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Original Article

DETERMINATION OF IRBESARTAN AND SINENSETIN SIMULTANEOUSLY BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY AND THEIR APPLICATION TO DRUG INTERACTION STUDY IN RAT PLASMA

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ABSTRACT

Objective: To determine and validate of irbesartan and sinensetin simultaneously by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and application of this method to study a pharmacokinetic interaction of irbesartan and ethanol extract of *Orthosiphon stamineus* herba in rat plasma.

Methods: The irbesartan and sinensetin were simultaneously extracted from plasma by protein precipitation with acetonitrile. Samples containing irbesartan and sinensetin were analyzed by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with C_{18} column Acquity® (100 mm × 2.1 mm), 1.7 µm particle size column at 40 °C. The gradient system of mobile phase composition was a mixture of acetonitrile and 0.1% formic acid (40:60 v/v), which was pumped at a flow rate of 0.3 ml/min. Mass detection was performed on Waters Xevo Triple Quadrupole equipped with an electro spray ionization (ESI) source at positive ion mode in the multiple reaction monitoring (MRM) mode. Irbesartan was detected at *m/z* 429.1>205.9, sinensetin was detected at m/z 373>342.9 and losartan as an internal standard was detected at *m/z* 423.05>404.9.

Results: The method was validated according to EMEA guidelines which showed good reproducibility and linearity of 0.99, the lower limit of quantification (LLOQ) were 25 ng/ml and 250 ng/ml for irbesartan and sinensatin, respectively. The precision (% CV) values of within-run and between-run analysis is 9.3-5.25% and 1.52–5.47% (for irbesartan), 1.52–5.09% and 2.47–9.14% (for sinensetin) whereas the accuracy (% diff) of both irbesartan and sinensetin were less than 20%. Stability studies revealed that irbesartan and sinensetin have been stable for 24 h at room temperature, 24 h in the autosampler, 3 freeze-thaw cycles, and at least 30 d at-20 °C. The validated method was applied to evaluate pharmacokinetic interactions of irbesartan and ethanol extract of *Orthosiphon stamineus* herba in rat plasma.

Conclusion: The developed LC/MS-MS method is valid to evaluate irbesartan and sinensetin simultaneous *in vitro* and showed good selectivity, linearity, accuracy, precision, matrix effect, and stability. The method was successfully applied to study the pharmacokinetics interaction of irbesartan and *Orthosiphon stamineus* herba in rat plasma.

Keyword: Validated method, LC-MS/MS, Drug interaction, Irbesartan, *Orthosiphon stamineus*

INTRODUCTION

Irbesartan is an angiotensin II receptor type 1 antagonist that works on the renin-angiotensin-aldosterone system [1]. Sinensetin was one of the bioactive marker compounds from *Orthosiphon stamineus* [2], that is used in traditional medicine for diuretic, antidiabetic, antihypertensive, anti inflammation and antitumor, antimicrobial [3]. Herbs preparation in combination with a synthetic drug taken simultaneously may interact with the synergistic effect result or can increase the side effect [4]. So the drug level in plasma must be measured in order to study the interaction between herb and drug. To support clinical investigations, a reliable analytical method with adequate sensitivity is necessary. Several HPLC and LC-MS/MS methods have been conducted previously for the determination of irbesartan in biological sample such as plasma or urine [5,6] but the combination of irbesartan and sinensetin simultaneous analysis has not been reported. The present study describes a simple, rapid, precise, and accurate LC-MS/MS method for determining irbesartan and sinensetin in human plasma *in vitro* and its application in the pharmacokinetic study of irbesartan and ethanol extract of *Orthosiphon stamineus* in rat plasma.

MATERIALS AND METHODS

Chemicals and reagents

Irbesartan was obtained from Sigma-Aldrich, Losartan Potassium from Ipca Labs Limited, and sinensetin from Chem Facs. Acetonitrile and methanol were HPLC-grade and were purchased from Merck. The other chemicals and reagents were analytical grade.

