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Determination of rebamipide in human plasma by a validated liquid chromatography-tandem mass spectrometry: Application in pharmacokinetics research

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Abstract: A new method for the determination of rebamipide in human heparin sodium plasma by LC-MS was established and its methodology was validated. In this method, protein precipitation method was used to pretreat the samples, and the rebamipide-d4 isotope of rebamipide was used as the internal standard. In the multi reaction monitoring mode, the electrospray ion source was used as the ionization technology, and LC-MS was used for detection and analysis. The liquid chromatographic conditions were: 00B-4605-AN (Kinetex® XB-C18 100A 50mm × 2.1mm, 5μm); mobile phase A: 0.1% FA and 1 mM NH_4FA aqueous solution, mobile phase B: 0.1% FA and 1mM NH_4FA 90% ACN solution, flow rate: 0.300mL/min, injection volume: 10uL, column temperature: 30°C, collection time: 3 min, injector temperature control: 5°C. The retention time of rebamipide and rebamipide-d4 were 1.32min and 1.31min, respectively. The lower limit of quantification was 1ng/mL, and the calibration map of rebamipide in the concentration range of 1 to 800ng/mL was linear (R^2>0.990, n=11). The CV% values of the inter and intra batch precision of the method were both less than 15.0%. This method has been successfully applied to pharmacokinetic studies to evaluate the main pharmacokinetic parameters of rebamipide.

Keywords: LC-MS, rebamipide, method validation, pharmacokinetic.

INTRODUCTION

Rebamipide, 2-(4-chlorobenzamido)-3-(2-oxo-1,2-dihydroquinolin-4-yl)propanoic acid, is a gastric protective agent that has multiple effects on the gastrointestinal tract (He *et al*., 2022), such as peptic ulcer disease, chronic gastritis, increased gastric acid production in the acute phase, erosive gastritis, prevention of mucosal damage caused by non steroidal anti-inflammatory drugs (NSAIDS), gastric ulcers after helicobacter pylori eradication treatment, and ulcerative colitis (Akagi *et al*., 2019; Hou *et al*., 2020; Andreev *et al*., 2019). There are recent literature reports that rebamipide eye suspension can also be used for the treatment of dry eyes (Sakane *et al*., 2019; Malhotra *et al*., 2022). Rebamipide was synthesized in Japan in the late 1980s as a compound with optical activity (Udhida *et al*., 1986; Otsubo *et al*., 1991). In addition to Japan, rebamipide is currently used in clinical practice in countries such as South Korea, China, India, Thailand, Indonesia, Vietnam, Malaysia, and Egypt. The initial pharmacological effect of rebamipide is to play an important role in gastric mucosal cell damage by increasing endogenous prostaglandin synthesis and scavenging oxygen free radicals. In addition, there are various mechanisms that have beneficial effects on gastric mucosa: inhibiting neutrophil activation and inflammation, regulating genes for cell apoptosis, inhibiting tyrosine nitration (Xu *et al*., 2021; Zvyaglova *et al*., 2020; Hou *et al*., 2020; Andreev *et al*., 2020). Currently, it has been reported that some researchers have used high-performance liquid chromatography combined with fluorescence detectors to detect the content of rebamipide in human plasma (Jeoung *et al*., 2004; Manglani *et al*., 2006; Sonawane *et al*., 2011). Although this method can successfully quantify the content of rebamipide, it has a long detection time, which is unfavorable for drug development and research. In order to fully support clinical research on rebamipide, we established and validated a rapid, liquid mass tandem method for determining plasma levels of rebamipide. In recent years, high performance liquid chromatography tandem mass spectrometry detection technology has been widely used in drug analysis, and the pharmaceutical industry has an increasing demand for shortening drug development time and improving sensitivity (Decosterd *et al*., 2020; Zhu *et al*., 2020; Mastrogianni *et al*., 2023). In this paper, the isotope internal standard of rebamipide-d4 was used as the internal standard to determine the content of rebamipide in human heparin sodium plasma using LC-MS technology and perform methodological validation. This method requires only 3 minutes of detection time has high sensitivity and has a linear range sufficient to cover the blood concentration of rebamipide from absorption to elimination. Both rebamipide and rebamipide-d4 can be detected within 1.3 minutes. In practical applications, it can achieve rapid, accurate, and precise detection results. The in vivo data in this article provide a valuable

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application insight for the pharmacokinetic study of rebamipide (Narala *et al*., 2019; Cho *et al*., 2009; Ngo *et al*., 2017).

