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Selective, sensitive and simple LC–APCI–MS method for the quantitation of Alpha-Tocopheryl Nicotinate in human plasma

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ABSTRACT

A sensitive and specific method using liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (LC–APCI–MS) has been developed and validated for the identification and quantification of Alpha-Tocopheryl Nicotinate in human plasma. A simple liquid–liquid extraction procedure was followed by injection of the extracts onto a C_{18} column with isocratic elution and detection using a single quadrupole mass spectrometer in selected ion monitoring (SIM) mode. The method was tested using six different plasma batches. Linearity was established for the concentration range 2–1500 ng/ml, with a coefficient of determination (r) of 0.9998 and good back-calculated accuracy and precision. The intra- and inter-day precision values were less than 15% and the deviations were within ± 5.0%. The lower limit of determination was reproducible at 0.5ng/ml with 0.5 ml plasma. The proposed method enables the unambiguous identification and quantification of Alpha-Tocopheryl Nicotinate for pre-clinical and clinical studies.

1. Introduction

Vitamin E (*alpha*-tocopherol) is a fat soluble chemical found in the diet in varying amounts [1], and it can be isolated from natural sources (plants, vegetables and meat) or can be made in the laboratory [2]. Therefore, vitamin E is sold commercially as a natural or synthetic preparation. The esterified forms of vitamin E such as alpha tocopherol acetate, alpha tocopherol succinate and alpha tocopherol nicotinate are made in the laboratory and are also sold commercially. Alpha-Tocopheryl Nicotinate (ATN), 2R-(2R*(4R*,8R*))-3-pyridinecarboxylic acid, 3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethy-ltridecyl)-2H-1-benzopyran-6-yl ester, is a well-known lipid-soluble antioxidant [3,4].

To date, some assays for the determination of ATN in preparations and biological samples utilizing normal-phase HPLC with UV [5], reversed-phase HPLC with UV [6–8], HPLC-IR [9] and so on. Although these methods gave high chromatographic resolution and good peak shape, they showed poor sensitivity with the lower limit of quantification (LLOQ) that ranged from 50 to 200 ng/mL. Plasma concentration of ATN was less than 50 ng/mL after a single oral dose of 400 mg ATN capsules to healthy volunteers. Thus, the methods mentioned above were inadequate for pharmacokinetic study of ATN in healthy volunteers. Further, these published methods [5–9] are not ideal for large number of sample determination because they need long total chromatographic run time. Stoggl and coworkers [5] have reported a sensitive method to identified the vitamin E constituents in the femtomole range by HPLCAPCI–IT–MS, however, this method cannot be used to quantitative determination of vitamin E for its poor linearity and reproducibility.

In order to fully evaluate the pharmacokinetics of ATN in pre-clinical or clinical studies, it was necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. The potentially large numbers of samples in pre-clinical studies need a rapid and reliable assay. An ideal method should have simple sample preparation, fast on-column separation, and sensitive and specific detection. Liquid chromatography coupled with mass spectrometry (LC–MS) has become an analytical tool that meets most of the above needs. In the present work, isolation of ATN was achieved by a single-step liquid–liquid extraction into ethyl acetate, followed by solvent evaporation, re-dissolution of the residue, and injection onto the chromatographic column. An atmospheric pressure chemical ionization (APCI) interface, suitable for analysis of low-polarity compounds, was used because it exhibited more sensitivity and better reproducibility for ATN compared with an ESI source. The analytical procedure was fully validated and successfully used to assess the pharmacokinetics of ATN in healthy volunteers.

