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Liquid chromatography–tandem electrospray mass spectrometry method for determination of serial chiral novel anticholinergic compounds of phencynonate in rat plasma

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Abstract

A sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed for the determination of serial chiral novel anticholinergic compounds of phencynonate in rat plasma. After a simple protein-precipitation using methanol, the post-treatment samples were separated on a CAPCELL UG120 column with a mobile phase of a mixture of methanol and water (35:65) containing 0.1% formic acid. The serial chiral analytes and internal standard (IS) were all detected by the use of selected reaction monitoring mode (SRM). The method of all serial chiral analytes developed was validated in rat plasma with a daily working range of 0.5–100 ng/ml with correlation coefficient, $R^2 \geq 0.99$ and a sensitivity of 0.5 ng/ml as lower limit of quantification, respectively. This method was fully validated for the accuracy, precision and stability studies for all serial chiral analytes. The method proved to be accurate and specific, and was applied to the pharmacokinetic study of serial chiral novel anticholinergic compounds of phencynonate in rat plasma.

Keywords: Anticholinergic compounds; Serial chiral analytes; Liquid chromatography–tandem electrospray mass spectrometry; Rat plasma; Quantification

1. Introduction

The ETH6(R S), ETH7(R S), ETH15(R S) and ETH16(R S) are the serial chiral novel anti-cholinergic compounds of phencynonate developed by our Institute of Pharmacology and Toxicology in China. Pharmacological evaluation has proved that they can prevent acute motion sickness with an efficacy similar to that of scopolamine [1–14]. Determination of the pharmacokinetic profile of the serial chiral novel anti-cholinergic compounds is important for gaining better understanding of their mechanism of action and fast screening the compound with good pharmacokinetic characteristics. Because of the low efficiency dose of them, a sensitive analytical method is needed for their determination in blood after oral administration. Liu et al. [10] has developed a gas chromatographic method with mass spectrometry to determination phencynonate in animal blood and the sample preparation and extraction and analytical procedure were time-consuming. The method reported here was validated to ensure proper quantification of the serial chiral novel anticholinergic compounds in rat blood with a concentration limit of 0.5 ng/ml in rat blood. This method was sensitive, specific and very fast so that it can be applied to determine the low concentrations of the serial chiral novel anti-cholinergic compounds in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

The ETH06, ETH07, ETH15, ETH16 and naloxone hydrochloride, the internal standard (IS), were kindly supplied by the Beijing Institute of Pharmacology and Toxicology of China. The purity of ETH06, ETH07, ETH15 and ETH16 and the IS were all more than 99%. Their chemical structures are shown in Fig. 1. Methanol used was of HPLC grade and was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Formic acid was of HPLC grade and purchased from Sigma-Aldrich (Shanghai, China). Water was triply distilled. The above solutions were filtered through 0.2 μm (organic) or 0.45 μm

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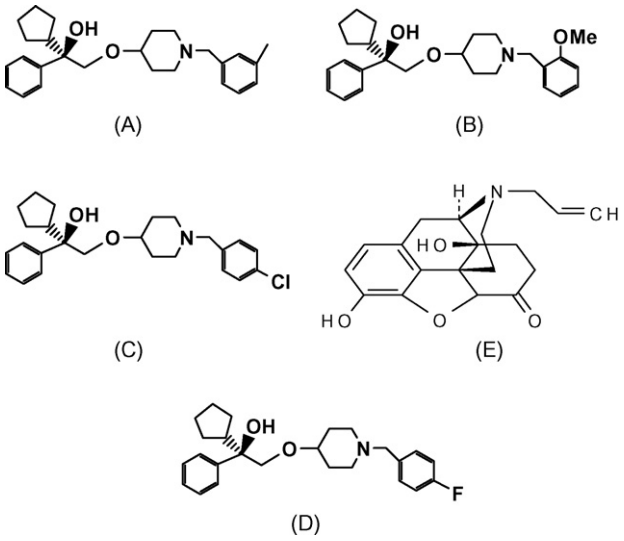


Fig. 1. The chemical structures of ETH06 (A), ETH07 (B), ETH15 (C) AND ETH16 (D) and the internal standard naloxone hydrochloride (E).