Instrument and chromatographic conditions

The chromatography was performed on C_{18} column Acquity BEH *(Bridged Ethylene Hybrid) Waters* (100 mm × 2.1 mm), 1.7µm at a temperature of 40 °C. The gradient mobile phase composition was a mixture of acetonitrile and 0.1% formic acid (40:60 v/v), which was pumped at a flow rate of 0.3 mL/min. Mass spectrometric detection was performed on Waters Xevo TQD Triple Quadrupole (Waters, Milford, USA) using multiple reaction monitoring (MRM), ESI+

Preparation of standard solutions and quality control samples

Primary stock solutions of irbesartan (1 mg/ml), sinensetin (1 mg/ml) and losartan potassium (1 mg/ml) were prepared in methanol. Then, diluted with methanol to obtain the certain concentration. Human plasma calibration standards of irbesartan were prepared by spiking an appropriate amount of the working standard solutions into drug-free human plasma. The concentration range of irbesartan in calibration curve was 25–2000ng/ml and Quality Control (QC) samples were prepared at three concentrations that were low (75ng/ml), medium (800ng/ml), and high (1500ng/ml). The range concentration of sinensetin in calibration curve was 250-20000 ng/ml and QC samples were prepared at three concentrations low (750ng/ml), medium (8000ng/ml), and high (15000 ng/ml).

Sample preparation

150µl of plasma containing certain concentrations of irbesartan and sinensetin were added 50µl of the internal standard working solution (5µg/ml) vortex-mixed for 30 Sec. Three parts of acetonitrile (400µl) were added to the precipitate protein in plasma, vortex-mixed for 2 min and centrifuged at 10.000 rpm for 10 min. A 5µl aliquot of the supernatant was injected into the LC-MS/MS system.

Validation of method

The validation parameters were linearity, selectivity, accuracy, precision, matrix effect, and stability. The method was validated in accordance with EMEA, 2011.

Pharmacokinetic study

The male *Spraque Dawley* rats weighing 200-250 gram were acclimatized for two weeks in order to observe their good health and suitability. The rats were given 500 mg/kg body weight of ethanol extract of *O. stamineus* orally for six days and on the 7th day were given 40 mg/kg body weight of irbesartan orally concomitantt with extract. Approximately 400 µl blood samples were collected into heparinized tubes via sinus orbitalis at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 8, 12, 24 and 34 h after drug administration, then centrifuged at 10000 rpm for 10 min to separate the plasma. The plasma samples were stored at-30 ° C prior to analysis.

The study protocol was approved by The Ethic Committee, Faculty of Medicine University of Indonesia with 07/H2. F1/ETIK/2015 reference number for notice of approval.

RESULTS

Optimization method

Mass detection was performed on Waters Xevo TQD equipped with electrospray ionization (ESI) source at positive ion mode in the multiple reaction monitoring (MRM) modes. The following operational parameters of the MS detector were optimized: MS ion mode, Precursor and product ions cone and collision energies are presented in table 1. Irbesartan was detected at *m/z* 429.1>205.9, sinensetin was detected at m/z 373>342.9 and losartan was used as internal standard at *m/z* 423.05>404.9

Table 1: Result of optimization detection of MS/MS

Compound	Parent (m/z)	Daughter (m/z)	Cone (V)	Collision (V)
Irbesartan	429.10	206.90	40	24
Losartan	423.05	404.9	35	11
Sinensetin	373.00	342.9	50	25

The full spectrum scan was dominated by protonated molecules [M+H]+m/z 429.10 for irbesartan and 373.00 for sinensetin, and the major fragmentations observed in each product spectrum were at m/z 206.90 and 342.90 (fig.1 and fig. 2).