2. Materials and methods

2.1. Chemicals and reagents

ATN reference standard (Fig. 1, 99.7% purity) and alpha tocopherol acetate (ATA, Fig. 1) reference standard (99.3% purity) were

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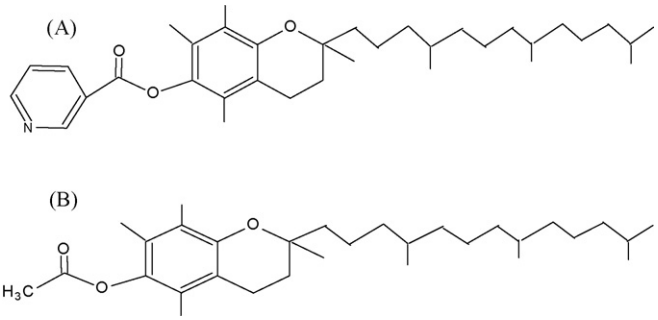

Fig. 1. Molecular structures of ATN (A) and ATA (B, internal standard).

purchased from Sigma; Methanol was chromatography pure grade and purchased from Merck (Merck Company, Germany). Other chemicals were all of analytical grade. Deionized water was distilled before use. Other reagents were used as received.

2.2. Instrumentation and operating conditions

Liquid chromatography was performed using a Shimadzu LC-10AD HPLC system equipped with an autosampler (SIL-HTc). The HPLC was coupled to a Shimadzu LCMS-2010A quadrupole mass spectrometer with an APCI interface. Data acquisition and processing were accomplished using Shimadzu LCMS solution Software for the LCMS-2010A system. Chromatographic separation was achieved on a Shim-pack stainless-steel column (C_{18}, 5 μm, 150 mm × 4.6 mm I.D., Shimadzu) at 40 °C. The isocratic mobile phase consisted of methanol: 0.05% formic acid (95:5 v/v) at a flow rate of 1.0 ml/min. The APCI source was used in positive ionization mode. The $[M+H]^+$ ions of ATN (*m*/*z* 536.15) and of ATA (*m*/*z* 473.15) were selected as ions for SIM detection. The quantification was performed using peak areas. The MS operating conditions were optimized as follows: drying gas 2.5 l/min, APCI temperature 400 °C, Curved Desolvation Line (CDL) temperature 200 °C, block temperature 200 °C, probe voltage + 4.0 kV, interval time 0.2 s.

2.3. Preparation of stock solutions

The stock solutions of ATN and ATA (IS) were prepared and the concentrations calculated after correcting for purity. The primary stock solution of ATN was prepared by dissolving 10.0 mg of ATN in 10 ml methanol to produce a concentration of 1.0 mg/ml, and was stored at 4 °C. The internal standard stock solution was prepared by dissolving 10.0 mg of ATA in 10 ml methanol to produce a concentration of 1.0 mg/ml, and was also stored at 4 °C. This solution was further diluted with methanol to prepare the internal standard working solution containing 2.0 μg/ml of ATA. Working solutions of ATN at 10, 1 and 0.05 μg/ml were prepared daily in methanol by appropriate dilutions of the stock solution.

2.4. Calibration curves

Calibration curves were prepared by spiking different 0.2 ml samples of blank plasma with appropriate volumes of one of the above-mentioned working solutions, to produce the calibration standards equivalent to 2, 5, 10, 20, 50, 100, 200, 500, 1000 and 1500 ng/ml of RAN. Each sample also contained 2.0 μg/ml of the internal standard. In each calibration run, a plasma blank sample (no IS) was also analyzed. Calibration curves were prepared by determining the best-fit of peak area ratios *R* (peak area of analyte/ peak area of internal standard) versus concentration (*x*), and fitted to the equation $R = bx + a$ by unweighted least-squares regression.

2.5. Preparation of quality control samples

Quality control (QC) samples were prepared at three different concentration levels, low limit (5 ng/ml), middle level (100 ng/ml) and a high level (1000 ng/ml). QC samples were prepared daily by spiking 0.2 ml plasma samples with appropriate volumes of a standard solution to produce the stated final concentrations of ATN and 2.0 μg/ml of internal standard after independent weighing standards of ATN and preparing working solution by a research fellow apart from the analyst. The extraction and analytical procedures were those described below.