(water) membranes. All other reagents and chemicals were of analytical grade.

2.2. Instrumentation

Surveyor AS HPLC system with Surveyor LC Pump and TSQ Quantum Discovery MAX triple–quadruple mass spectrometer (Thermo, USA) equipped with ESI source was used. The chromatography was performed on a CAPCELL UG120 column (100 mm × 2.0 mm i.d., 5 μm, Janpan) at ambient temperature. A guard column was used to protect the analytical column. The mobile phase was composed of methanol and water (35:65, v/v), containing 0.1% formic acid, which was pumped at a flow-rate of 0.2 ml/min. The sample injection volume was 10 μl. The detection was performed in positive ionization mode. Nitrogen was used as a sheath gas and ultra-high purity helium as the dampening gas in the Q_2. The ESI source voltage was set at 4.5 kV and sheath gas flow-rate and auxiliary gas flow-rate were 10 and 2 psi, respectively; capillary temperature 320 °C. The Q_1 and Q_3 peak widths were set to 0.7 FWHM. When running collision-induced dissociation (CID), the pressure was set to 1.5 mTorr. For selected reaction monitoring (SRM) mode, the following transitions were recorded: ETH6(R) *m/z* 394 $[M+H]^+ \rightarrow$ *m/z*105@-36 eV, ETH7(R) *m/z* 410 $[M+H]^+ \rightarrow$ *m/z*121@-34 eV, ETH15(R) *m/z* 386 $[M+H]^+ \rightarrow$ *m/z*180@-26 eV, ETH16(R) *m/z* 398 $[M+H]^+ \rightarrow$ *m/z*109@-38 eV; ETH6(S) *m/z* 394 $[M+H]^+ \rightarrow$ *m/z*105@-36 eV, ETH7(S) *m/z* 410 $[M+H]^+ \rightarrow$ *m/z*121@-34 eV, ETH15(S) *m/z* 386 $[M+H]^+ \rightarrow$ *m/z*180@-26 eV, ETH16(S) *m/z* 398 $[M+H]^+ \rightarrow$ *m/z*109@-38 eV; IS *m/z* 328 $[M+H]^+ \rightarrow$ *m/z*310@-35 eV. The LC system and mass spectrometer were controlled using the Thermo Finnigan Chemstation software (Xcalibur version 1.4). Data was processed using the IS method of plotting peak area ratios versus relative analyte/IS concentration with a weighting factor $1/x^2$.

2.3. Materials

Male Wister rats, weighing 200 ± 20 g, were obtained from Animal Center o f Academy Military Medical Sciences (Beijing, China) and were housed under standard laboratory conditions with free access to water and feed. Experiments were done after the approval from the local animal care committee. Drug-free plasma was obtained from the healthy rats and stored at −20 °C and then thawed room temperature for use in calibration curves and quality control samples.

