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Simultaneous determination of multiple angiotensin type 1 receptor antagonists and its application to high-throughput pharmacokinetic study: Cassette dosing versus cassette analysis

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Abstract

A rapid and sensitive high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ESI–MS/MS) detection was developed for the simultaneous determination of multiple angiotensin type 1 receptor antagonists (AT1RAs) WX472, WX581, 1b and telmisartan in rat plasma for the purpose of high-throughout pharmacokinetic screening. The method was operated under selected reaction monitoring (SRM) mode in the positive ion mode. The analytes and the internal standard (pitavastatin) were extracted from 100 μL rat plasma under acidic conditions by liquid–liquid extraction with ethyl acetate. The analytes and internal standard were baseline separated on a Gemini analytical column (3 μm, 150 mm × 2.0 mm) with the adoption of a gradient elution using acetonitrile and 0.05% aqueous formic acid. The standard curves were linear in the concentration ranges of 4.5–900 ng/mL for WX472, 5–1000 ng/mL for WX581 and 0.5–100 ng/mL for 1b and telmisartan. Intra- and inter-batch precisions (R.S.D.%) were all within 15% and the method assessed a quite good accuracy (R.E.%). Recoveries were found to be >65% for all the compounds and no obvious matrix effects were found. This method has been successfully applied to the high-throughput pharmacokinetic screening study for both cassette dosing and cassette analysis of four compounds to rats. Significant drug–drug interactions were observed after cassette dosing. The study suggested that cassette analysis of pooled samples would be a better choice for the high-throughput pharmacokinetic screening of angiotensin type 1 receptor antagonists.

Keywords: LC–MS/MS; Pharmacokinetic screening; Cassette analysis; Cassette dosing

1. Introduction

The development of new therapeutic drugs is a long and costly process. With the introduction of combinatorial chemistry, genomics, parallel synthetic and proteomics, high-throughput screening and other technologies have increased the speed of lead generation. However, identification of lead compounds, which have good pharmacokinetic and toxicological profiles in animals, appears crucial in the selection of viable targets from millions of compounds at the early stage. This leads to the birth of novel concepts for performing pharmacokinetic studies, i.e., cassette dosing and cassette analysis.

Cassette dosing (N-in-one) is a technique that a number of analytes are administered to a single animal and analytes are quantitated simultaneously. It is common to study four to six compounds at a time in rats to rapidly acquire preliminary pharmacokinetic information. Based on the pharmacokinetic results in cassette dosing, promising compounds are then studied individually to acquire more accurate pharmacokinetic parameters. Although cassette dosing seems to be a good way to simulta-

Abbreviations: AT1RAs, angiotensin type 1 receptor antagonists; LC–MS/MS, high performance liquid chromatography-electrospray tandem mass spectrometry; QC, quality control; LLOQ, lower limit of quantification; SRM; selective reaction monitoring; LLE, liquid–liquid extraction; CMC-Na, sodium carboxymethyl cellulose; PK, pharmacokinetic; IS, internal standard.

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neously screen many compounds, the potential for drug–drug interactions is a serious problem. The potential drug–drug interactions are usually raised from enzyme induction, pharmacological and toxicological effects on organ blood flows and clearances, heteroactivation of clearance pathways and competition for clearance pathways, plasma protein binding and net absorption [1–7]. As for cassette analysis, the samples are combined only when they are ready to be assayed. Samples from the same time point for all compounds are pooled and simultaneously analyzed. This one-in-one in vivo approach is devoid of potential drug–drug interactions as N-in-one in vivo pharmacokinetics [8,9]. However, it dose not provide the most important advantage of cassette dosing, such as the reduction in animal handling and use.

WX472 and WX581 are angiotensin type 1 receptor antagonists (AT1RAs) which are the first major innovation in hypertension management in over a decade. AT1RAs have been proposed as alternatives to the more traditional angiotensin converting enzyme (ACE) inhibitors because they selectively block the renin–angiotensin system and their specificity of action and excellent side-effect profile provide good conditions for patient compliance as well as high effectiveness [10,11]. WX472 and WX581 are designed and synthesized by application of the principle of bioisosterism and hybrid approach, and the IC_{50} of antagonistic activity is assessed by the ability of the target compounds inhibiting AT1 induced contraction of a rabbit's aortic ring. They are derived from losartan and irbesartan, respectively. The IC_{50} of irbesartan, losartan, WX472 and WX581 are 7.9, 9.1, 1.2 and 5.6 nmol/L. The pharmacokinetic properties of 1b and telmisartan have been studied and here they are chosen as standards to monitor the pharmacokinetic study of WX472 and WX581.

The main aim of the present work is to introduce a sensitive LC–MS/MS method using LLE and pitavastatin as internal standard for the simultaneous determination of AT1RAs in rat plasma which increases the efficiency of ionization and simplifies the mobile phase. Secondly, the LC–MS/MS method was applied to generate oral pharmacokinetic profiles of these candidate compounds in male Sprague–Dawley rats to illustrate the applicability of high-throughput method concept and compare the PK data of cassette analysis and cassette dosing.