2.6. Extraction procedure

QC samples, calibration standards, and clinical plasma samples, were extracted employing a liquid–liquid extraction technique. To each tube containing 0.2 ml plasma, 20 μl of the 2 μg/ml solution of internal standard and 1.0 ml of ethyl acetate were added, and the mixture was then vortexed for 2 min. The samples were then centrifuged for 10 min at 40,000 × *g*. The organic layer was removed and evaporated under a stream of nitrogen at 40 °C. The residue was re-dissolved in 200 μl methanol. An aliquot of 20 μl was injected into the LC–MS system.

2.7. Method validation

The specificity of the method was tested by screening six different batches of blank human plasma. Each blank sample was tested for interferences in the SIM channels using the proposed extraction procedure and chromatographic/MS conditions, and the results were compared with those obtained for an aqueous solution of the analyte at a concentration near to the LLOQ (lowest limitation of quantitation).

The matrix effect on the ionization efficiency of analyte and IS was evaluated by comparing the peak areas of analyte and IS dissolved in blank sample extract (i.e., the final solution obtained from blank plasma after extraction and reconstitution) with those for ATN and IS dissolved to the same concentrations in methanol. Three different concentration levels of ATN (5, 100 and 1000 ng/ml) with 200 ng/ml of internal standard were evaluated by analyzing five samples at each concentration level. The blank plasma used in this study was obtained as six different batches. If the peak area ratios for the plasma extracts versus clean methanol solutions were <85 or >115%, a matrix effect was implied.

Linearity was tested for the concentration range 2.0–1500.0 ng/ml. For the determination of linearity, standard calibration curves containing at least ten points (non-zero standards) were used. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. For a calibration run to be accepted, four out of ten non-zero standards including the LLOQ and ULOQ (up limitation of quantitation) were required to meet the following acceptance criteria: no more than 15% deviation at LLOQ and no more than 10% deviation for standards above the LLOQ. The acceptance criterion for the correlation coefficient was ≥ 0.998, otherwise the calibration curve was rejected. Five replicate analyses were performed on each calibration standard. The samples were run in order from low to high concentration.

The intra-day precision and accuracy of the assay were measured by analyzing five spiked samples of ATN at each QC level

(5, 100 and 1000 ng/ml). The inter-day precision and accuracy was determined over three days by analyzing 15 QC samples ($n = 5$ for each concentration level) each day. The precision was within 15%, and accuracies (deviation values) were required to be within 15% of the actual values.

The extraction yield (absolute recovery) was determined by comparing the ATN /IS peak areas obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post-extraction to the same nominal concentrations (5, 100 and 1000 ng/ml). The recovery of IS was also determined similarly.

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantification (LLOQ), and was required to meet the following criteria: LLOQ response should be 10 times that of the average noise level in the SIM chromatogram, and be identifiable, discrete and reproducible within a precision of 10%. Samples at the concentration of 1.0 ng/ml were investigated as the LLOQ, and the reproducibility and precision were determined at this concentration.

2.7.1. Short-term temperature stability

Stored spiked plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation (around 6 h). These samples were then analyzed as described above.

2.7.2. Post-preparative stability

The autosampler stability was conducted by re-analyzing extracted QC samples kept under the autosampler conditions (4 °C) for 24 h.

2.7.3. Freeze–thaw stability

QC plasma samples containing ATN were tested after three freeze (−20 °C) and thaw (room temperature) cycles. Long-term stability of ATN in human plasma was studied for a period of 10 days employing QC samples at the three different levels. If in this stability study the analyte was found to be unstable at −20 °C it should be stored at −70 °C. The stability of the ATN and internal standard working solutions were evaluated by testing their validity over 6 h at room temperature. This stability of working solutions was expressed as for percentage recovery.