2.4. Sample preparation

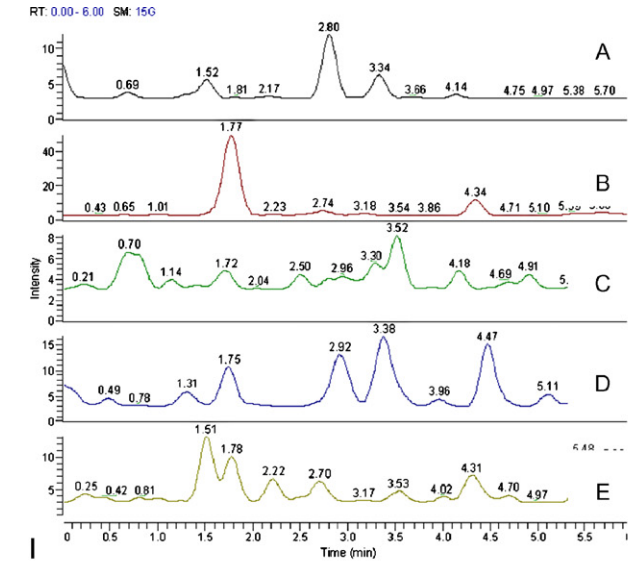
Stock solutions were prepared by dissolving 10 mg of ETH6(R), ETH7(R), ETH15(R), ETH16(R), ETH6(S), ETH7(S), ETH15(S), ETH16(S) and the IS in 10 ml of HPLC-grade methanol respectively. Working standard solution was prepared by serial diluting of the stock solution using the methanol. Quality control (QC) samples were also prepared in the same way, using a separately weighed stock solution. All solutions were stored at 4 °C before use.

Blood samples (0.1 ml) were spiked with 10 μl mixture of ETH6(R), ETH7(R), ETH15(R), ETH16(R) and 10 μl of IS stock solution (100 ng/ml). Then methanol was added to precipitate protein. The combined samples were vortex-mixed for 1 min and centrifuged at 10,000 rpm for 10 min. The upper organic phase 100 μl was transferred to HPLC auto-sampler vials, and then10 μl was injected into the LC–MS/MS system.

Sample preparation of the ETH6(S), ETH7(S), ETH15(S), ETH16(S) is the same as the ETH6(R), ETH7(R), ETH15(R), and ETH16(R).

