Development of a UPLC-MS–MS Method for Quantitative Determination of BYYT-25 in Rat Plasma and Its Application to a Pharmacokinetic Study

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Abstract

A highly sensitive and rapid method for the analysis of BYYT-25 ([E]-2-{4-[(tetrahydropyrrole) methyl] benzylidene}-5, 6-dimethoxy-2, 3-dihydroinden-one) in rat plasma using UPLC–MS–MS was developed. The procedure involved a simple liquid–liquid extraction of BYYT-25 and BYYT-8 (internal standard) and separation by UPLC on a Waters BEH C18 column (50 mm × 2.1 mm, 1.7 µm) with a mobile phase consisting of ammonium formate buffer solution (20 mmol/L ammonium formate, 0.2% formic acid)–acetonitrile (76:24, v/v) at a flow rate of 0.25 mL/min. Detection was performed by positive ion electrospray ionization in MRM mode (BYYT-25, 368.3 → 297.3; IS, 384.3 → 297.3.). Specificity, linearity, accuracy, precision, and stability were evaluated. Retention times of BYYT-25 (1.7 min) and internal standard (1.4 min) suggest the potential for high-throughput of the proposed method. The validated method was successfully applied to investigate the pharmacokinetics of BYYT-25 in rats after oral administration of BYYT-25.

Introduction

Alzheimer's disease (AD) is one of the most severe health problems of the aged (1). Acetyl-cholinesterase (AChE) inhibitors are the first and the most developed group of drugs approved for AD symptomatic treatment. BYYT-25 [(E)-2-(4-((tetrahydropyrrole) methyl) benzylidene)-5, 6-dimethoxy-2, 3-dihydroinden-one, see Figure 1] is one of the 2-phenoxy-indan-1-one derivatives tested as acetyl-cholinesterase inhibitors, synthesized by Sheng et al. (2). It has exhibited high AChE inhibitory activity (IC_{50} = 2.66 µM) in in vitro experiments, and the molecular docking study indicated that it was nicely accommodated by AChE. Now, many works have been done for preclinical application of BYYT-25 in the research institute. For example, pharmacodynamic studies (2.0 mg/kg, i.g.) in mice indicated that BYYT-25 had a significant effect on improving spatial learning and memory and toxicology studies in mice suggested that it had low toxicity (LD_{50} was 53 mg/kg) (2).

The commercial success of a new chemical entity (NCE) depends not only on its pharmacological activity but also on its absorption, distribution, metabolism, and excretion (ADME) properties (3). So, it is necessary to develop an excellent assay method for a pharmacokinetic study of the drug at earlier stages of research and development.

For a comprehensive understanding of the pharmacokinetics of BYYT-25, it is crucial to develop a reliable method for the determination of BYYT-25 in rat plasma. The recent introduction of ultra-performance liquid chromatography (UPLC) offers the possibility of significant improving efficiency of the chromatographic separation through the utilization of columns packed with smaller diameters particles (1.7 µm) that can withstand high backpressures compared to the conventional HPLC instrumentation. A mass spectrometer (MS) was introduced as a high sensitive and special instrument for analysis. In this study, therefore, the aim was to develop a sensitive and rapid UPLC-ESI–MS–MS method for the determination of BYYT-25 in rat plasma and test its potential for high-throughput analysis.

Experimental

Chemicals and reagents

BYYT-25 (HPLC purity > 99.0%) and BYYT-8 [as internal standard (IS) HPLC purity > 98.0%] were provided by the Department of Medicinal Chemistry (College of Pharmaceutical Sciences, Zhejiang University, China). Acetonitrile (Excellence, Tedia, Phoenix, AZ) and formic acid (Tedia, Phoenix, AZ) were of

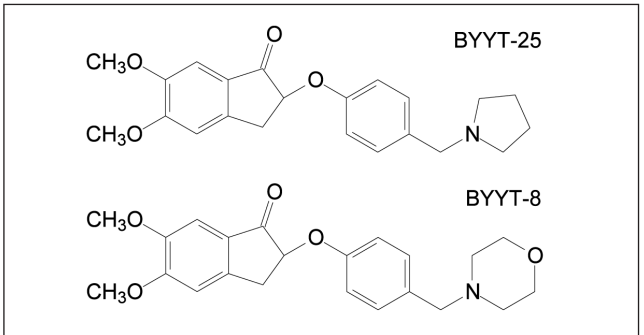

Figure 1. Structures of BYYT-25 and BYYT-8 (IS).