A new calibration curve was generated to assay samples in each analytical run, and was used to calculate the concentration of ATN in the unknown samples in that run. The calibration was obtained halfway through each run. In order to monitor the accuracy and precision of the analytical method, a number of QC samples were prepared to ensure that the method continued to perform satisfactorily. In this case the QC samples were prepared in duplicate at each of the three concentrations (5, 100 and 1000 ng/ml), and were analyzed together with the processed test samples at intervals based on the total number of samples per batch.

3. Clinical study design

This was an open randomized, balanced, two-period cross-over study in 24 Chinese healthy volunteers. Each volunteer received in random order, single oral dose of 400 mg ATN test capsules or reference capsules in cycle. Blood samples (2 ml) for assay of plasma concentration of ATN were collected at the time of 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0,10.0, 12.0, 24.0 and 36.0 h after oral administration of the capsules. They were put into lithium heparin tubes and immediately were centrifuged at 3000 × *g* for 10 min. The plasma obtained was frozen at−20 °C in coded polypropylene tubs until analysis.

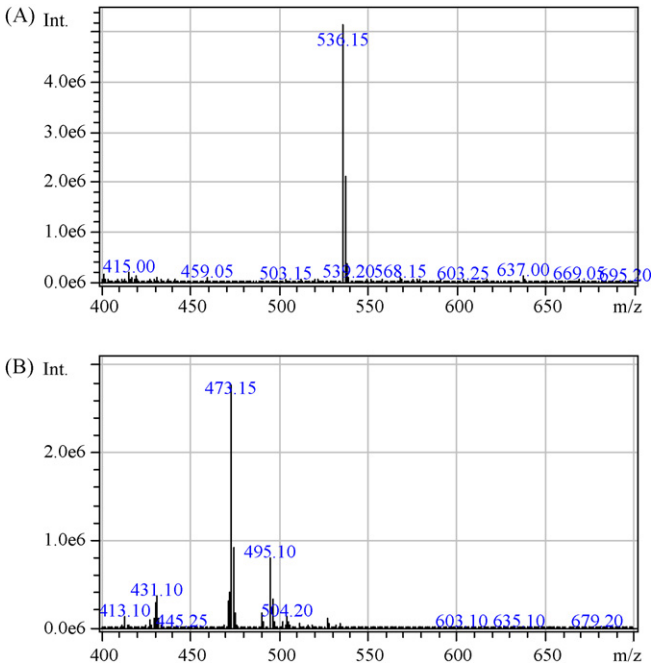

Fig. 2. Positive ion APCI mass spectrum of ATN (A) and ATA (B, IS).

4. Results and discussion

4.1. Selection of IS

It is necessary to use an IS to obtain high accuracy when a mass spectrometer is used as the HPLC detector. ATA was adopted as IS because of its similarity in its retention and ionization characteristics with those of the analyte, and because of the minimal endogenous interferences in the SIM channel for ATA ($[M+H]^+$ at m/z 473.15). Before quantitative determination the ATN and IS, we scanned the IS (1 ug/ml) by the isocratic mobile phase consisted of methanol:water (95:5 v/v) and the signal of $[M+H]^+$ was found higher than $[M+Na]^+$ (seen Fig. 2). In order to enhancing ionization efficiency, 0.05% formic acid was added to mobile phase during our analysis to supply H^+ enough for ionization. The results from our current and earlier studies indicated that $[M+Na]^+$ can be neglected when the formic acid was added in mobile phase because the signal of $[M+H]^+$ was 100 times higher than other ion form.

ATA appears in many dietary supplements and in a number of foods as an antioxidant. We have measured baseline levels of the ATA in clinical trial patients, but we have not found ATA because it may lower than lowest limitation of quantitation.

4.2. Sample preparation

Liquid–liquid extraction was advantageous because this technique not only extracted the analyte and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Ethyl acetate, trichloromethane and diethyl ether were all tested as extraction solvent, and ethyl acetate was finally adopted because of its high extraction efficiency.