2.5. Method validation

Calibration samples in plasma were prepared by mixing solutions of ETH6(R), ETH7(R), ETH15(R), ETH16(R) (or ETH6(S), ETH7(S), ETH15(S), ETH16(S))and IS with rat blank plasma to form a concentration series of 0.5, 2, 10, 40, 100 ng/ml ETH6(R), ETH7(R), ETH15(R), ETH16(R) (or ETH6(S), ETH7(S), ETH15(S), ETH16(S)) and 10 ng/ml IS. Intra-day accuracy and precision (each $n=5$) were tested by analysis of the QC samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of the same samples over five consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. The precision and accuracy of the method were calculated as the relative standard deviation (R.S.D.) and the percentage deviation of observed concentration from theoretical concentration, respectively. The extraction recovery was determined by calculating the ratio of the area of extracted compound from drug-free plasma spiked with known amounts of serial chiral analytes to the area of compound added at the same concentrations to mobile phase solution. To evaluate the three freeze-thaw cycle stability and room temperature matrix stability, six replicates of QC samples at three levels were subjected to three freeze-thaw cycles or were stored at room temperature for 48 h before processing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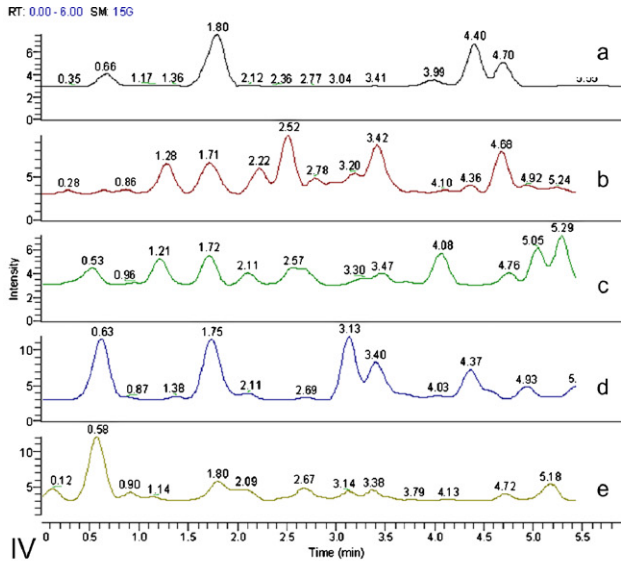
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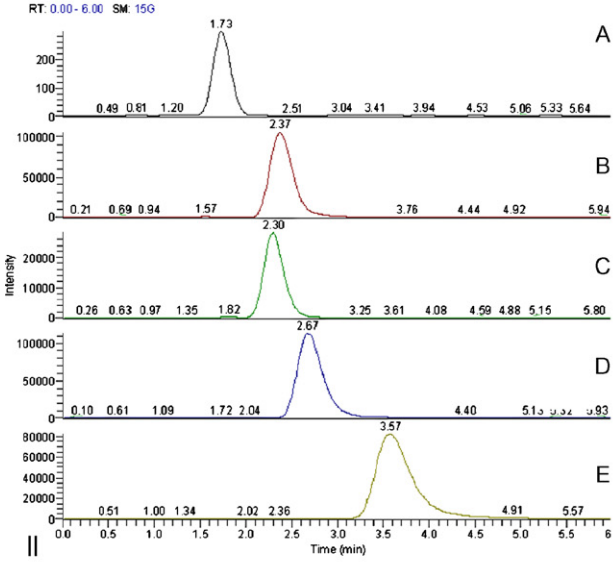
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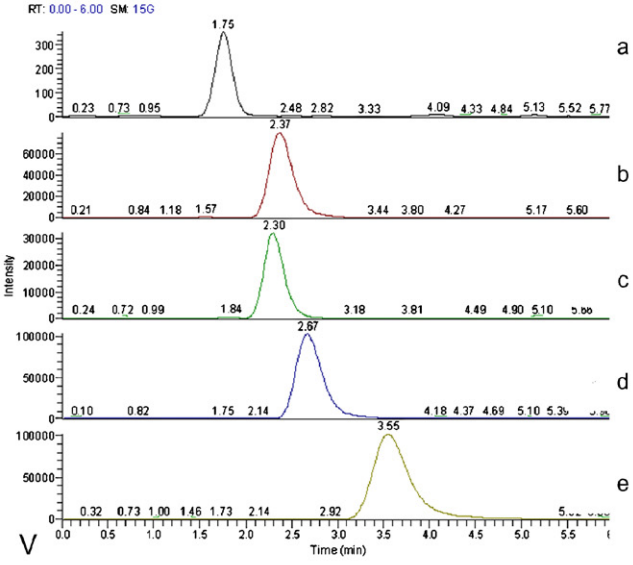
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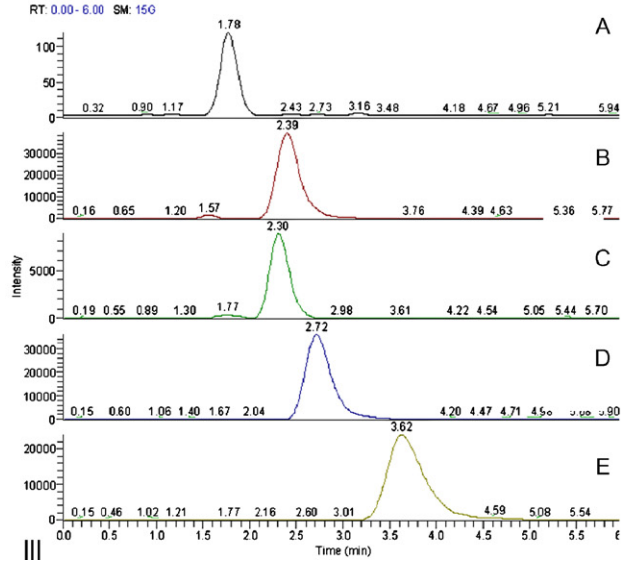
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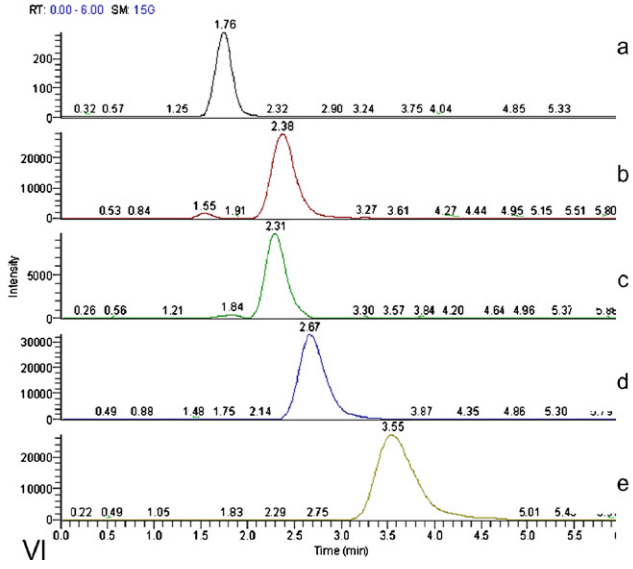
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Intensity
Time (min)
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respectively. Six replicates of QC samples were processed and stored in autosampler for 24 h, then assayed to assess processed sample stability. Stability is considered acceptable if the mean value is within 15% of the theoretical value at each concentration.