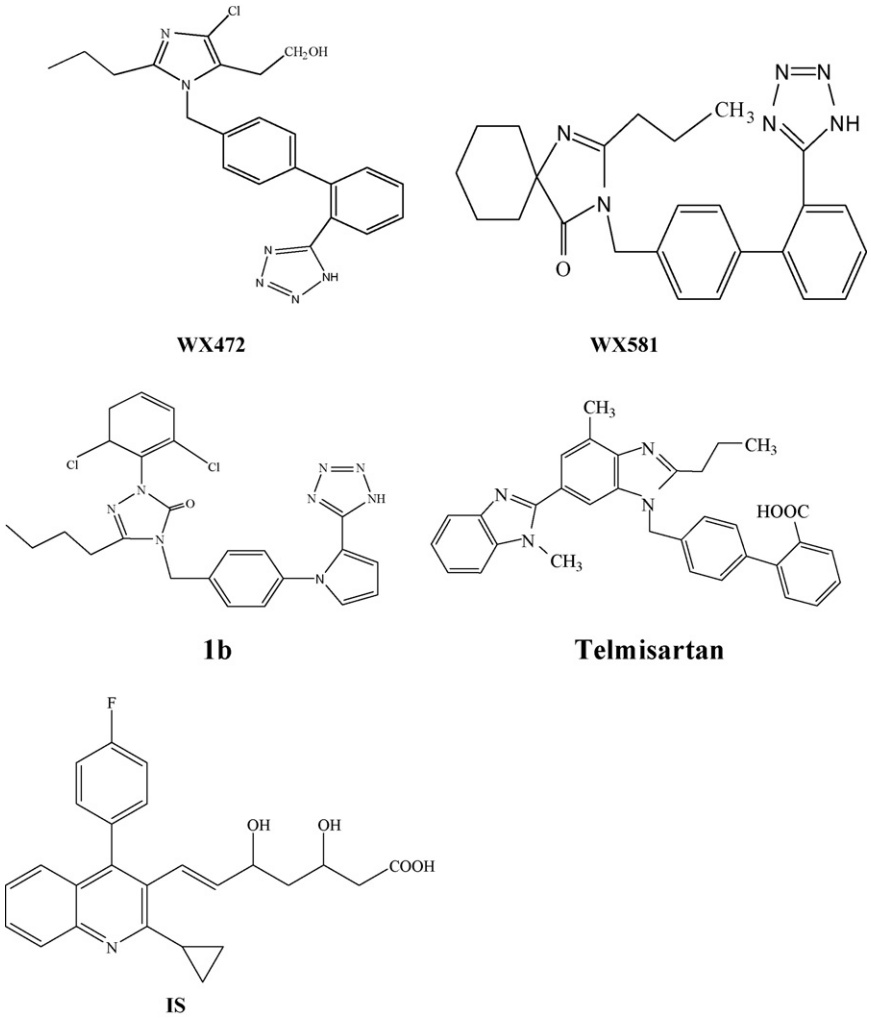

Fig. 1. Chemical structures of AT1RA compounds and pitavastatin (IS).

2. Experimental

2.1. Material and methods

2.1.1. Chemicals and reagents

The AT1RAs under study were—telmisartan: 4-[(2-*n*-propyl-4-methyl-6-(1-methylbenzimidazole-2-yl)-benzimidazole-1-yl)methyl]-biphenyl-2-carboxylicacid, 1b: 5-*n*-butyl-4-{4-[2-(1H-tetrazole-5-yl)-1H-pyrrol-1-yl]phenylmethyl}-2,4-dihydro-2-(2,6-dichloridephenyl)-3H-1,2,4-triazol-3-one, WX472: 2-propyl-4-chloro-5-(hydroxyethyl)-1-[[2-(1H-tetrazol-5-yl) biphenyl-4-yl]methyl]imidazole, WX581: 2-propyl-3-[4-[2-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1,3-diazaspiro[4,4] dec-1-en-4-one. 1b (MW = 508), WX472 (MW = 422), WX581 (MW = 428), were kindly provided by the Department of Medicinal Chemistry of China Pharmaceutical University. Telmisartan (MW = 514) and pitavastatin (internal standard, MW = 421), were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of all chemicals was above 99% and their chemical structures are shown in Fig. 1. HPLC grade acetonitrile was obtained from Fisher Scientific (Inc., USA). Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Ethyl acetate and other chemicals and solvents used were of analytical grade.

2.1.2. Instrumentations and operating conditions

LC experiments were conducted using a Finnigan Surveyor^{TM} HPLC system (Thermo Electron, San Jose, CA, USA). Separation of analytes was achieved using a Gemini ana-