4.3. Separation and specificity

Positive ion APCI mass spectra of ATN and ATA are shown in Fig. 2. The major ions observed for ATN and ATA were both $[M+H]^+$ (m/z 536.15, 473.15). Examples of SIM chromatograms for extracts of spiked blank plasma are shown

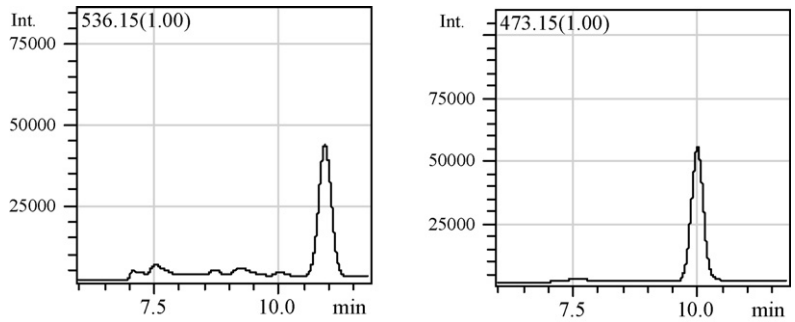

Fig. 3. The SIM (+) chromatograms obtained for extracts of blank plasma spiked with ATN at 200.0 ng/ml and ATA (IS) at 250.0 ng/ml. The retention times of ATN and the IS were 10.8 and 10.0 min, respectively.

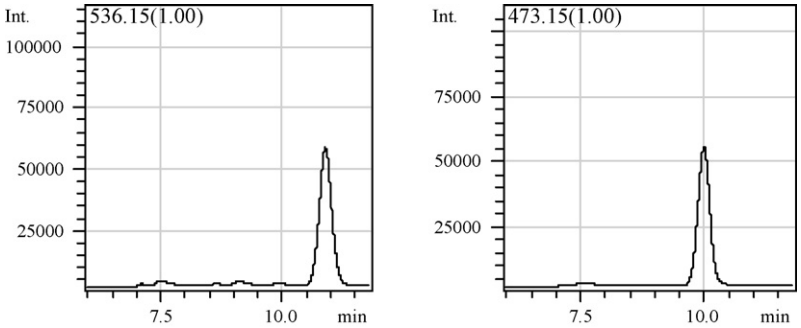

Fig. 4. The SIM(−) chromatogram for plasma sample of a healthy volunteer at 8 h after oral administration 400 mg ATN capsule. The retention times of ATN and the ATA (IS) were 10.8 and 10.0 min, respectively. The concentration of ATN and ATA were 262.3 and 250.0 ng/ml, respectively.

Table 1
Results of five representative calibration curves for LC–MS determination of ATN

Added concentration (ng/ml)	2.0	5.0	10.0	20.0	50.0	100.0	200.0	500.0	1000.0	1500.0
	1.92	4.68	10.90	19.20	50.40	102.3	210.0	496.1	975.2	1512.0
	2.10	4.80	9.87	20.06	48.63	100.0	189.2	510.2	1010.0	1498.3
Back-calculated concentration (ng/ml)	2.00	5.13	9.70	20.40	48.80	97.4	197.5	493.2	980.0	1464.0
	1.90	4.80	10.20	19.70	51.40	103.2	202.1	501.4	1000.0	1503.9
	1.86	5.09	9.80	19.20	51.20	106.0	212.1	495.2	1040.0	1536.0
Mean	1.96	4.90	10.09	19.71	50.09	101.8	202.2	499.2	1001.0	1502.8
R.S.D. (%)	4.87	4.05	4.83	2.68	2.61	3.20	4.63	1.37	2.60	1.73
Mean accuracy (%)	97.80	98.00	100.94	98.56	100.17	101.78	101.09	99.84	100.10	100.19

in Fig. 3.The retention times of ATN and the IS were 10.8 and 10.0 min, respectively. The total HPLC–MS analysis time was 11.3 min per sample. No interferences were observed for ATN or IS in blank plasma samples because of the high selectivity of the SIM mode, and no ionization suppression effects were found under the developed sample preparation and chromatographic conditions. The SIM chromatograms obtained for an extracted plasma sample of a volunteer at