2.6. Pharmacokinetic studies

The LC–MS/MS method was successfully applied to pharmacokinetic studies of the serial chiral analytes in Wister rats. Diet was prohibited for 12 h before the experiment while water was taken freely. Blood was collected from the orbital vein of the rats before and after receiving a single oral dose of the mixture of the serial chiral analytes including ETH6(R), ETH7(R), ETH15(R), ETH16(R) or ETH6(S), ETH7(S), ETH15(S), ETH16(S) (0.4 mg/kg, respectively). Approximately 0.25 ml blood was collected in heparinized tubes before drug administration and post-dose at 10, 30, 60, 120, 240, 480, 720, and 1440 min, respectively. Five experiments using five rats were done for each compound in the pharmacokinetic study. All blood samples were sealed and stored at −20 °C until analysis. The blood samples were extracted as above.

3. Results and discussion

3.1. Method development

HPLC–MS/MS is a powerful technique and now is widely used in biological analysis. Sample preparation plays a key role for determination of analytes in biological samples. After several trials, a protein precipitation using methanol extraction method was found to be suitable for the determination of serial chiral analytes in rat plasma. The method was adopted and proved to be reliable for sample preparation in this experiment. Methanol rather than other solvent was selected as protein-precipitation for compatibility with the mobile phase to produce symmetric peak shapes for the analytes and IS. This procedure produced a clean chromatogram for plasma sample.

A CAPCELL UG120 column was used for the chromatographic separation and a guard column was used to protect the analytical column. Other chromatographic conditions, especially the composition of mobile phase, were optimized through several trails to obtain good resolution and symmetric peak shapes, as well as a short run time. It was found that a mixture of methanol and water (35:65, v/v) with 0.1% formic acid could achieve these goals and was finally adopted as the mobile phase for the chromatographic separation. An internal standard is necessary for the determination of analytes in biological samples and naloxone was found to be optimal for an IS.

3.2. Selectivity

The selectivity of the method was assessed by analyzing six different plasma extracts. As exemplified in Fig. 2, no interfering peaks were observed at the retention time and/or at the specific detection window of each analyte. The above rapid method gave very good selectivity for the analysis of serial chiral analytes and IS in the blank plasma. The retention time (RT) was short that makes it suitable for routine analysis.