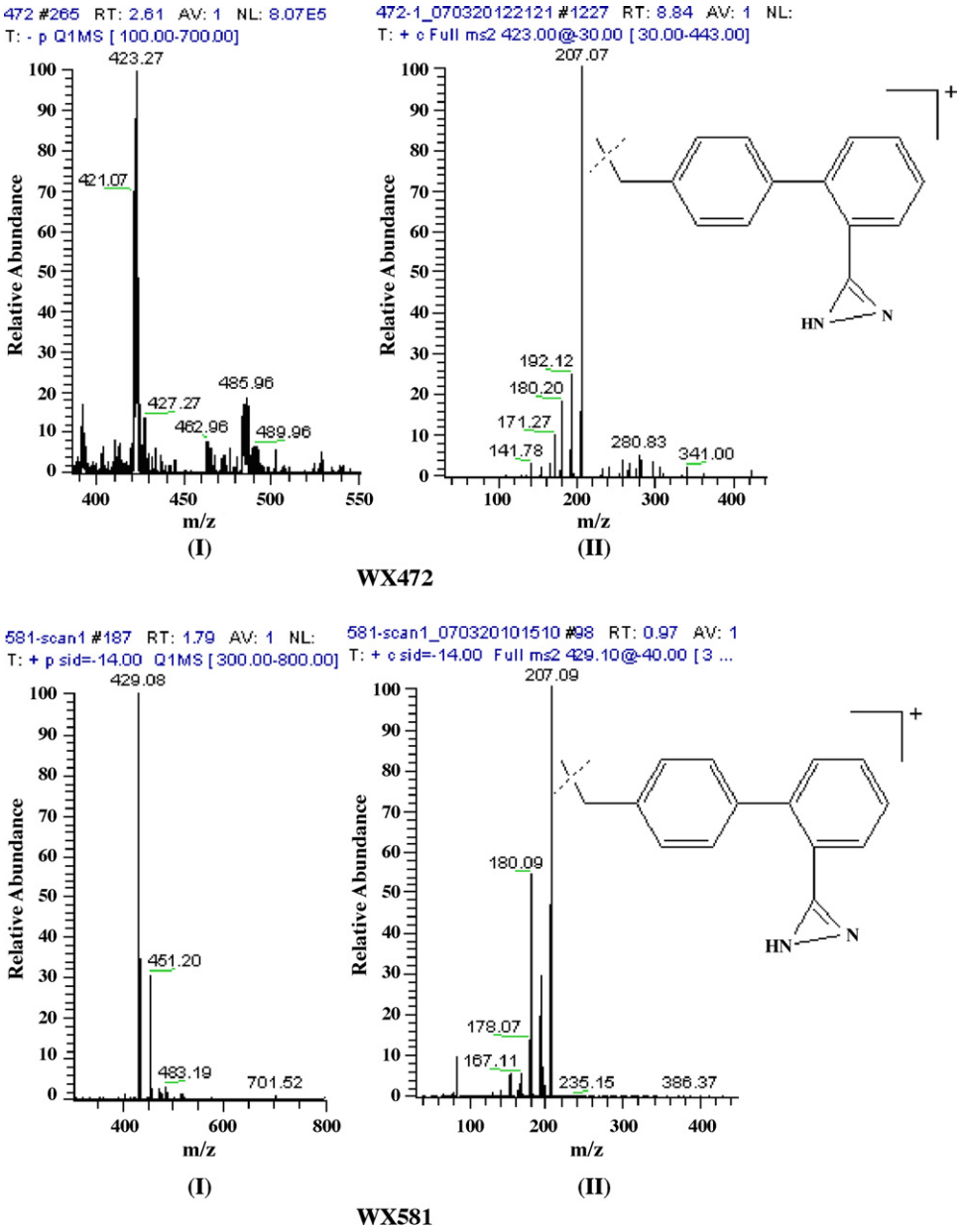

Fig. 2. Mass spectrum of AT1RA compounds and IS obtained from full scan. Showing the parent ions (I), corresponding abundant product ions (II) and the product ion structures for the WX472, WX581, 1b, telmisartan and IS.

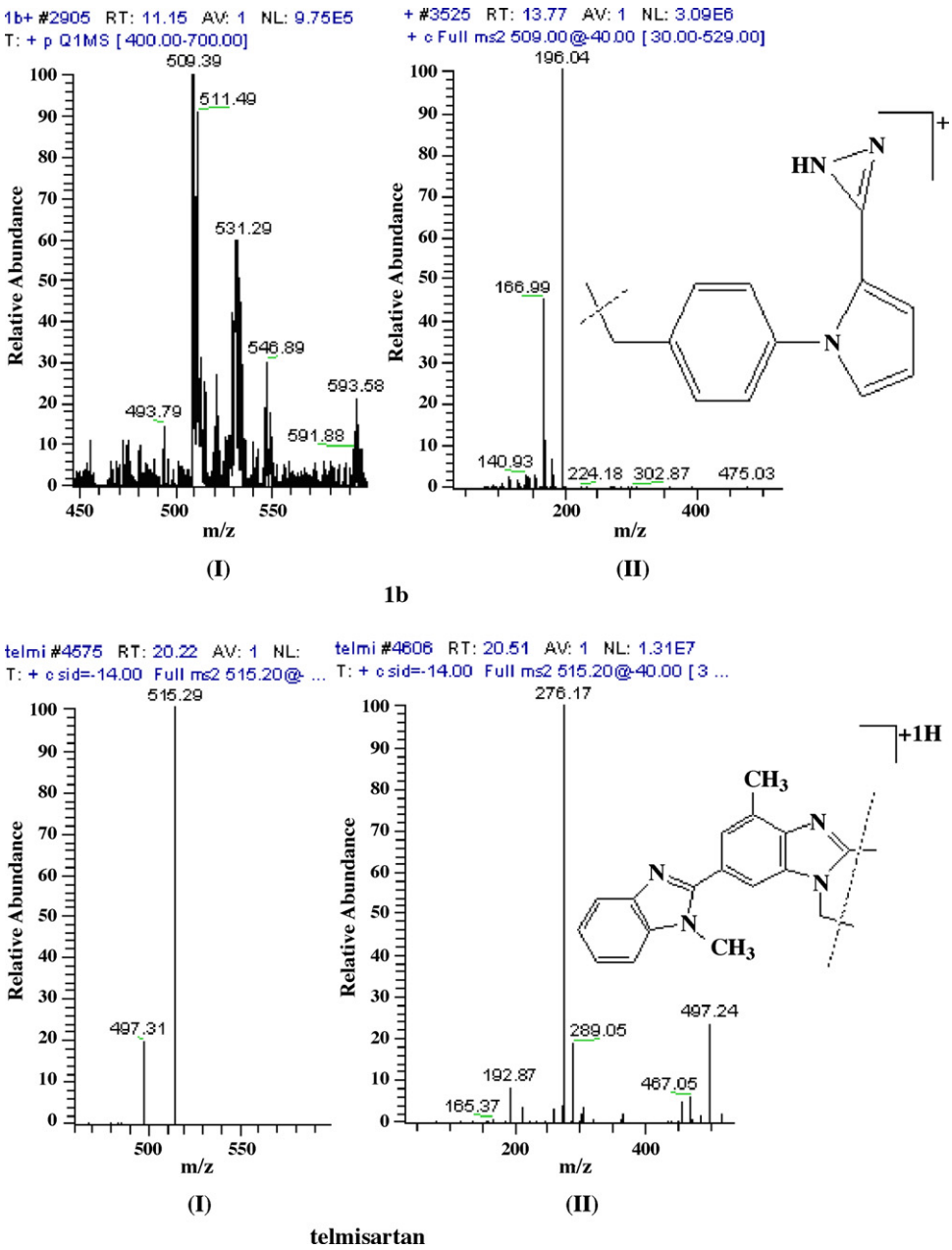

Fig. 2. (*Continued*)