Table 3
Recoveries of ATN from human plasma ($n = 5$) observed at the 3 QC sample concentrations

Added (ng/ml)	Recovery (mean ± S.D.) (%)	R.S.D. (%)
5.0	89.09 ± 9.01	10.11
100.0	91.63 ± 6.46	7.05
1000.0	90.23 ± 3.17	3.51

Table 2
Results obtained in tests of the inter- and intra-day precision and accuracy of the method for determination of ATN ($n = 5$ at each of the 3 QC concentrations)

Added concentration (ng/ml)	Intra-day			Inter-day								
	5	100	1000	1d			2d			3d		
				5	100	1000	5	100	1000	5	100	1000
	5.0	103.1	979.7	5.0	95.9	999.0	5.0	104.8	1003.0	5.0	103.1	979.7
	4.9	95.1	1030.8	5.2	99.3	1000.2	5.1	108.1	1038.7	5.0	95.2	1011.1
Back-calculated Concentration (ng/ml)	4.9	96.4	979.5	5.0	101.4	987.5	4.9	101.2	1030.1	4.8	97.3	979.5
	5.1	107.8	1001.8	5.1	97.9	1005.3	5.2	101.1	971.6	5.0	107.9	1032.4
	4.9	105.3	978.4	5.5	96.5	1014.9	5.2	103.4	1004.8	5.0	108.2	978.4
Mean	5.0	101.5	994.0	5.2	98.2	1001.4	5.1	103.7	1009.6	5.0	102.3	996.2
R.S.D. (%)	0.10	5.56	22.75	0.22	2.23	9.97	0.13	2.91	26.35	0.11	5.98	24.50
Mean accuracy (%)	1.96	5.47	2.29	4.28	2.27	1.00	2.59	2.80	2.61	2.11	5.84	2.46

Table 4
Data obtained in stability tests of ATN in human plasma at the 3 QC concentrations ($n = 5$)

	5.0 (ng/ml)		100.0 (ng/ml)		1000.0 (ng/ml)	
	Recovery (mean ± S.D.) (%)	R.S.D. (%)	Recovery (mean ± S.D.) (%)	R.S.D. (%)	Recovery (mean ± S.D.) (%)	R.S.D. (%)
Short-term stability	92.17 ± 6.71	7.28	96.43 ± 4.05	4.20	90.56 ± 5.72	6.32
Freeze and thaw stability	92.08 ± 6.45	7.00	93.82 ± 3.18	3.39	89.08 ± 4.48	5.03
Long-term stability	90.43 ± 2.39	2.64	88.51 ± 3.33	3.76	90.51 ± 4.21	4.65
Post-preparative stability	90.91 ± 7.42	8.16	99.65 ± 7.26	7.29	97.94 ± 6.68	6.82

8 h after oral administration of 400 mg ATN are depicted in Fig. 4.

The purpose of the investigation was to develop a specific, sensitive and simple procedure for the determination of ATN. HPLC–APCI–MS has several advantages for the analysis of ATN. The combination of HPLC (under the isocratic conditions described) with APCI–MS leads to a shorter run time and yields both higher selectivity and sensitivity than HPLC.

4.4. Method validation

The method exhibited good linear response for the concentration range 2.0–2000.0 ng/ml, with a coefficient of determination of 0.9998. Results of five representative calibration curves for LC–MS determination of ATN are given in Table 1.

Data for intra- and inter-day precision of the method for ATN determined from analyses of QC samples at concentrations of 5.0, 100.0 and 1000.0 ng/ml, are presented in Table 2.