MATERIALS AND METHODS

The ethical approval process of this study was in accordance with the GCP, the Declaration of Helsinki, and relevant domestic laws and regulations. The trial protocol, informed consent forms, and recruitment materials were reviewed at the Medical Ethics Committee meeting of the First Affiliated Hospital of Xiamen University. The trial was developed in accordance with ICH GCP and NMPA GCP guidelines and applicable regulations for data management and statistical analysis. At the same time, according to the technical guidelines for human Bioequivalence research of chemical generics with pharmacokinetic parameters as end points issued by NMPA, healthy subjects were recommended for this study. According to the FDA Food Effects and Feed Bioequivalence Study and the Technical Guidelines for Human Bioequivalence Studies of Chemical generics with Pharmacokinetic parameters as endpoints, it is recommended that this trial should be conducted in healthy subjects under fasting/postprandial conditions.

Chemicals

Rebamipide (purity: 97.49%), supplier: SINCO PHARMACHEM; Internal standard rebamipide-d4, supplier: TLC Pharmaceutical Standard, purity: 95.6%; human heparin sodium blank whole blood was collected from healthy volunteers recruited by the BE/I phase clinical trial center of the First Affiliated Hospital of Xiamen University; DMSO, supplier is Xilong Science Co., Ltd., AR level; The CAN supplier is MERCK and the level is HPLC; NH4FA, supplier is Aladdin, AR grade; The FA supplier is Sigma with ACS level.

Instruments and workstations

The HPLC is Agilent 1290 Infinity II, Agilent Technology Co., Ltd; Mass spectrometry, API 4000, Applied Biosystems/Scienx; Ion source: ESI. H2O-I-2-UV-T series integrated water machine (Sartorius, Germany); Oscillator: MX-S, Scilogex; DG-2500R, Shanghai Bajiu Industry; Centrifuge 5810R, Eppendorf; Balance MSA6.6S-0CE, CPA225D, Sartorius; Ultrasonic cleaning machine GT SONIC-D20, Gutor; Trigger Thermo; Data acquisition software: Analyst, version 1.6.3; Data processing software: Analyst, version 1.6.3, Watson LIMS 7.5.

High Performance Liquid Chromatography Conditions

Chromatographic column: 00B-4605-AN (Kinetex® XB-C18 100 Å, 50mm ×2.1mm, 5μm); mobile phase A aqueous solution of 0.1% FA and 1mM NH4FA, mobile phase B: 0%ACN solution of 0.1% FA and 1mM NH4FA, flow rate: 0.300mL/min, injection volumn: 10uL, column temperature: 30°C, acquisition duration: 3min, temperature control of sample injector: 5°C.

Mass spectrum conditions

IonSpray Voltage: 5500V; CUR: 25.00; GS1: 35.00, GS2: 40.00; TEM: 500.00; ihe: ON; EP: 10.00; CXP: 12.00; Q1: Unit, Q3: Unit, CAD: 8.00, model: ESI, Positive, MRM, DP: 70(rebamipide),74(rebamipide-d4), Collision Cell Exit Potential: 26(rebamipide), 26(rebamipide-d4), Dwell Time(msec): 100; Transitions: 371.100/216.200 (rebamipide), 375.100/216.200 (rebamipide-d4).

Solution preparation

Diluent: acetonitrile: water (V: V=50:50)

Standard curve stock solution and working solution: An appropriate amount of rebamipide was precisely weighed and completely dissolved in DMSO to prepare a standard curve stock solution with a final concentration of 1mg/mL and refrigerated at 2-8°C. The standard curve working solution with concentration ranging from 20.0 to 16000ng/mL was obtained by dilution of the standard curve reserve solution (Zhao *et al*., 2014).

Quality control sample stock solution and working solution: Proper amount of rebamipide was precisely weighed and completely dissolved in DMSO to prepare a sample stock solution with final concentration of 1.00mg/mL and refrigerated at 2-8°C. Sample working solution ranging from 20.0 to 32000ng/mL was obtained by dilution of the standard curve stock solution.

Internal standard stock solution and working solution: Proper amount of rebamipide d4 was precisely weighed in amber glass bottles and completely dissolved with DMSO to prepare the final concentration of 1.00mg/mL internal standard stock solution. A working solution of internal standard with a concentration range of 300ng/mL was obtained by diluting the standard curve reserve solution (Schmidt *et al*., 2022).