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HPLC grade, ammonium formate and ethyl acetate (Sinopharm Chemical Regent Co., Ltd., Shanghai, China) was of analytical grade. Distilled water and doubly distilled water in the laboratory were used throughout the study.

Sample collection

Rat blood samples were collected by tail vein into tubes containing sodium heparin as anticoagulant before and after BYYZ-25 administration. After centrifugation at 1,200 × *g* for 10 min, plasma was transferred into a new tube and stored at –20°C until analysis.

Sample preparation

In this study, a conventional liquid–liquid extraction method was developed to isolate analytes from plasma samples. After 100 µL of IS solution was transferred into a 1.5 mL plastic test tube and dried in vacuum, 100 µL of plasma sample was transferred into the same test tube and vortex-mixing, and then 1.0 mL of ethyl acetate was added. The analyte and IS were extracted from plasma by vortex-mixing for 1 min. Then the sample was centrifuged at 16,000 × *g* for 8 min and 950 µL of the organic layer was transferred into another test tube and evaporated to dryness in vacuum. Finally, the residue was dissolved in 100 µL mobile phase and centrifuged at 16,000 × *g* for 15 min, 7 µL of supernatant was injected into chromatographic system for analysis.

Equipments and chromatographic conditions

The UPLC system was an Acquity UPLC system (Waters, Milford, MA). The detection system was a TQ detector, equipped with an electro-spray interface operated in triple quadrupole mode. The chromatographic separation was carried on a 1.7 µm BEH C18 column (50 mm × 2.1 mm, Waters) with a mobile phase consisting of ammonium formate buffer solution (20 mmol/L ammonium formate, 0.2% formic acid)–acetonitrile (76:24, v/v) at a flow rate of 0.25 mL/min. The column and autosampler tray temperature were kept constant at 40°C and 4°C, respectively. The mass spectrometer was set at positive ion multiple reactions monitoring (MRM) mode. The multiple reaction monitoring transitions for analytes and IS were as follows: BYYT-25, 368.3 → 297.3; IS, 384.3 → 297.3. Nitrogen was used as the desolvation gas with the settings of 550 L/h. Other settings were as follows: dwell time, 0.5 s; source temperature, 140°C; desolvation gas temperature, 350°C; capillary voltage, 3200 V. The collision gas was argon gas; collision energy was 23 eV. Masslynx V4.1 (Waters) was used for instrument control, data acquisition and data processing.

Preparation of standard solution and quality control samples

A stock solution of BYYT-25 with a concentration of 2500 µg/mL was prepared by dissolving BYYT-25 in methanol. A series of standard working solutions with concentrations in the range of 4–1000 ng/mL for BYYT-25 were obtained by further dilution of the stock solution with methanol. Effective concentrations in plasma samples were 0.4, 1.0, 4.0, 10.0, 40.0, and 100.0 ng/mL. The quality control (QC) samples were similarly prepared at concentrations of 0.9, 9.0, and 90.0 ng/mL with blank rat plasma. A 12 ng/mL IS working solution was prepared by diluting the stock solution of BYYT-8 with methanol. All the solutions were stored at 4°C and were brought to room temperature before use.

Method validation

The limit of detection (LOD) was defined as the amount that could be detected with a signal-to-noise ratio (S/N) of 3. The limit of quantitation (LOQ) was used as the lowest concentration of the standard curve (S/N = 10). Plasma samples were quantified by using the ratio of the peak area of analyte to that of IS as the assay parameter. Standard curves representing peak area ratios versus analyte concentrations were described in the form of $y = a + bx$. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. The accuracy, precision and matrix effect were calculated by determining QC samples at high, middle and low concentration levels. The accuracy was expressed as (mean measured concentration) / (spiked concentration) × 100% and the precision as relative standard deviation (RSD%). The absolute recovery of analyte at three QC concentration levels were determined by comparing the peak areas obtained from QC samples, extracted from plasma, with the un-extracted standard working solutions at the same concentration in the same solvent.