lytical column (150 mm × 2.0 mm, 3 μm, Phenomenex, USA). The column and autosampler tray temperature were set at 40 and 4 °C, respectively. Separation of the analytes was carried out using a gradient elution program. The best mobile phase was found to be acetonitrile–0.05% aqueous formic acid at a flow rate of 0.2 mL/min. The mobile phase consisted of A (0.05% aqueous formic acid) and B (acetonitrile) with linear gradient elution. The gradient cycle consisted of an initial 1 min isocratic segment (60% A and 40% B) increasing solvent B to 70% within 1.5 min and maintained from 2.5 to 7.5 min, then changing back to 40% solvent B at 7.6 min and maintaining up to 10.5 min for column equilibration. The flow rate was 0.2 mL/min during the whole gradient cycle.

The mass detection was conducted using a Finnigan TSQ Quantum Discovery max system (Thermo Electron) equipped with an electrospray ionization source. Data acquisition was performed with Xcalibur 1.2 software (Thermo Finnigan, USA). Peak integration and calibration were performed using LCQuan software (Thermo Finnigan). Mass spectrometer was operated in positive ion mode. The optimum ion source parameters were as follows: auxiliary gas pressure 6 Arb; sheath gas pressure 17 Arb; ion transfer capillary temperature 310 °C; ion spray voltage 4100 V. Collision energies were set at 25, 35, 38, 35 and 35 eV for WX472, WX581, 1b, telmisartan and pitavastatin (IS), respectively. In order to find the most abundant ion, AT1RAs and the internal standard were separately scanned under the Q1 MS full scan mode to determine the parent ion, and under the Q1/Q3 (MS/MS) product ion scan mode to locate the most abundant product ion. The scan results are shown in Fig. 2. Quantification was thus performed using selected reaction monitoring (SRM)

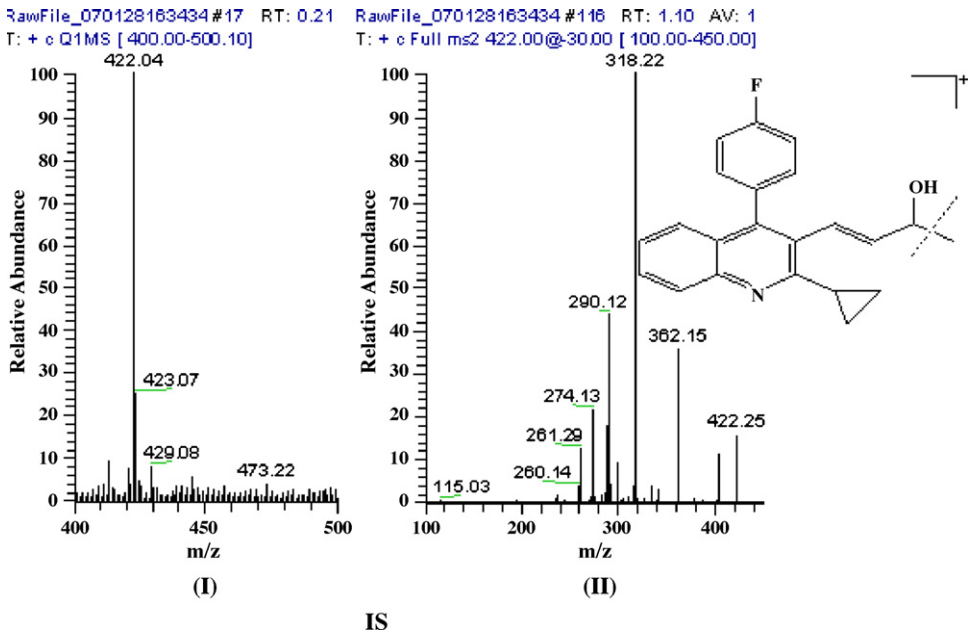

Fig. 2. (*Continued*).

of *m/z* 423 → 207 for WX472, *m/z* 429 → 207 for WX581, *m/z* 509 → 196 for 1b, *m/z* 515 → 276 for telmisartan and *m/z* 422 → 318 for pitavastatin (IS), respectively, with a scan time of 0.2 s per transition. The tuning parameters were optimized for pitavastatin and AT1RAs by direct infusing a solution containing 5 μg/mL of each analytes at a flow rate of 20 μL/min into the mobile phase (0.2 mL/min) using a flow injection system.

2.1.3. Preparation of stock solutions, calibration curves and quality control samples

The stock solutions at the concentration of 0.3, 0.5, 0.5, 0.5 and 1.0 mg/mL were prepared in methanol for WX472, WX581, 1b, telmisartan and IS, respectively. The AT1RA solutions were further serially diluted with methanol to obtain mixed standard working solutions at the concentrations of 45.0, 90.0, 180.0, 450.0, 900.0, 1800.0, 4500.0 and 9000.0 ng/mL for WX472, 50.0, 100.0, 200.0, 500.0, 1000.0, 2000.0, 5000.0 and 10000.0 ng/mL for WX581, 5.0,10.0, 20.0, 50.0, 100.0, 200.0, 500.0 and 1000.0 ng/mL for 1b and telmisartan. The IS working solution contained 5 μg/mL of pitavastatin. All the solutions were stored at 4 °C and were brought to room temperature before use. Calibration curves were prepared by spiking blank rat plasma with mixed working solutions to obtain concentrations of 4.5, 9.0, 18.0, 45.0, 90.0, 180.0, 450 and 900 ng/mL for WX472, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 500.0 and 1000.0 ng/mL for WX581 and 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL for 1b and telmisartan. Calibration curves were constructed by plotting the peak area ratios R of analytes to internal standard versus analytes concentration (x), and fitted to the equation $R = bx + a$ by weighted linear regression ($1/x$).