Preparation of quality control sample solution: appropriate amount of sample working solution was transferred to the blank matrix and diluted to the concentration as follows: Solutions of Lower limit of quantification (LLOQ QC)(1.00ng/mL), limit of quantification (LOQ)(3.00ng/mL), Geometric Mean Quality Control (GMQC)(30.0ng/mL), Mean Quality Control (MQC)(400ng/mL), High Quality Control (HQC) (600ng/mL) and Diluent Quality Control (DQC) (1600ng/mL) were prepared fresh on the same day.

Sample pretreatment process

At the corresponding position in the 96-well plate, 100μL sample (standard curve sample, quality control sample, test sample) was added, followed by 50.0μL internal standard working solution, and 50.0μL 50%ACN was added instead for blank sample. An additional 300μL acetonitrile was added and the mixture was mixed for 10 minutes. Centrifugation was performed at 4000 rpm for 5 min at 4°C. 100μL of the supernatant was transferred to a new 96-well plate, another 100μL of ultrapure water was

added, the plate was sealed and the plates were shaken for 10 minutes.

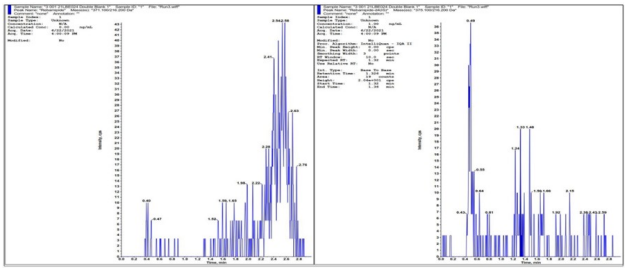

Fig. 1: Double blank matrix

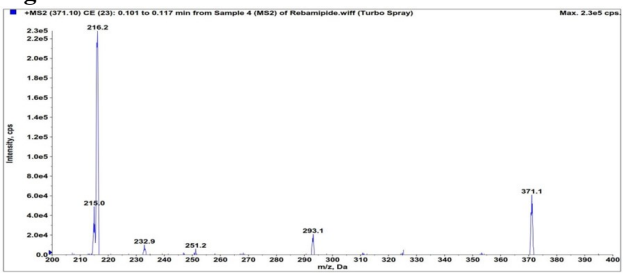

Fig. 2: Rebamipide ion scanning diagram

Calibration Procedure

Chromatograms of samples were collected and analyzed by Analyst Version1.6.3. The software was used to automatically integrate the test object and internal standard in the sample to obtain the peak area. Watson LIMS (7.5) was used to perform linear regression on the standard curve data, with a weighting coefficient of 1/X2. Watson LIMS (7.5) software was used to obtain the concentration values of each sample calculated by linear equation.

System suitability

The signal-to-noise ratio (SNR) of rebamipide and internal standard, the peak area ratio of rebamipide and internal standard and the CV% of retention time were measured by continuous injection of six LLOQ concentration levels.

Specificity

It was evaluated by analyzing a blank human heparin sodium plasma sample without analyte and internal standard compared to a standard curve sample with the lower limit of quantification, with six consecutive injections. The interference of internal standard to analyte was evaluated by adding internal standard (without analyte) to the blank matrix to the working concentration of internal standard and the lower limit of quantification standard curve sample (LLOQ). Analyte interference with the internal standard was assessed by adding rebamipide (without internal standard) to the blank matrix to the upper limit of quantification concentration (ULOQ) level for three consecutive injection needles.

Standard curve and lower limit of quantification

Preparation of the standard curve: appropriate amount of the standard curve working solution was transferred to the blank matrix and diluted into solutions with concentrations of 1.00, 2.00, 5.00, 50.0, 200, 500, 680 and 800ng/mL. The solutions were prepared fresh on the same day.