3.3. Linearity

The calibration curve of serial chiral analytes in rat plasma was constructed in the range 0.5–100 ng/ml. The regression equations of the curve were calculated as follows: $y = 5248.99 + 12706.6x$ ($R^2 = 0.9904$, W: $1/\chi^2$) for ETH6(R), $y = 2710.8 + 16765.8x$ ($R^2 = 0.9939$, W: $1/\chi^2$) for ETH7(R), $y = 7461.11 + 16351.2x$ ($R^2 = 0.9943$, W: $1/\chi^2$) for ETH15(R), $y = -2419.29 + 4624.85x$ ($R^2 = 0.9934$, W: $1/\chi^2$) for ETH16(R); $y = -1005.52 + 16946.76x$ ($R^2 = 0.9980$, W: $1/\chi^2$) for ETH6(S), $y = -6146.71 + 17688.4x$ ($R^2 = 0.9936$, W: $1/\chi^2$) for ETH7(S), $y = -7821.14 + 15577x$ ($R^2 = 0.9953$, W: $1/\chi^2$) for ETH15(S), $y = -2434.6 + 3985.03x$ ($R^2 = 0.9911$, W: $1/\chi^2$) for ETH16(S). Where x was concentration of serial chiral analytes (ng/ml) and y was the ratio of serial chiral analytes peak area to IS peak area. It showed good liner relationships between the ratio and the concentrations. The assay proved to be linear and acceptable. Good linearity was observed over the concentration ranges of 0.5–100 ng/ml for rat plasma.

3.4. Precision and accuracy

The precision and accuracy of the method were assessed in rat plasma by performing replicate analyses of spiked samples against calibration standards. The procedure was repeated on the same day and for different days on the same spiked standard series. The intra-day and inter-day precision and accuracy of the method are presented in Table 1. The data indicates that the precision and accuracy of the method are acceptable.

The lower limit of quantification was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±15% and a precision below 15% were obtained. The present LC–MS/MS method offered an LLOQ of 0.5 ng/ml in rat plasma sample. Under the present LLOQ of 0.5 ng/ml, the serial chiral analytes concentration can be determined in plasma samples after a single oral dose of 0.4 mg mix of ETH6(R) ETH7(R), ETH15(R), ETH16(R) or mix of ETH6(S), ETH7(S), ETH15(S), and ETH16(S), respectively, which is sensitive enough for the pharmacokinetic study of the serial chiral analytes.

Fig. 2. Representative MRM chromatograms: (I, IV) blank plasma sample without analytes added, (II, V) blank plasma sample with analytes (10 ng/ml) added, (III, VI) unknown sample drawn at 30 min after oral administration of 0.4 mg analytes. SRM of m/z 394 → m/z105@-36 eV for ETH6(R), (B) SRM of m/z 410 → m/z121@-34 eV for ETH7(R), (C) SRM of m/z 386→m/z180@-26 eV for ETH15(R), (D) SRM of m/z398 → m/z109@-38 eV for ETH16(R), (E) SRM of m/z328 → m/z310@-35 V for IS. (a) SRM of m/z 394 → m/z105@-36 eV for ETH6(S), (b) SRM of m/z 410 → m/z121@-34 eV for ETH7(S), (c) SRM of m/z 386 → m/z180@-26 eV for ETH15(S), (d) SRM of m/z398 → m/z109@-38 eV for ETH16(S). (e) SRM of m/z328 → m/z310@-35 V for IS.

Table 1
Precision and accuracy of the LC–MS/MS analysis of the serial chiral analytes