In order to establish a reliable method for the quantitative analyses of BYYT-25 in blood, the stability studies utilizing different procedures were performed. All stability tests were performed with QC samples at three different concentrations (0.9, 9, and 90 ng/mL) in triplicates for each concentration. The short-term storage stability at room temperature for 2 and 10 h and after three freeze–thaw cycles was investigated. The QC samples stored for 5 and 30 days at –20°C were analyzed to investigate the long-term storage stability. The stability of BYYT-25 during the storage in the auto-sampler at room temperature was assessed by injecting a sample at different time intervals (0, 6, and 24 h).

Application

The present method was used to determine the rat plasma concentrations of BYYT-25 after oral administration of BYYT-25. The studies were approved by the Animal Ethics Committee of Zhejiang University. All data were subsequently processed with the computer program DAS 2.0 (Practical Pharmacokinetic Program, China) to determine the compartment models and pharmacokinetic parameters. Six male Sprague–Dawley rats (200–220 g) were obtained from the Center for Experimental Animal, Zhejiang Academy of Medical Sciences (Hangzhou, China). The rats were kept in this laboratory for at least 3 days before use and had free access to the standard laboratory food and water. After a 12 h fast, but with free access to water, the rats were given by intragastric administration at a dose of 5 mg/kg. Then, 0.25 mL blood samples were collected from the tail vein at 0, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 5, 6, 8, 12, and 24 h, and immediately centrifuged to separate 100 µL plasma. The obtained plasma samples were stored at –20°C until analysis.

Results and Discussion

Mass spectrometry

The MS–MS parameters were optimized to get maximum response for BYYT-25 in the positive ion mode. Under ESI conditions, the protonated molecular ions $[M+H]^+$ with *m/z* 368.3 and

m/z 384.5 were identified as the major peaks for BYYT-25 and the IS, respectively. The precursor ions were subjected to collision-induced dissociation with argon gas to produce fragment ions. For BYYT-25, the most intense product ions observed were the ions with *m/z* 297.3, which was chosen as the product ion for monitoring BYYT-25. For the IS, BYYT-8, the product ion with *m/z* 297.3 was also the most abundant ion. Based on these observations, the MS–MS transitions selected were: 368.3 → 297.3 for BYYT-25 and 384.3 → 297.3 for BYYT-8, respectively.

Development of the sample extraction procedure and UPLC conditions

To compare the extraction ratio of BYYT-25 in plasma different organic solvents (such as ethyl acetate, chloroform, diethyl ether, dichlormethane) have been used. It was found that ethyl acetate is the best (data not shown) extraction solution for both BYYT-25 and IS due to the highest recovery and low ion suppression. BYYT-25 and the IS were separated from matrix components using a BEH C18 column and a mobile phase consisted of ammonium formate buffer solution (20 mmol/L ammonium formate, 0.2 % formic acid)–acetonitrile (76:24, v/v) in the ratio of 0.25 mL/min under isocratic conditions. The UPLC conditions were optimized to eliminate interferences from the matrix and to elute the analyte and IS within a short run time (< 2.5 min, show in Figure 2), thereby decreasing the total time for sample analysis. The method showed good detection level (LOD = 0.2 ng/mL) for BYYT-25 in plasma. The typical chromatogram of BYYT-25 and IS are presented in Figure 2. Under the described chromatographic conditions, a good separation between this drug and IS was achieved and no obvious interferences from endogenous plasma substances were observed. The retention time of BYYT-25 and IS were 1.7 min and 1.4 min, respectively.

Matrix effect

The matrix effects for BYYT-25 at concentrations of 0.9, 9.0, and 90.0 ng/mL were 5.7%, 6.4%, and 7.1%, respectively (shown in Table I). The matrix effects for IS (12 ng/mL in plasma) were 4.9%. These results showed that ion suppression or enhancement from plasma matrix was negligible in the present condition.

Linearity and lower limit of quantitation

A six-point standard curve was generated over the range of 0.4–100 ng/mL, and the resultant curve of area ratio (area BYYT-25/area IS) versus nominal BYYT-25 concentration was interpolated using a least-squares linear regression. The regression equation of curve regression parameters are y = 0.1299x + 0.0055, $r^2 > 0.9999$, and the precision of six point on standard curves < 14.4%. The precision was expressed as the relate standard deviation (RSD) and the accuracy was measured as percent analytical recovery relative to the nominal concentration. The limit of quantitation is 0.4 ng/mL (RSD% = 6.7%, *n* = 5).