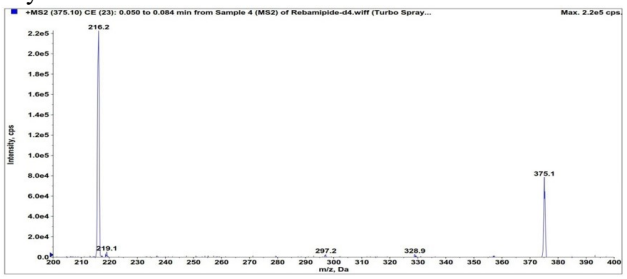

Fig. 3: Rebamipide-d4 ion scanning diagram

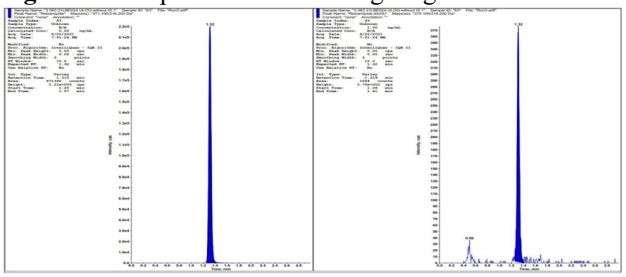

Fig. 4: ULOQ without IS

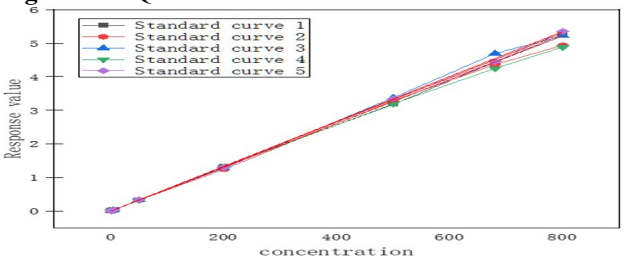

Fig. 5: Rebamipide standard curve

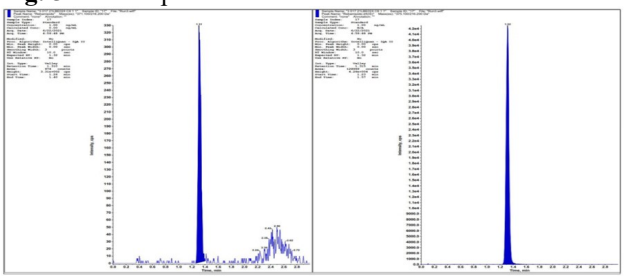

Fig. 6: LLOQ spectrogram

Precision and accuracy

Rebamipide precision and accuracy were assessed by repeated testing (n=6) of samples. Different working solutions were added to human heparin sodium plasma to prepare the following concentrations: LLQC sample (1.00ng/mL), LQC sample (3.00ng/mL), GMQC sample (30.0ng/mL), MQC sample (400ng/mL), HQC sample (600ng/mL). The precision was evaluated by calculating the coefficient of variation (%CV) of the detection concentration. The accuracy was investigated by calculating the deviation between the mean of the detected concentration and the theoretical concentration (calculated as the mean %Diff).

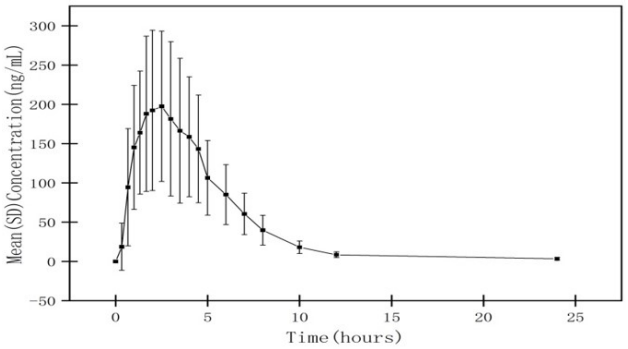

Fig. 7: Subject mean(sd) blood concentration-time curve (fasting)

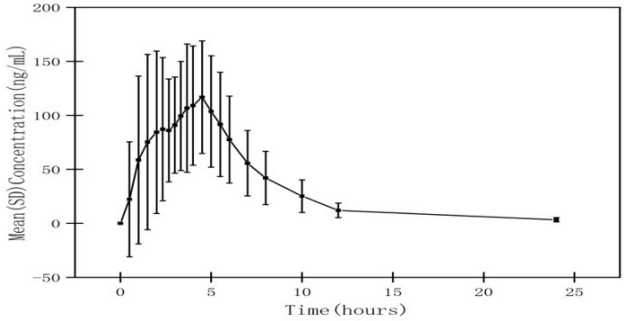

Fig. 8: Subject mean blood concentration-time curve (After the meal)

Extraction recovery rate

The recovery of analyte and internal standard was determined by the concentration levels used in the assay: six LQC samples (3.00ng/mL), six MQC samples (400ng/mL), and six HQC samples (600ng/mL) were prepared, and 18 blank samples containing neither analyte nor internal standard were simultaneously extracted. After the matrix sample was extracted, the analyte and internal standard were added to the extraction solution at the same concentration as the low, medium and high extraction samples. Extraction recovery was assessed by comparing the peak area of a single QC sample that was extracted with analyte and internal standard and the average peak area of a blank extract sample to which analyte and internal standard were added.