Quality control (QC) samples, which were used both in pre-study validation and during the PK study, were prepared by spiking control rat plasma with prepared analyte standard working solutions to obtain concentrations of 9.0, 90.0, 900.0 ng/mL for WX472, 10.0, 100.0, 1000.0 ng/mL for WX581, 1.0, 10.0, 100.0 ng/mL for 1b and telmisartan.

2.1.4. Extraction procedure

QC samples, calibration standards and rat plasma samples were extracted employing a LLE technique. To each tube containing 100 μL plasma, 10 μL of 5 μg/mL of IS and 10 μL of 0.5 mol/L HCL, then 1.0 mL of ethyl acetate were added, and the mixture was then vortexed for 3 min. The samples were then centrifuged for 10 min at 20,000 rpm. 0.8 mL of the upper organic layer was removed and evaporated to dryness using evaporator at 45 °C. The residue was dissolved in 200 μL mobile phase. An aliquot of 10 μL was injected into the LC–MS/MS system.

As for cassette analysis group, the rat plasma samples from different compounds were evenly pooled into one tube and a volume of 100 μL of pooled sample was processed as procedures in Section 2.1.4 described previously.

2.2. Method validation

The method was fully validated for its specificity, linearity, lower limit of quantification (LLOQ), accuracy and precision. The intra-batch precisions and accuracies of the assay were measured by analyzing five spiked samples of AT1RAs at each QC level. The inter-batch precisions and accuracies were determined over three days by analyzing 15 QC samples ($n = 5$ for each concentration level) each day. The precisions were expressed by relative standard error (R.S.D.%), and accuracies by relative error (R.E.%). The precisions should within 15%, and accuracies were required to be within 15% of the actual values.

The extraction recoveries were determined by comparing the peak areas of AT1RAs 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.

The matrix effects on the ionization efficiency of analytes and IS were evaluated by comparing the peak areas of analytes and IS dissolved in blank sample extract (i.e., the final solution obtained from blank plasma after extraction and reconstitution) with those for AT1RAs and IS dissolved to the same concentrations in methanol. Three different concentration levels of AT1RAs were evaluated by analyzing five samples at each concentration level. If the peak area ratios for the plasma extracts versus clean methanol solutions were <85 or >115%, a matrix effect was implied.

The LLOQs were considered as the concentration that produced a signal-to-noise (S/N) over 10. The stability of AT1RAs in rat plasma was assessed by analyzing the QC samples at three concentration levels. QC samples for the long-term and freeze/thaw stability studies were frozen before analysis at −20 °C for 2 weeks followed by three freeze/thaw cycles, each of which contained storage at −20 °C for 24 h and thawing at 37 °C. The short-term stability of AT1RAs in plasma at ambient temperature was assessed by processing and analyzing the QC samples after storage for 6 h on the laboratory bench. The post-preparative stability of AT1RAs in processed samples which had been kept in the autosampler at 4 °C was investigated by repeated injection of QC samples after a period of 24 h. The stability of the working solutions of AT1RAs and the IS was evaluated by testing their validity over 6 h at room temperature; this stability of working solutions was expressed in the same way as for percentage recovery.

2.3. Application to pharmacokinetic study

15 male Sprague–Dawley rats (190–210 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China, certificate No. SCXK-2003-0002) and housed with free access to food and water. The animals were maintained on a 12-h light:12-h dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22–24 °C) and ca. 60% relative humidity. The rats were fasted for 12 h before the pharmacokinetics study. AT1RAs were dosed at 2 mg/kg and resolved in 0.5% CMC-Na suspension. Both cassette dosing and cassette analysis were performed based on the need of the research projects. For cassette dosing group, 4 compounds were mixed together and dosed to three rats. For cassette analysis groups, the 4 compounds were dosed to rats separately and then pooled before extraction. About 0.25 mL of blood samples via the postocular vein were collected into heparinized tubes before dosing and at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 h after dosing. Blood samples were centrifuged immediately to obtain plasma (100 μL) and were stored at −20 °C until analysis.

The area under the curve (AUC) was calculated by linear trapezoidal method. Maximum plasma concentration (C_{max}) and the time-to-maximum concentration (T_{max}) were estimated by visual values of semi-logarithmic plots of the concentration–time curves. Half-life time ($T_{1/2}$) was calculated as $T_{1/2} = 0.693/k_e$. Elimination rate constant (k_e) was calculated by linear regression of the final log-linear part of the drug concentration–time curve.

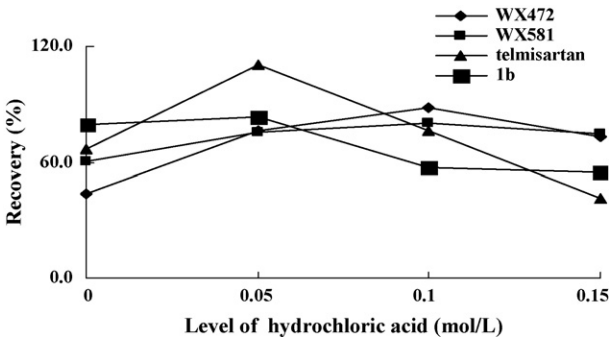

Fig. 3. Effect of different levels of hydrochloric acid on recoveries of AT1RAs from rat plasma.

