and salt on the extraction efficiencies. Conditions: as with Fig. 3, donor and acceptor phases' pH 1 and 10, respectively.

3.1.6. Effect of the stirring rate

Magnetic stirring facilitates the mass transfer process and reduces the time required to reach thermodynamic equilibrium. So, it has been used universally to improve microextraction efficiency [59]. In this work, extraction was performed at the maximum magnetic stirrer performance, 1250 rpm. After utilizing the acceptor phase for back-extraction, this effect was studied at three different stirring rates: 0 (static case), 400 and 650 rpm. Faster stirring speeds were avoided due to the production of vortex flow in the membrane phase that could reduce stability of the microdrop. In this step, agitation of donor phase induces convection in the organic membrane. The obtained results show that the agitation improves the extraction efficiency of the target analyte significantly. Thus, a stirring rate of 650 rpm was used in the subsequent studies.

3.1.7. Extraction and back-extraction time profiles

LLLME system is an equilibrium based process that involves mass transfer from donor phase to organic layer and subsequently from organic layer to acceptor phase. The main objective in microextraction techniques is to achieve sufficiently high extraction efficiency within a relatively short period of time [60].

For the first equilibrium, extraction time was studied in the range of 0.5–6.5 min, while the time for back-extraction process was kept constant for 9.0 min. As shown in Fig. 5, the relative peak area was enhanced with the increase in exposure time up to 4.5 min and remained constant afterwards. An extraction time of 4.5 min therefore, was selected as the optimum.

The time of back-extraction was also investigated in the range of 3.0–12.0 min. As demonstrated in Fig. 5, the analytical signal increases quickly with sampling time of 7.5 min and after that the rate of increase slows down. It can be seen when the extraction time was set at 7.5 min, satisfactory extraction efficiency was achieved while equilibrium in the extraction of the analyte was not reached even at 12.0 min. Since it is not considered practicable for exposure time to be excessively long to allow equilibrium to occur, it

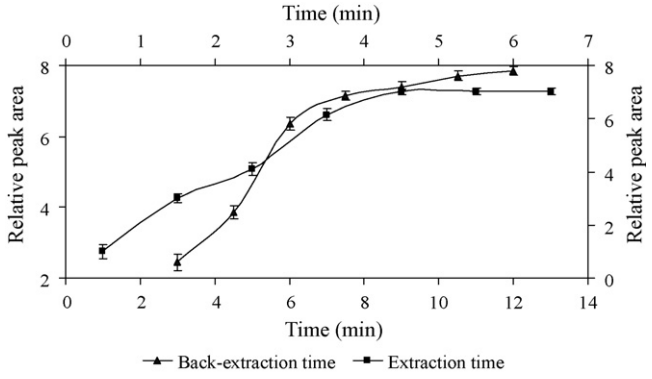

Fig. 5. Effect of the extraction and back-extraction time profiles on the LLLME efficiency. Conditions as with Fig. 4, stirring rate of back-extraction: 650 rpm and sample solution temperature: 45 °C.

Table 1
Quantitative data obtained after the LLLME–HPLC-UV determination of AT in human plasma.

Parameter	Analytical feature
Limit of detection (LOD based on S/N = 3, ng mL^{-1})	0.4
Limit of quantification (LOQ based on S/N = 10, ng mL^{-1})	1
Linear range (LR, ng mL^{-1})	1–500
Regression coefficient (r^2)	0.996
Enrichment factor (EF, at concentration level of 50 ng mL^{-1})	187
Extraction recovery (%) (ER)	22.9

should be just long enough for the extraction rate to have slowed to improve precision [12,61,62]. Furthermore, droplet lifetime cannot be too long due to drop dissolution or loss. Hence, a back-extraction time of 7.5 min appeared to be an appropriate value.

3.2. Evaluation of the method performance

To access the practical applicability of the LLLME method, the optimized conditions were adopted in the evaluation of linear range (LR), correlation coefficient (r^2), limit of detection (LOD, S/N = 3), limit of quantification (LOQ, S/N = 10), enrichment factor (EF) and also extraction recovery % (ER). An external calibration curve was plotted using plasma samples spiked with known concentrations of AT. For each level, three replicate extractions were performed. The results are summarized in Table 1. As shown, the LR is enough to cover the possible concentration of AT in various real samples. LOD and LOQ values imply a high sensitivity of the developed method.

For the EF calculation, three replicate extractions were performed from the aqueous solution containing 50 ng mL^{-1} of the analyte. EF was calculated as the ratio of the final analyte concentration in the acceptor phase and its concentration in the original solution. The AT standard solutions were prepared in acceptor phase and a calibration curve was drawn in the concentration ranges of 0.5–25 μg mL^{-1} with three replicate direct injections. The actual concentration of AT in acceptor phase was calculated from the non-extractive calibration curve and the EF value was determined.

ER% was calculated based on the following equation:

$$ER\% = (C_{acceptor} \times V_{acceptor}/C_{donor} \times V_{donor}) \times 100$$
$$= EF \times (V_{acceptor}/V_{donor}) \times 100$$

where $C_{acceptor}$ and C_{donor} are the analyte concentration in acceptor and donor phase, respectively. Moreover, $V_{acceptor}$ and V_{donor} are the volume of the acceptor and donor phase, respectively.