Compounds	Theoretical concentration (ng/ml)	*n*	Recovery (%)	Within-day precision (R.S.D., %)	Between-day precision (R.S.D., %)	Accuracy percent error (%)
ETH6(R)	2	5	96.8	6.5	6.8	−8.7
	10	5	98.2	5.3	4.2	6.2
	40	5	98.4	2.5	3.6	4.8
ETH7(R)	2	5	92.3	8.6	9.6	12.5
	10	5	94.6	5.4	7.7	10.0
	40	5	95.0	3.5	8.3	−5.7
ETH15(R)	2	5	93.1	5.8	9.3	7.8
	10	5	94.2	4.6	10.5	−9.1
	40	5	95.6	10.2	2.5	2.3
ETH16(R)	2	5	89.2	5.9	12.6	−13.4
	10	5	90.5	4.7	8.2	10
	40	5	95.3	4.1	7.5	6.1
ETH6(S)	2	5	88.6	3.2	8.5	9.9
	10	5	89.8	4.4	7.6	8.5
	40	5	92.1	1.2	5.3	−7.4
ETH7(S)	2	5	88.7	9.2	8.8	11
	10	5	85.9	6.4	7.9	−9.4
	40	5	91.8	1.9	2.3	−2.4
ETH15(S)	2	5	90.6	13.5	8.8	5.4
	10	5	96.7	12.4	9.0	8.6
	40	5	97.3	5.2	7.3	5.4
ETH116(S)	2	5	86.7	14.1	10.1	−6.9
	10	5	89.5	6.1	9.2	7.4
	40	5	92.8	7.6	4.8	2.8

3.5. Recovery

The extraction recovery was determined for five replicates of rat plasma spiked with low, medium and high concentrations of serial chiral analytes. The results are summarized in Table 1. The data indicates that the recovery of serial chiral analytes from rat plasma was concentration-independent in the concentration range evaluated.

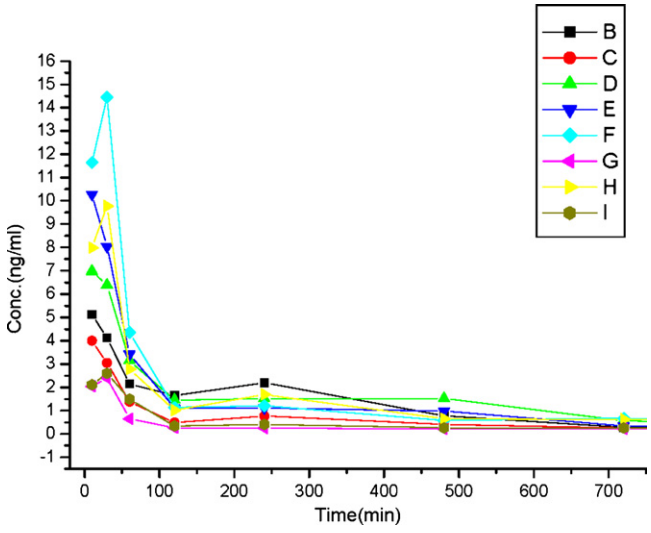

Fig. 3. Pharmacokinetic profile of the serial chiral analytes following administration of a single oral dose (0.4 mg/kg) to rats. (ETH6(R)-B, ETH7(R)-C, ETH15(R)-D, ETH16(R)-E, ETH6(S)-F, ETH7(S)-G, ETH15(S)-H, ETH16(S)-I).

3.6. Stability

The stability of the analytes in rat plasma under different conditions was evaluated as follows: Firstly, allowed to stand at ambient temperature for at least 48 h before extraction; Secondly, allowed to stand at ambient temperature in the autosampler for at least 24 h after extraction; Thirdly, subjected to three freeze-thaw cycles for at least 3 days.

3.7. Pharmacokinetics of the serial chiral analytes

The LC–MS/MS method showed satisfactory results for the determination of the serial chiral analytes in rat plasma and can be successfully used for the pharmacokinetic study of the serial chiral novel anticholinergic compounds of phencynonate following oral administration to rats. The plasma concentration–time profiles for the serial chiral analytes are shown in Fig. 3.

4. Conclusion

A method was developed for quantification of serial chiral novel anticholinergic compounds of phencynonate in rat plasma by HPLC–MS/MS. The method was rapid and identification and quantification could be done at the same time. The protein

precipitation using methanol extraction method was used and the extraction recovery exceeded 90%. The assay had a good sensitivity and repeatability to fit the need of determination. The selectivity of this method was shown to be excellent, with no interference from other analytes. The LC–MS/MS method has been successfully applied to further pharmacokinetic studies.

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