3. Results and discussions

3.1. Method optimization

The choice of positive or negative ion mode was evaluated at the early stage of method development. Although MS signal of 1b was comparable in the positive and negative ion mode, other compounds (telmisartan, WX472 and WX581) had high MS signal in positive ion mode. Therefore, the positive ion mode was finally selected. The full scan mass spectra of $[M+H]^+$ and the ion structures for the product ion [12–15] are shown in Fig. 2. A number of commercially available column, including Diamond, Luna and Gemini analytical columns were tested and Gemini analytical column provided sharper peak shape.

It is necessary to use an IS to obtain good accuracy and precision when a mass spectrometer is used as the HPLC detector. Irbesartan was once tested as the internal standard due to its similar structure. However, WX581 and irbesartan had the same molecular weight and their parent ions, products ions and the retention time were very close. Pitavastatin was adopted as IS finally because of the similarity of its retention time and ionization characteristics with those of the analytes, and because of the minimal endogenous interferences in the SRM channel for pitavastatin (m/z 422 → 318). And as to the extracting power of the internal standard, the recent study had shown that the acidic extracting method reached a satisfactory recovery of pitavastatin [16].

LLE is advantageous because this technique not only extracted the analytes and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Ether, trichloromethane and ethyl acetate were all tested as extraction solvent, and ethyl acetate was finally adopted because of its high extraction efficiency. Since AT1RAs are acidic compounds, the acidic environment during extraction can ensure their existing as predominant unionized forms ready for liquid–liquid extraction. Addition of hydrochloric acid at different levels was tested and 0.05 mol/L was the best choice. The result is shown in Fig. 3.

Under the current chromatographic conditions, all analytes were rapidly eluted within 8.0 min. Addition of 0.05% aqueous formic acid to the aqueous phase was found to be an important factor for acquiring the high sensitivity.

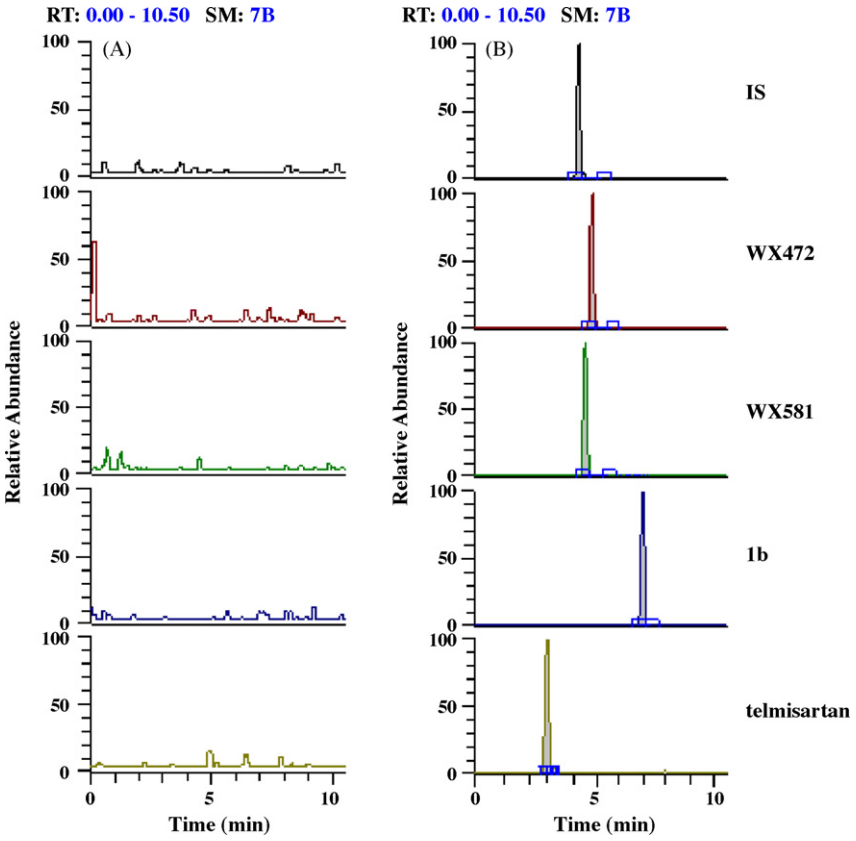

Fig. 4. Representative SRM chromatograms of AT1RAs and IS (A) A blank rat plasma; (B) a plasma sample 1 h after oral-dosing of 2 mg/kg AT1RAs by cassette dosing.

3.2. Method validation

3.2.1. Selectivity

Under the current optimized HPLC and MS/MS conditions, WX472, WX581, 1b, telmisartan and IS were baseline separated chromatographically with the retention time of above 4.92, 4.70, 7.01, 3.15 and 4.42 min, respectively. Fig. 4 shows the representative chromatograms of the blank rat plasma (a), and the plasma sample obtained at 1 h post-dosing of AT1RAs (b), supporting the high selectivity of this method.

3.2.2. Linearity of calibration curves

The method exhibited good linear response for the concentration range 0.5–100 ng/mL for 1b and telmisartan, 4.5–900 ng/mL for WX472 and 5–1000 ng/mL for WX581. The residuals improved by weighted (1/x)

Table 1
The inter-batch and intra-batch precision and accuracy of the assay for the AT1RAs in rat plasma ($n = 5$)