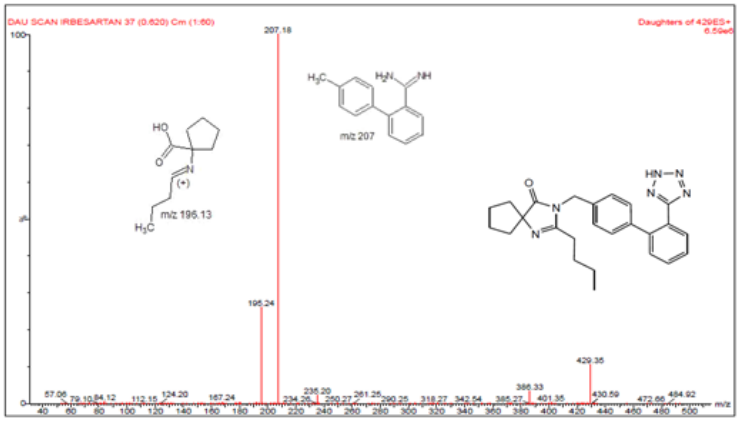

Fig. 1: Fragmentation of ion mass spectra of irbesartan

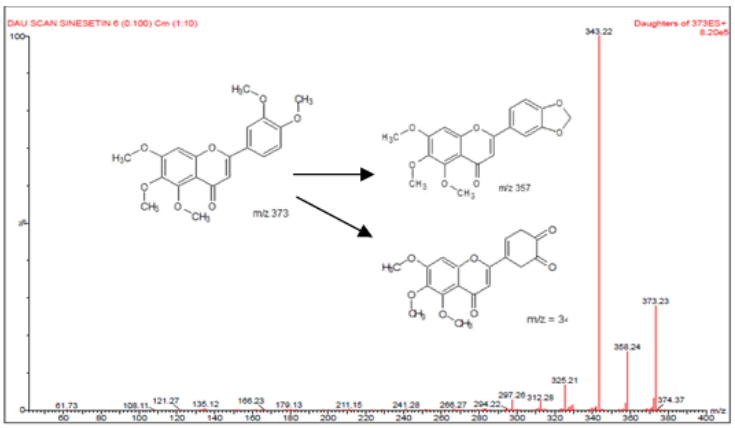

Fig. 2: Fragmentation ion mass spectra of sinensetin

Chromatogram of irbesartan, sinensetin and losartan simultaneously is given in fig. 3. The chromatography condition as follows: coulom C18 Acquity BEH (1,7 µm, 100 mm×2.1 mm), with mobile phase acetonitrile: 0.1% formic acid (40:60), gradient system, flow rate 0.3 ml/m, detection with ESI^+, mode analisis MRM (*Multiple Reaction Monitoring*) at m/z 429.1>205.9 for irbesartan, 423.05>404.9 for losartan and 373>342.9 for sinensetin, injected volume 5 µl.

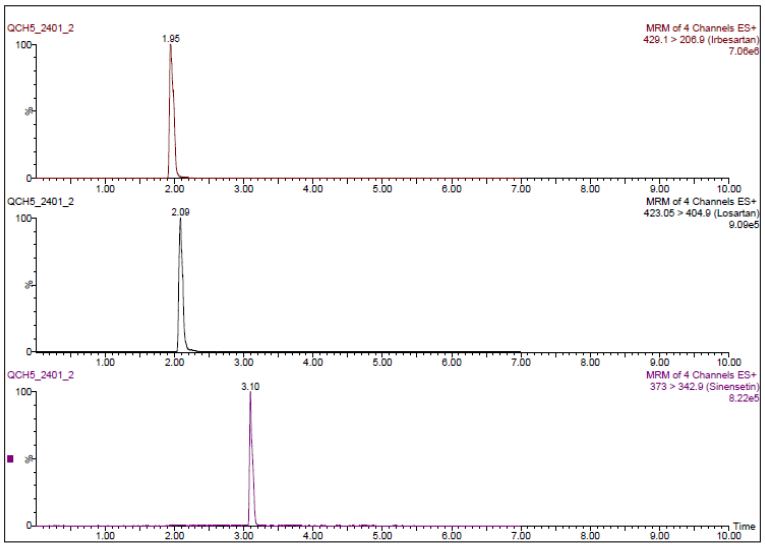

Fig. 3: Chromatogram of irbesartan, sinensetin and losartan simultaneously

Validation assay

Calibration curve and lower limit of quantification (LLOQ)

The linearity of each calibration curve was determined by plotting the peak area ratio (*Y*) of analyte to internal standard (analyte/IS) versus the nominal concentration (*X*) of irbesartan.

The calibration curves were linear over the concentration range of 25-2000ng/ml for irbesartan and 250-20000ng/ml for sinensetin with a correlation coefficient of 0.99. The correlation coefficient from five replicate calibration curves on different days was more than 0.99. The lower limit of quantification (LLOQ) was 25ng/ml (irbesartan) and 250ng/ml (sinensetin) with a coefficient of variation of less than 20%.