Table I. Absolute Recovery, Method Recovery, and Matrix Effect for the Three Analytes in Spiked Rat Plasma Samples (*n* = 3)

	Absolute recovery		Method recovery		Matrix effect	
Spiked conc. (ng/mL)	recovery %	RSD %	recovery %	RSD %	recovery %	RSD %
0.9	90.9	2.4	99.3	3.6	94.3	2.4
9	87.7	3.2	93.5	6.0	93.6	3.3
90	88.2	6.5	101.3	5.6	92.9	2.4

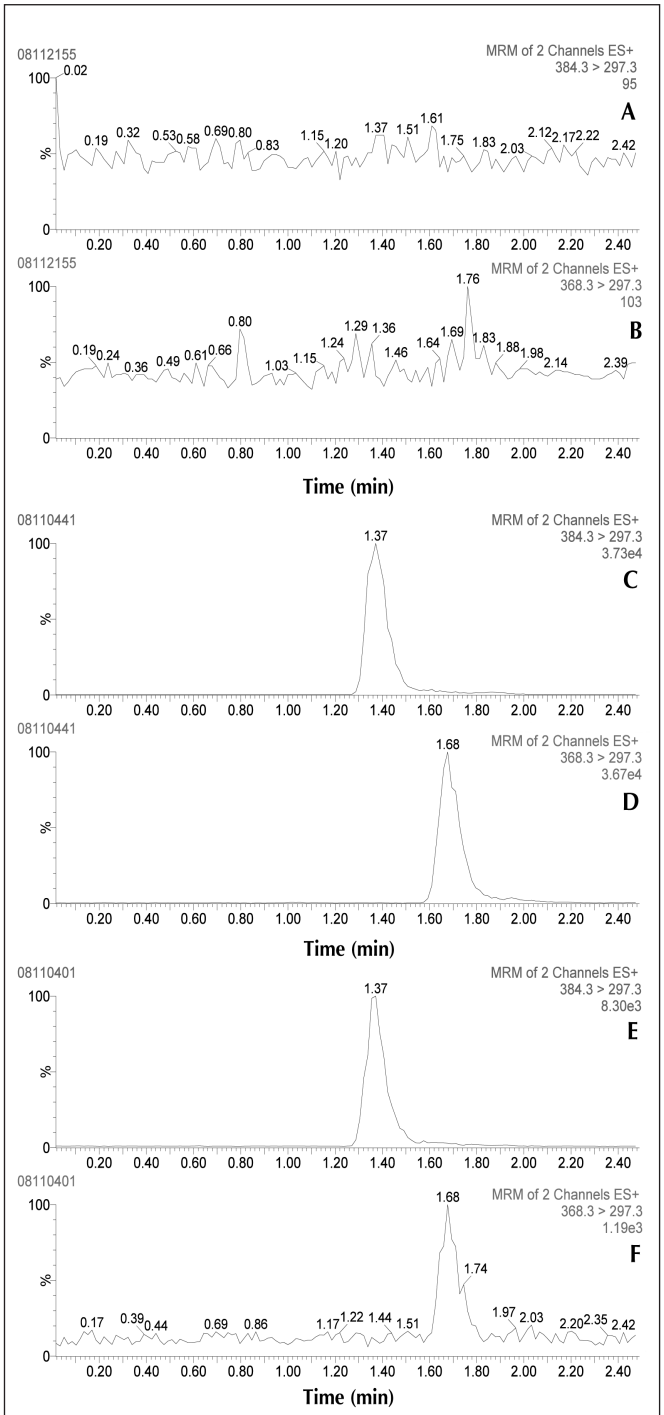

Figure 2. UPLC–MS–MS chromatograms of BYYT-25. Blank rat plasma sample (A and B); real sample at 120 min after a single oral administrating of 5 mg/kg BYYT-25 (C and D); LOQ of BYYT-25 in rat plasma (0.4 ng/mL) (E and F). Peaks of 1.37 and 1.68 min are IS and BYYT-25, respectively.

Extraction recovery, accuracy and precision

The extraction recoveries of BYYT-25 in rat plasma were shown in Table II. At three concentration levels of BYYT-25, the extraction recoveries were all more than 87%. The extraction recovery of IS was approximately 86%. Table II summarizes the intra- and inter-day precisions and accuracies of BYYT-25 at different concentration levels. As shown in Table II, the intra- and inter-day accuracies were within the range of 97.0–99.6% and 96.1–98.4%, respectively. The intra- and inter-day precisions (RSD%) were all less than 7.9%. The results demonstrated that the values were all within the acceptable range (< 15%) and the method was accurate and precise.