Matrix effects

Normal plasma matrix effects: Six individual plasma samples were used to investigate the matrix effect. After extraction, analytes and internal standards were added to blank plasma samples to achieve final concentrations that were consistent with LQC, MQC and HQC injection concentrations, respectively (3 aliquots per concentration level and per blank matrix)." LQC, MQC and HQC reference solutions containing analyte and internal standard were prepared at 3 parts per concentration. Matrix effects (expressed as matrix effect factor MF) of analytes and internal standards were calculated by comparing the peak areas of analytes and internal standards in matrix samples and reference solutions, respectively. Internal standard normalized matrix effect was calculated by the peak area ratio of analyte to internal standard. In this experiment, the internal standard normalized matrix effect was used to calculate (De *et al*., 2017; Rudzki *et al*., 2018; Nasiri *et al*., 2021; Raposo *et al*., 2021;).

The matrix effect of hemolyzed plasma was investigated by adding high and low quality control samples (6 samples each) of analytes to hemolyzed plasma and comparing with the standard curve and quality control samples prepared from routine plasma. Heparin-sodium hemolyzed plasma was prepared: (human whole blood treated by vigorous shaking and fragmentation of red blood cells was mixed with normal blank matrix (mixed) in a volume ratio of 2:98 to give 2% hemolyzed matrix).

The matrix effect of hyperlipidemic plasma was investigated by adding high and low quality control samples (6 samples each) of analytes to hyperlipidemic plasma and comparing with the standard curve and quality control samples prepared from conventional plasma. Heparin-sodium hemolyzed plasma was prepared: (human whole blood treated by vigorous shaking and fragmentation of red blood cells was mixed with normal blank matrix (mixed) in a volume ratio of 2:98 to give 2% hemolyzed matrix).

Stability

The stability of the isotope internal standard does not need to be investigated. During the entire validation process, any internal standard solution must pass the specificity test, that is, within the channel of the analyte (rebamipide), no interference peak greater than 20.0% of the mean value of the LLOQ peak area will be generated, and the specificity test results should be ensured to meet the entire validation process.

STATISTICAL ANALYSIS

The method was applied to the pharmacokinetic study, and the subjects were directly detected after a single oral dose of 0.1g of drug before and after meals. The relationship between the average plasma concentration of rebamipide and time was plotted, and the pharmacokinetic parameters of the subjects were calculated according to the actual sampling time using a non-compartmental model (Castaman *et al*., 2020; Chen *et al*., 2020).

The $AUC_{0-\infty}$ was the area under the concentration-time curve from zero to infinity. AUC_{0-t} was the area under the blood concentration-time curve from point 0 to the last time point t; Cmax is the maximum concentration, Tmax is the time to reach the maximum concentration, T1/2 is the elimination half-life, λz h-1 terminal elimination rate constant, Q1 is the first quartile, Q2 is the third quartile, and GM is the geometric mean.

Table 1: System suitability results

Sample ID	Retention time of the analyte	Retention time of the analyte CV%	Internal standard retention time	Internal standard retention time CV%	Area ratio	Area ratio CV%
1	1.319	0.2	1.314	0.2	0.291857	1.9
2	1.316		1.312		0.291693	
3	1.316		1.319		0.299853	
4	1.317		1.311		0.300560	
5	1.316		1.313		0.300560	
6	1.310		1.314		0.302476	

Table 2: Interference of blank matrix on compound and internal target

Drug response value					Internal standard response value				
ID	Blank matrix peak area	LLOQ peak area-1	LLOQ peak area-2	Interference %	ID	Blank matrix peak area	LLOQ peak area-1	LLOQ peak area-2	Interference %
1	0	974	1000	0.0	1	15	128969	137383	0.0
2	0			0.0	2	6			0.0
3	0			0.0	3	0			0.0
4	0			0.0	4	0			0.0
5	0			0.0	5	0			0.0
6	0			0.0	6	0			0.0

Table 3: Interference of internal standards with analytes and interference of analytes with internal standards

Drug response value					Internal standard response value				
ID	QC0	LLOQ peak area-1	LLOQ peak area-2	Interference %	ID	ULOQ without IS	ULOQ peak area-1	ULOQ peak area-2	Interference %
1	10	974	1000	1.0	1	1159	130699	136386	0.8
2	0			0.0	2	1044			
3	8			0.8	3	1172			
4	0			0.0					
5	0			0.0					
6	5			0.5					

Table 4: T-test results of the significance of the difference between standard curve intercept and zero value

ID	Linear range	Linear equation	r2	intercept		t-statistic	DF	P value
				SD	SEM			
1	1-800 ng/mL	Y=0.0065α-0.0030	0.9997	0.004	0.002	2.63936	4	0.05762
2		Y=0.0063α+0.0106	0.9986					
3		Y=0.0067α+0.0026	0.9986					
4		Y=0.0062α+0.0026	0.9993					
5		Y=0.0066α-0.0023	0.9998					