Selectivity

The selectivity was evaluated by analyzing blank plasma sample and blank plasma spiked with irbesartan, sinensetin and internal standard (losartan). The result showed that there is no interference endogenous compound from the blank plasma of the six different sources, whereas the % diff both of irbesartan and sinensetin were less than 20%.

Carry over

Carry over value after high concentration injection was 2.86% for irbesartan and 16.98% for sinensetin from LLOQ response while the carry over of internal standard was 0.44%. The value of the carry over-fulfilled the acceptance criteria for analyte<20% and for the internal standard of<5%.

Table 2: Accuracy and precision of irbesartan

Analyte	Actual concentration (ng/ml)	Mean measured concentration (ng/ml)+SD (n=5)	Precision (% CV)	Accuracy (% diff)
within-run	LLOQ (25)	22.42±2.09	9.3	(3.52–18.44)
	QCL (75)	72.52±1.92	2.65	(0.18-6.14)
	QCM (800)	711.50±37.38	5.25	(5.51–11.41)
	QCH (1500)	1467.53±47.33	3.23	(7.19–13.52)
between-run	LLOQ (25)	23.03±1.26	5.47	(0.82–19.92)
	QCL (75)	72.87±2.03	2.79	(4.24–14.71)
	QCM (800)	712.77±10.83	1.52	(7.87–14.51)
	QCH (1500)	1424.86±76.52	5.37	(2.87–14.97)

Table 3: Accuracy and precision of sinensetin

Analyte	Actual concentration (ng/ml)	Mean measured concentration (ng/ml)+SD (n=5)	Precision (% CV)	Accuracy (% diff)
within-run	LLOQ (250)	206.67±4.48	2.17	(14.36–19.03)
	QCL (750)	619.98±9.40	1.52	(14.11–14.97)
	QCM (8000)	7283.45±370.94	5.09	(4.48–14.47)
	QCH (15000)	14838.00±677.31	4.56	(4.18–14.52)
between-run	LLOQ (250)	229.11±20.94	9.14	(3.50–19.92)
	QCL (750)	766.94±42.15	5.49	(2.81–14.56)
	QCM (8000)	8535.52±210.76	2.47	(0.38–13.97)
	QCH (15000)	14764.25±629.04	4.26	(0.03–14.36)

Precision and accuracy

Precision and accuracy were calculated by within run and between run variation of QC sample in five replicates at four concentrations as shown in table 2 and table 3. The precision (% CV) values of within-run and between-run analysis is 9.3-5.25%and 1.52–5.47% (for irbesartan), 1.52–5.09% and 2.47–9.14% (for sinensetin) whereas the accuracy (% diff) of both irbesartan and sinensetin were less than 20%. The within-run and between-run precision and accuracy values indicate the adequate reliability and reproducibility of the method within the analytical range.

Matrix effect

Ion increasing effects due to matrix constituents were observed which the value of MF on the low concentration (QCL) was 1.13 (irbesartan) and 1.06 (sinensetin), whereas the % CV of both irbesartan and sinensetin were less than 15%, but there were no significant variations in matrix effects between the different blank source.

Stability test

The stability test of irbesartan and sinensetin in plasma was evaluated under different temperature and storage condition and was performed at QCL and QCH in three replicates. The result of stability test showed irbesartan and sinensetin have been stable for 24 h at room temperature, 24 h in the autosampler, 3 freeze-thaw cycles, and at least 30 d at-20 ° C (stability data in table 4).

Application to interaction pharmacokineticcs

The method described above was successfully applied to pharmacokinetics interaction study of irbesartan and ethanol extract of *O. stamineous* (EKK) in five male rats after an oral administration of the irbesartan (40 mg/kg) and extract of *O. stamineus* (500 mg/kg). The pharmacokinetic profiles are in fig.4 and the result of the pharmacokinetic parameter has been summarized in table 5.