AT1RAs	Spiked concentration (ng/mL)	Intra day			Inter day		
		Determined concentration (mean ± S.D., ng/mL)	R.S.D. (%)	R.E. (%)	Determined concentration (mean ± S.D., ng/mL)	R.S.D. (%)	R.E. (%)
WX472	9.0	9.8 ± 0.2	2.4	109.0	10.1 ± 1.0	9.5	112.1
	90.0	91.2 ± 4.5	4.9	101.5	96.3 ± 7.1	7.4	107.0
	900.0	888.7 ± 37.3	4.2	98.7	905.3 ± 49.6	5.5	100.6
WX581	10.0	9.5 ± 0.8	8.7	94.7	10.4 ± 1.3	12.0	104.2
	100.0	94.1 ± 4.2	4.5	94.1	89.6 ± 3.6	4.0	89.6
	1000.0	1016.6 ± 38.6	3.8	101.7	1000.6 ± 78.0	7.8	100.1
	1.0	1.1 ± 0.1	11.3	110.0	0.9 ± 0.1	10.7	92.5
1b	10.0	10.6 ± 0.6	5.5	105.9	10.6 ± 1.2	11.6	105.8
	100.0	96.6 ± 5.9	6.1	96.6	100.4 ± 7.7	7.7	100.4
Telmisartan	1.0	1.0 ± 0.1	10.5	94.5	0.9 ± 0.1	14.8	91.6
	10.0	10.5 ± 0.7	6.7	105.2	9.7 ± 0.9	9.6	97.5
	100.0	104.5 ± 4.7	4.5	104.5	107.8 ± 11.5	10.7	107.8

Table 2
Recovery and matrix effect of AT1RA compounds from rat plasma ($n = 5$, mean ± S.D.)

AT1RAs	Spiked concentration (ng/mL)	Recovery (mean ± S.D., %)	Matrix effect (mean ± S.D., %)
WX472	9.0	76.5 ± 12.5	103.8 ± 11.4
	90.0	76.2 ± 6.4	103.3 ± 11.9
	900.0	65.1 ± 2.2	103.1 ± 13.7
WX581	10.0	76.3 ± 8.2	89.1 ± 8.4
	100.0	74.3 ± 5.4	100.2 ± 13.0
	1000.0	67.5 ± 1.9	99.4 ± 11.3
1b	1.0	83.7 ± 10.8	95.9 ± 12.8
	10.0	97.1 ± 14.9	94.3 ± 10.9
	100.0	83.8 ± 5.8	102.1 ± 8.5
Telmisartan	1.0	110.1 ± 8.4	104.2 ± 19.0
	10.0	110.8 ± 15.3	94.5 ± 10.3
	100.0	109.3 ± 13.3	108.8 ± 13.5

least-squares regression. The linear equation for the concentration versus the peak area was $y = 0.00169x - 0.00402$ ($r = 0.997$) for WX472, $y = 0.0168x + 0.0274$ ($r = 0.999$) for WX581, $y = 0.00119x + 0.000154$ ($r = 0.999$) for 1b, $y = 0.0007083x - 0.000267$ ($r = 0.996$) for telmisartan.

LLOQs were as follows: 1.8 ng/mL for WX472, 0.5 ng/mL for 1b and telmisartan, 5 ng/mL for WX581 (S/N > 10).

3.2.3. Precision and accuracy

Intra- and inter-batch precisions and accuracies of AT1RAs are shown in Table 1. In this assay, the intra- and inter-batch precisions ranged from 2.4 to 14.8% and the accuracies ranged from 89.6 to 112.1% for WX472, WX581, 1b and telmisartan, respectively, for each QC level. These results indicated that the present method had a satisfactory accuracy, precision and reproducibility.

3.2.4. Recovery and matrix effect

Extraction recoveries and matrix effects calculated from spiked plasma samples at QC concentration levels (9, 90, 900 ng/ml for WX472, 10, 100, 1000 ng/ml for WX581, 1, 10, 100 ng/ml for 1b and telmisartan) after the LLE procedure are collected in Table 2.

The mean absolute recoveries (after comparison of values at the three concentration level studied) did not depend on concentration level and were more than 65% for all the analytes ($n = 5$).

The matrix effects of these four AT1RA compounds were within 89–110% which indicated that no marked endogenous matrix effects were found.

3.2.5. Stability

The compounds in methanol were proved to be stable for at least 3 months at 4 °C when protected from light. The analytes reconstituted in the starting mobile phase were also stable in the autosampler for at least 24 h. No significant degradation in plasma samples after storage at −20 °C for 6 months. Furthermore, rat plasma samples of four AT1RA compounds were stable after three freeze–thaw cycles.

3.3. Pharmacokinetic data from cassette analysis and cassette dosing

The developed method was applied to the simultaneous determination of WX472, WX581, 1b and telmisartan in rat plasma samples using the high-throughput pharmacokinetic method. It was reported that AT1RAs had a relative long half-life [17], so in our study rat blood samples were collected up to 72 h. The pharmacokinetic parameters determined from the high-throughput methods for the four compounds are shown in Table 3. Mean

Table 3
Pharmacokinetic parameters for AT1RAs after oral administration using cassette dosing and cassette analysis ($n = 3$, mean ± S.D.)

		C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	AUC (ng/mL h)
Cassette dosing ($n = 3$)	WX472[a]	121.38 ± 19.00	1.67 ± 0.58	2.98 ± 0.44	793.94 ± 383.44
	1b[a]	31.73 ± 7.23	1.33 ± 0.58	7.92 ± 1.91	225.69 ± 94.24
	Telmisartan[b]	233.97 ± 77.54	6.00 ± 0.00	8.50 ± 0.98	1253.06 ± 356.47
	WX581[c]	520.67 ± 112.53	24.00 ± 0.00	20.63 ± 1.42	21858.33 ± 4124.32
Cassette analysis ($n = 3$)	WX472[a]	212.78 ± 125.35	3.00 ± 1.73	4.81 ± 1.73	2643.03 ± 1240.65
	1b[a]	76.41 ± 14.29	0.50 ± 0.00	3.43 ± 1.10	518.64 ± 186.81
	Telmisartan[b]	272.38 ± 57.19	4.00 ± 0.00	9.60 ± 1.80	1778.35 ± 428.79
	WX581[c]	769.96 ± 117.48	24.00 ± 0.00	24.22 ± 3.55	32861.52 ± 9854.28

[a] AUC_{0-24h}.
[b] AUC_{0-48h}.
[c] AUC_{0-72h}.