The lower limit of quantitation (LLOQ) for ATN was found to be 2.0 ng/ml and the lower limit of detection (LLOD) was 0.5 ng/ml. The extraction recovery determined for ATN was precise and reproducible (see Table 3). The extraction recovery of the IS was > 85%.

4.4.1. Stability

Table 4 summarizes the data from the short-term, freeze–thaw, and long-term stability, as well as for the post-preparative test of ATN. The short-term stability indicated reliable stability behavior under the experimental conditions of the analytical runs. The results of the freeze–thaw stability test indicated that the analyte was stable in human plasma for three cycles when stored at −20 °C and thawed to room temperature. The post-preparative stability of QC samples showed that ATN was stable in the sample extract when kept at 4 °C in the autosampler for 24 h. The findings from the long-term test indicate that storage of plasma samples containing ATN at 20 °C is adequate when maintained for 10 days. Thus, no stability-related problems are expected during the routine analyses for the pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the working solutions was tested at room temperature. Based on the results obtained, these working solutions were stable over 6 h.

4.5. Results of pharmacokinetic study

The method has been successfully used for the determination of plasma concentrations of ATN in a randomized cross-over bioequivalence study of following single oral administration of 400 mg ATN capsule each in the pharmacokinetics study. The procedure developed was sensitive enough to permit quantitative analysis of ATN in plasma with acceptable accuracy and precision over a period of 36 h after a single oral administration. The mean plasma concentration–time profiles and the major pharmacokinetic parameters have been represented in Fig. 5.

In this study, the test capsules produced by Jiangxi Huiren Pharma. were found to be bioequivalent to the reference one (purchased from Zhejiang Anglikang Pharma) in terms of rate and extent of absorption. Furthermore, there was no adverse event during the course of the study. Thus, the assay procedure for ATN in plasma samples demonstrated the linearity, precision and sensitivity desired for the pharmacokinetic studies of this drug.

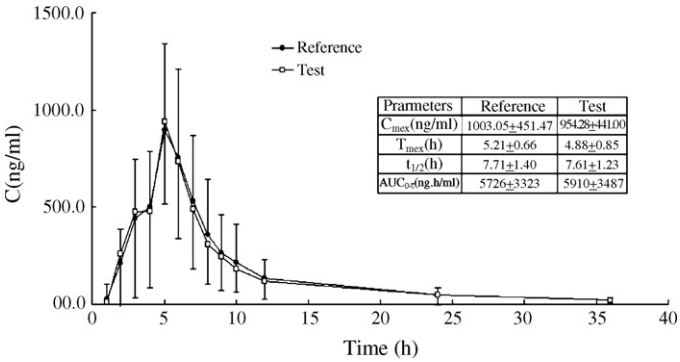

Fig. 5. Mean drug plasma concentration–time curve of ATN in 24 volunteers after oral administration 400 mg ATN capsule.

5. Conclusion

The objective of this work was to develop a sensitive, simple, selective and cost effective method for estimation of ATN in human plasma, especially in the absorption and elimination phase after oral administration of 400 mg formulation. The method employs a simple inexpensive liquid–liquid extraction for sample preparation with quantitative recovery for ATN and IS.

APCI was used for ionization during our analysis. Usually, APCI is preferred over ESI for the analysis of compounds of low polarity and of low molecular weights. APCI is known to be somewhat less mild than ESI due to the fact that the evaporation of mobile phase is supported by a heated nebulizer (350–400 °C). During the APCI process, the mobile phase acts as a reactant gas to ensure ionization. The LC mobile phase in the optimized method contained HCOOH at a concentration of 0.01% which is commonly used, which dramatically increased the APCI efficiency of the analytes and decreased the matrix effects. This is a particularly important finding because increased MS detection sensitivity often relies on the design of new ion sources or mass spectrometers. Thus, we developed a non-instrumental technique for improving the sensitivity limit of MS detection. Furthermore, the applicability of the method was demonstrated in a pilot pharmacokinetic study in human and animals.

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