Sampling process

Thirty-six healthy male and female subjects (29 males and 7 females) who did not smoke or drink alcohol were selected from the local population to evaluate the pharmacokinetics of rebamipide (Zvyaglova *et al*., 2020; Hou *et al*., 2020). The subjects participated and signed the informed consent form and the process of obtaining the informed consent form met the requirements of GCP. Volunteers were over 18 years of age and had a body mass index between 18.0 and 27.9 kg/m2. Body weight ≥50 Kg for men and ≥45Kg for women. Each subject received a single oral dose of 0.1g.

This trial was divided into fasting and postprandial subjects. Fasting subjects were required to fast overnight for at least 10h before administration of the drug, followed by administration of the drug. Subjects were given a standard lunch 4h after the dose and a standard dinner 10h later, while they were forbidden to drink water for an hour before and after the dose. Postprantal subjects

Table 5: Precision and accuracy within and between batches

Experiment number	LLOQ QC	Accuracy deviation %	LQC	Accuracy deviation %	GMQ C	Accuracy deviation %	MQC	Accuracy deviation %	HQC	Accuracy deviation %
1#	0.963	-3.7	3.04	1.3	30.7	2.3	413	3.3	572	-4.7
	0.809	-19.1	2.80	-6.7	30.0	0.0	396	-1.0	581	-3.2
	0.871	-12.9	3.16	5.3	30.1	0.3	420	5.0	609	1.5
	0.880	-12.0	2.82	-6.0	31.0	3.3	417	4.3	597	-0.5
	0.877	-12.3	2.99	-0.3	31.2	4.0	394	-1.5	574	-4.3
	0.917	-8.3	2.84	-5.3	31.3	4.3	421	5.3	603	0.5
Average	0.886	NA	2.94	NA	30.7	NA	410	NA	589	NA
within-run SD	0.0513	NA	0.145	NA	0.556	NA	12.1	NA	15.7	NA
within-run% CV	5.8	NA	4.9	NA	1.8	NA	3.0	NA	2.7	NA
within-run Accuracy deviation %	-11.4	NA	-2.0	NA	2.3	NA	2.5	NA	-1.8	NA
2#	0.978	-2.2	3.07	2.3	29.8	-0.7	384	-4.0	571	-4.8
	0.812	-18.8	2.83	-5.7	29.5	-1.7	376	-6.0	577	-3.8
	0.974	-2.6	3.12	4.0	30.2	0.7	383	-4.3	568	-5.3
	1.05	5.0	3.01	0.3	30.3	1.0	380	-5.0	600	0.0
	0.957	-4.3	2.79	-7.0	30.8	2.7	383	-4.3	569	-5.2
	1.04	4.0	3.09	3.0	30.7	2.3	392	-2.0	557	-7.2
Average	0.969	NA	2.99	NA	30.2	NA	383	NA	574	NA
within-run SD	0.0854	NA	0.141	NA	0.504	NA	5.29	NA	14.4	NA
within-run %CV	8.8	NA	4.7	NA	1.7	NA	1.4	NA	2.5	NA
within-run Accuracy deviation %	-3.1	NA	-0.3	NA	0.7	NA	-4.3	NA	-4.3	NA
3#	0.981	-1.9	2.87	-4.3	28.2	-6.0	403	0.8	584	-2.7
	1.01	1.0	3.06	2.0	30.3	1.0	387	-3.3	590	-1.7
	0.860	-14.0	2.98	-0.7	31.5	5.0	376	-6.0	577	-3.8
	1.04	4.0	2.98	-0.7	30.4	1.3	412	3.0	577	-3.8
	1.01	1.0	3.03	1.0	31.1	3.7	403	0.8	570	-5.0
	1.05	5.0	2.91	-3.0	30.0	0.0	387	-3.3	586	-2.3
Average	0.990	NA	2.97	NA	30.3	NA	395	NA	581	NA
within-run SD	0.069	NA	0.0714	NA	1.15	NA	13.5	NA	7.31	NA
within-run %CV	7.0	NA	2.4	NA	3.8	NA	3.4	NA	1.3	NA
within-run accuracy deviation %	-0.8	NA	-1.0	NA	1.0	NA	-1.3	NA	-3.2	NA
between-run precision	0.948	NA	2.97	NA	30.4	NA	396	NA	581	NA
between-run SD	0.055	NA	0.118	NA	0.779	NA	15.3	NA	13.9	NA
between-run%CV	5.8	NA	4.0	NA	2.6	NA	3.9	NA	2.4	NA
between-run accuracy deviation %	-5.1	NA	-1.0	NA	1.3	NA	-1.0	NA	-3.2	NA