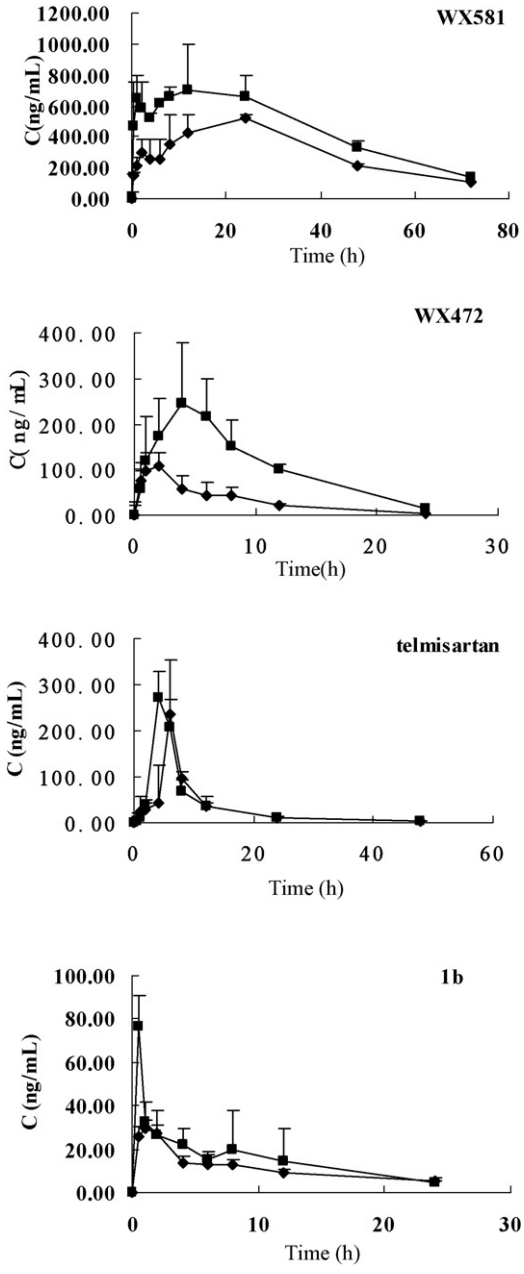

Fig. 5. Mean plasma concentration–time curves of WX472, WX581, 1b and telmisartan (means ± S.D., $n = 3$). (■) Cassette analysis; (♦) cassette dosing.

concentration–time curves of WX472, WX581, 1b and telmisartan are shown in Fig. 5. The cassette analysis PK data of 1b were consistent with the reports by Yan [18,19]. The half-life of telmisartan was reported at about 20 h which was longer than the results of cassette analysis [20,21]. These differences may be caused by the species difference and the lower dosage used in high-throughput screening study for AT1RAs. Fig. 6 represents the half-life values between cassette analysis and cassette dosing studies for 4 compounds. A poor correlation factor of 0.889 was observed between the data of cassette analysis and cassette dosing. The PK data obtained from cassette dosing were lower than that of cassette analysis, which indicated parameters would be potentially affected by the drug–drug interactions of the competition of oral absorption even when the lower dosage was chosen and had the great potential for a serious screening error. The research results suggested that it was preferred to choose cassette analysis method because it could avoid the drug–drug interactions and get the first hand accurate PK information as early as possible.

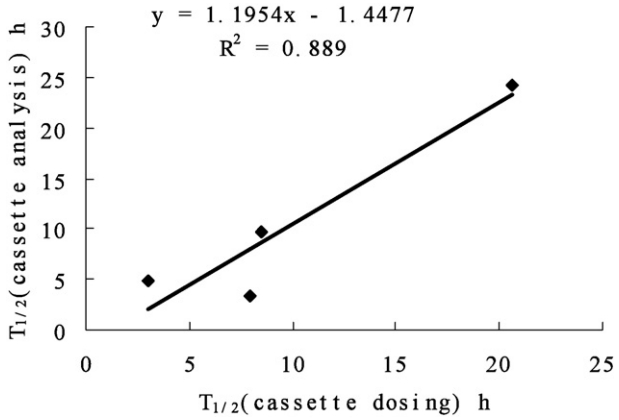

Fig. 6. The correlation of half-life values between cassette analysis and cassette dosing studies.

WX472 reached peak plasma concentration in rat at about 3 h, which indicated its rapid absorption. WX581 had an obvious double peak phenomenon with the first sharp peak at about 1 h and the second flat peak at about 24 h after oral administration. The factors contributing to the double peak phenomenon in the plasma concentration–time curve of WX581 will be further investigated. From the half-life time of WX472 and WX581, we knew that WX472 eliminated from plasma very quickly, whereas WX581 eliminated from plasma slowly. The results implied that WX581 had a better pharmacokinetic characteristic and might be a potentially new non-peptide angiotensin type 1 receptor antagonist candidate for the further research.

4. Conclusions

Overall, the present liquid–liquid extraction based on LC–MS/MS method was simple, sensitive and specific. The method was suitable for cassette dosing and cassette analysis which can quantitate four AT1RA compounds, WX472, WX581, 1b and telmisartan in rat plasma using pitavastatin as internal standard. In spite of the complex matrix, acceptable values of precision and accuracy were obtained by this method. Although the high-throughput method of cassette analysis needed more rats, it could avoid the drug–drug interactions and get the more exact pharmacokinetic data. Therefore, cassette analysis is suitable for the future research of in vitro assay such as Caco-2 permeability, metabolic stability, protein stability and so on.

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