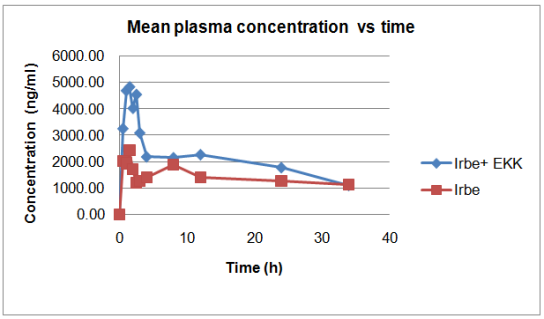

Fig. 4: The pharmacokinetic profile of irbe (irbesartan) alone (n=5) and irbe+EKK (irbesartan plus extract of *O. stamineus)* (n=5) after oral administration in male rats plasma

Table 4: Stability of irbesartan and sinensetin

Stability	Actual conc. (ng/ml)	Mean measured conc irbesartan (ng/ml)±SD (n=3)	%Diff.	Mean measured conc. sinenstin (ng/ml)±SD (n=3)	%Diff
Short term (24 h)	QCL	64.00±0.14	14.53-14.87	850.36±13.97	11.32-14.94
	QCH	1489.68±47.30	4.40-10.16	13978.51±595.1	8.34-14.86
Autosampler at 28 ° C (24 h)	QCL	75.36±5.09	4.18-7.35	850.76±8.99	12.28-14.46
	QCH	1475.48±34.98	5.71-10.07	13775.16±151.0	12.90-14.77
Freeze thaw from-30 ° C to 28 ° C	QCL	64.81±1.40	11.55-14.65	847.81±13.22	11.11-14.56
	QCH	1822.55±17.17	12.74-14.85	15491.34±150.4	4.06-12.11
Long term at-30 ° C (30 d)	QCL	64.80±0.95	12.32-14.84	642.60±3.01	13.93-14.73
	QCH	1363.08±1.26	14.72-14.87	14168.28±339.1	9.63-13.77

Note: QCL = Quality control low concentration (75 ng/ml for irbesartan and 750 ng/ml for sinensetin), QCH = Quality control high concentration (1500 ng/ml for irbsartan and 15000 ng/ml for sinensetin)

Table 5: The parameter pharmacokinetics of irbesartan

Parameter	Irbesartan	Irbesartan+Ethanol extract of *O. stamineus*
Cmax (ng/ml)	2426.20	4843.25
Tmax (hr)	1.5	1.5
$T_{1/2}$	23.82	32.07
AUC t_{-inf}	38609.62	39683.12
AUC $_{0-inf}$	79281.92	102688.07

DISCUSSION

To obtain good peak shape and good retention time, several combinations of mobile phase and flow rate were investigated. Acetonitrile: 0.1% formic acid (40:60) and flow rate 0.3 ml/min with the gradient system can separate the analytes with good peak and short retention time [fig. 3].

Liquid-liquid extraction (LLE) and protein precipitation method were performed to extract irbesartan and sinensetin simultaneously from plasma. Based on chromatogram and area of irbesartan and sinensetin showed that the protein precipitation with acetonitrile is better than protein precipitation with methanol and also liquid-liquid extraction.

The method has several advantages as compared to the methods reported in literature for irbesartan [5,6] such as simple sample preparation procedures by protein precipitation, short analysis time (5 min per sample), and high sensitivity which rendered for the purpose of its application to measure concentration-time profiles for interaction pharmacokinetic in rat plasma

The result of pharmacokinetics parameter showed that the plasma concentration of irbesartan and half time in the group combination of irbesartan and extract of *O. stamineus* was higher and longer than the group irbesartan alone. According to *in vitro* study shown, the *O. stamineous* extract is potent inhibitory activity against CYP2C9 with IC50 77.5±1.1 µg/ml [9], so the herb-drug interaction mechanism may be due to inhibition of CYP2C9, because irbesartan was also metabolized by CYP2C9.

CONCLUSION

The developed LC/MS-MS method is valid for irbesartan and sinensetin simultaneously *in vitro* and showed good selectivity, linearity, accuracy and precision, matrix effect and stability. The method is applicable for pharmacokinetic interaction study of irbesartan and *Orthosiphon stamineus* in rat plasma.

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CONFLICT OF INTERESTS

Declared None

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