Table 6: Extraction recovery rate of the analyte

Experiment number	HQC peak area			MQC peak area			LQC peak area		
	After extraction	Pre-extraction	Recovery %	After extraction	Pre-extraction	Recovery %	After extraction	Pre-extraction	Recovery %
1	516077	596122	86.9	349312	397616	88.2	2511	2835	84.7
2	514685	603235	86.7	350692	392494	88.6	2699	2971	91.1
3	509326	605148	85.8	333311	399889	84.2	2616	2842	88.3
4	532951	580298	89.8	370594	396668	93.6	2720	3048	91.8
5	528095	580583	89.0	362026	394406	91.4	2764	2933	93.3
6	542135	595927	91.3	360166	394571	91.0	2758	3146	93.1
Average	523878	593552	88.3	354350	395941	89.5	2678	2963	90.4
SD	12600	10800	2.12	12900	2650	3.26	97.7	121	3.32
%CV	2.4	1.8	2.4	3.6	0.7	3.6	3.6	4.1	3.7
Overall recovery %	89.4								
Overall% CV	1.2								

Table 7: Recovery rate of analyte

Experiment number	Concentration 300: ng/mL		
	Internal standard peak area after extraction	Pre-extraction Internal standard peak area	Recovery %
1	130938	148761	86.4
2	132228	148290	87.2
3	131818	148677	87.0
4	136898	151610	90.3
5	136953	150216	90.3
6	142211	158464	93.8
7	131303	156927	86.6
8	137269	147097	90.6
9	134278	155590	88.6
10	136152	151351	89.8
11	136056	148887	89.8
12	140965	148117	93.0
13	133980	155271	88.4
14	132064	156684	87.1
15	133607	151622	88.1
16	139809	152526	92.2
17	140395	147593	92.6
18	140062	150832	92.4
Average	135944	151584	89.7
SD	3630	3580	2.39
%CV	2.7	2.4	2.7
Average Recovery %	89.7		

Table 8: Matrix Effect results

Matrix effect						
Concentration	Rebamipide matrix effect	Precision of Rebamipide matrix effect	Internal standard Matrix effect	Precision of Internal standard Matrix effect	Internal standard working solution concentration level (all batch substrates tested) matrix effect precision	The precision of internal standard normalized matrix effect mean
LQC	1.058	2.3	1.024	0.9	2.0	1.7
MQC	1.059	1.5	1.057	1.4		
HQC	1.046	2.6	1.042	1.6		
Hemolytic plasma matrix effect			Hyperlipidemic matrix effect			
	Accuracy deviation %	Precision deviation%	Accuracy deviation %		Precision deviation%	
LQC	1.0	4.7	1.0		2.9	
HQC	-4.5	2.7	0.2		1.6	

Table 9: Pharmacokinetic parameters of Rebapite

Parameters	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-t} (h*ng/mL)	$AUC_{0-\infty}$ (h*ng/mL)	%AUC $_{\%Extrap}$	$\lambda_z h^{-1}$	$T_{1/2}$ (h)
Fasting							
Mean	2.36	254.97	1108.11	1140.28	2.64	0.1709	5.78
SD	1.08	72.45	321.28	332.37	4.33	0.0639	7.08
CV%	45.63	28.42	28.99	29.15	164.05	37.40	122.55
Min	1	123	652.28	677.57	0.32	0.0156	2.58
Max	4.5	418	1928.25	1963.27	26.49	0.2692	44.34
Median	2.00	260	1079.79	1124.64	1.62	0.1882	3.68
GM	2.13	244.69	1064.51	1094.64	1.72	0.1529	4.53
After the meal							
Mean	3.49	178.79	803.41	833.75	3.63	0.1751	5.04
SD	1.58	66.99	159.52	163.68	3.54	0.0704	3.39
CV%	45.21	37.47	19.86	19.63	97.56	40.21	67.24
Min	1	100	517.98	597.29	0.67	0.0453	1.76
Max	6	343	1126.06	1161.29	18.05	0.3934	15.31
Median	3.84	150.50	780.63	795.89	2.46	0.1814	3.82
GM	3.04	168.23	788.45	818.73	2.72	0.1587	4.37

were required to fast for more than 10h before eating, eat a high-fat meal 30min before drug administration, eat a standard lunch 4h after drug administration, and eat a standard dinner 10h after drug administration. Water was forbidden for 1h before and after drug administration.