Table II. Inter- and Intra-day Precision (RSD%) and Accuracy (%) for the Three Analytes in Spiked Rat Plasma Samples (*n* = 5)

Spiked conc. (ng/mL)	Inter-day repeatability		Intra-day repeatability	
	Accuracy %	RSD %	Accuracy %	RSD %
0.9	97.0	1.3	98.4	3.6
9	99.6	3.1	96.7	6.3
90	99.6	5.3	96.1	7.9

Stability

The stability tests of the analyte were designed to cover anticipated conditions for the preservation of the samples. The stability results showed that BYYT-25 spiked into rat plasma was stable for 10 h at room temperature, for 30 days at –20°C, and during three freeze–thaw cycles. The stability of BYYT-25 extracts in the sample solvent on autosampler was also observed over a 24 h period. The results of the stability experiments are shown in Table III and IV.

Table III. Stability Results of BYYT-25 Stored in Rat Plasma at Room Temperature and Treated Samples During the Storage in the Auto-sampler (*n* = 3)

Conc. of spiked (ng/mL)	Recovery after Storage at room temperature (% of 0 h)				Recovery after storage in the auto-sampler (% of 0 h)			
	2 h		10 h		6 h		24 h	
	Accuracy %	RSD %	Accuracy %	RSD %	Accuracy %	RSD %	Accuracy %	RSD %
0.9	97.3	9.9	99.1	14.0	100.2	8.2	96.9	8.4
9.0	98.9	9.3	97.4	8.8	99.5	6.1	98.2	4.3
90.0	98.2	2.8	99.5	3.6	98.7	4.8	99.4	2.6

Pharmacokinetic study

The validated analytical method was applied to the assay of BYYT-25 in rat plasma after a single oral administration to rat of 5 mg/kg BYYT-25. The mean plasma concentration versus time profile is presented in Figure 3. The main pharmacokinetic parameters of BYYT-25 in six rats were calculated. After oral administration of 5mg/kg BYYT-25, T_{max} and C_{max} of BYYT-25 were found to be 2.4 ± 1.3 h and 22.1 ± 6.1 ng/mL, respectively. Plasma concentration declined with $t_{1/2}$ of 6.4 ± 1.9 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 223.8 ± 30.3 and 245.4 ± 34.4 ng/mL h, respectively.

Table IV. Stability Results of BYYT-25 in Rat Plasma Stored at –20°C and Three Cycles of Freeze-Thaw (*n* = 3)

Conc. of spiked (ng/mL)	5 days		30 days		Three freeze–thaw cycles	
	Accuracy %	RSD %	Accuracy %	RSD %	Accuracy %	RSD %
0.9	96.8	14.1	99.0	11.5	88.9	11.7
9	98.3	0.3	96.9	7.7	91.2	3.1
90	96.8	0.4	87.3	0. 6	86.3	6.2

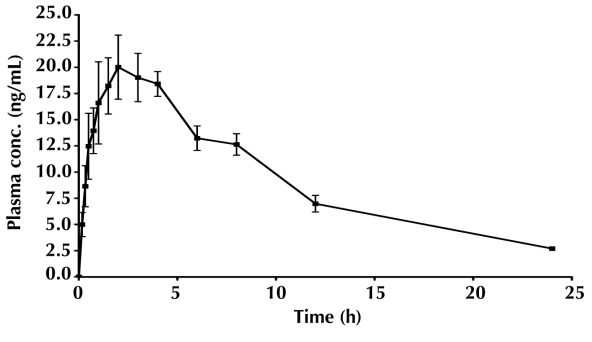

Figure 3. Mean plasma concentration-time profile of BYYT-25 after a single oral dose of 5 mg/kg BYYT-25 to six Sprague–Dawley rats.

Conclusion

In the present study, a sensitive and rapid UPLC-ESI–MS–MS method for the determination of BYYT-25 in rat plasma has been developed and validated. A simple liquid–liquid extraction procedure has been used to prepare the samples with a high recovery. The established assay has been proved to be precise, accurate, specific, and reproducible and has been successfully applied to the pharmacokinetic study of BYYT-25 in six SD rats.

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