The results of the intra-day and inter-day precision at three different concentration levels are presented in Table 2. As it is illustrated, the intra-assay RSD measured at 6 runs a day of a sample was relatively low (4.4–7.0%). Also, the inter-assay precision evaluated on different days with a total of 8 runs provided RSD values in the range of 4.9–7.7%.

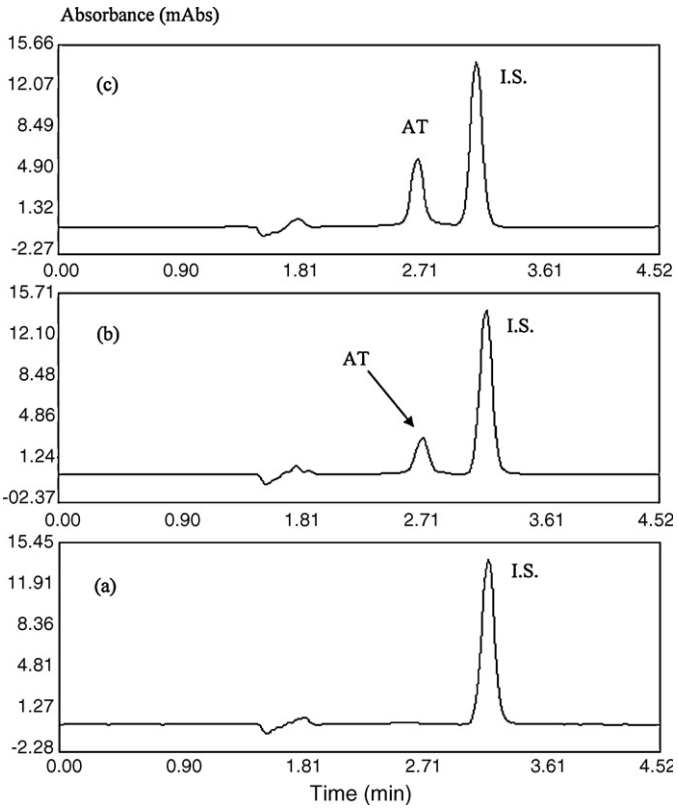

Fig. 6. HPLC-UV chromatograms obtained using developed method after oral administration of 40 mg single dose of AT at time (a) 0 h, (b) 2 h and (c) spiked sample.

Table 2
The intra-assay and inter-assay reproducibility of the method and instrument on plasma sample.

AT concentration (ng mL^{-1})	RSD (%)	
	Intra-day (n = 6)	Inter-day (n = 8)
5	7.0	7.7
50	5.8	6.4
250	4.4	4.9

As shown in Table 3, comparing LLLME–HPLC-UV with other analytical methods applied for the determination of AT in plasma samples, this technique along with its simplicity provided wider LR, lower LOD and an acceptable reproducibility.

3.3. Application

The developed protocol at optimal conditions was used to determine AT concentration in human plasma. Oral administration of a 40 mg single dose was performed to a healthy male volunteer. The concentration of AT 2 h after it was administrated was

Table 3
Comparison of different analytical methods applied for the determination of AT in plasma.

Analytical technique	LR[a] (ng mL^{-1})	LOD[b] (ng mL^{-1})	RSD[c] (%)	Reference
LLE–HPTLC[d]-UV	101–353.5[e]	30.3[e]	1.7–3.4	[63]
LLE–HPLC-UV	4–256	1	≤11.7	[45]
LLLME–HPLC-UV	1–500	0.4	4.4–7.7	Represented method

[a] Linear range.
[b] Limit of detection for S/N = 3.
[c] Relative standard deviation.
[d] High-performance thin layer chromatography.
[e] ng zone^{-1}.

found to be 12.3 ng mL^{-1}. This sample was spiked with AT standard (C_{added} = 10.0 ng mL^{-1}) to assess the matrix effects. LLLME is a non-exhaustive extraction procedure and the relative recovery (determined as the ratio of concentrations found in real sample and reagent water sample, spiked with the same amount of analyte), instead of the absolute recovery (used in exhaustive extraction procedures), was employed. The relative recovery value of 91% (C_{found} = 21.4 ng mL^{-1} with RSD% = 7.2, n = 6) was achieved, which indicated that the real matrix in our present context had little effects on the LLLME. Fig. 6 shows the HPLC-UV chromatograms at 0 and 2 h after administration, and also the spiked sample. The obtained chromatograms reveal that in spite of the complex matrix of the sample, due to the high sample clean up performance, almost no other components than the target analyte were recovered in the acceptor solution.

4. Conclusion

The results from this work show that the LLLME technique in combination with HPLC-UV is a valid means of enrichment and quantification of AT at trace level in human plasma. The established protocol demonstrates good sample clean up with high sensitivity and reproducibility. Moreover, the entire analytical process presents an economical and rapid way for the screening purpose. Simplicity of operation and significant increase in acceptor phase volume (which causes major enhancement in the extraction recovery), are the important advantages of the method over conventional droplet based LLLME. Excellent extraction efficiency is achieved almost independent of the matrix in the actual application. Putting all the benefits together, it possesses great potential to be employed in bio-analytical and pharmacokinetic studies.

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