Blood sample extraction and processing process: 4mL blood was taken into the blood vessel containing heparin sodium anticoagulant for each time. After collection, the blood samples were centrifuged within 90min and the blood samples were pretreated within 120min and stored in the refrigerator at -60°C.

RESULTS

System suitability results

As can be seen from table 1, the retention time CV% of the substance to be tested and the internal standard were both 0.2% and the peak area ratio CV% is 1.9%, which met the standard (the standard is ≤ 15.0%), and the validation results were good.

Specificity results

The interference of blank plasma matrix to the test substance was 0.0%, and the interference to the internal standard was 0.0%. The maximum interference of the internal standard to the test substance was 1.0%, and the interference of the test substance to the internal standard was 0.8%. Acceptance criteria were met.

Standard curve and LOQ results

The standard curve sample of rebamipide prepared as described in the experiment was injected into the LC-MS system for curve calibration. The linear relationship between the peak area signal of the analyte and the corresponding concentration is shown in fig. 5. The lower limit of quantification was determined to be 1ng/mL. To further demonstrate the accuracy of the calibration curve, we established five calibration curves over a period of 2 weeks, all with r2 values exceeding 0.99 and within ±15.0% of the theoretical value for each concentration level and standard concentration. The t-test was used to determine whether there was a significant difference between the experimental intercept of the above regression equation and the theoretical zero value (Elkady *et al*., 2020; Chen *et al*., 2016). The significance level of 0.05 was chosen to assume that the intercept was not significantly different from the 0 value, then the null hypothesis was true according to the result p-value of 0.05762, which was greater than 0.05, which means that the intercept of all regression lines was not significantly different from zero.

Precision (%CV), accuracy

As shown in table 5, the maximum intra-assay precision (except LLOQ QC quality control samples) was 4.9, and the intra-assay accuracy deviation was between -4.3 and 2.5; the maximum intra-assay precision of LLOQ QC quality control samples was 13.9, and the intra-assay accuracy deviation was between -11.4 and 4.0. All met the standard (deviation must be within ±15.0% (for LLOQ QC, within ±20.0%), precision must be ≤15.0% (for LLOQ QC, ≤20.0%). The maximum intra-assay precision (except LLOQ QC samples) was 4.0%, and the intra-assay accuracy deviation was between -3.2-1.3%. The maximum intra-assay precision of LLOQ QC samples was 11.8% and the intra-assay accuracy deviation was -3.7%, which met the standard (deviation must be within ±15.0% (for LLOQ QC, Within ±20.0%), precision must be ≤15.0% (for LLOQ QC, ≤20.0%). The above results show that the method has good precision and accuracy.

Extraction recovery results

For analytes and internal standards, the precision of recovery for each concentration level and for each concentration level must be within 15.0%. The results in table 6 and table 7 showed that the maximum recovery precision of rebabapide and internal standard was 4.1% and 2.7%, respectively, which was much lower than the standard value, indicating that the results were good.

Matrix Effect

Table 8 showed that normal plasma matrix effects: The precision of matrix effects at each concentration level of analyte (all matrices tested in batches) and the concentration level of internal standard working solution (all matrices tested in batches) was within 15.0% and the precision of the normalized mean value of matrix effects at the three concentration levels of analyte was within 15.0%. The precision of hemolytic plasma matrix effect and hyperlipidemia matrix effect were both within 15.0%. Good matrix effect indicates that the matrix had no interference with the object to be measured when detecting the analyte, which made the detection result more accurate.

Solution stability results

The reserve solution of DMSO was stable at room temperature for 27h and stable at 2-8°C for 35 days. The reserve solution with 50% acetonitrile as solvent was stable for 25h at room temperature and for 35 days at 2-8°C. Whole blood stroma was stable at room temperature for 2h. The plasma matrix remained stable for 24˚C at room temperature and up to 32˚C at -20˚C and -70˚C, and was stable after four freeze-thaw cycles. The prepared samples were stable at 2-8°C for 191h. In this experiment, the internal standard of the isotope of the object to be tested was used. As its properties were similar to those of the object to be tested, and it was proved in each analysis batch that the internal standard had no interference with the compound, the stability test of the internal standard-related solution was not carried out separately.

Pharmacokinetic study

Figs. 7, 8 and table 9 showed the various pharmacokinetic parameters of rebamipide for fasting and postprandial administration in 36 healthy subjects. Fasting and

postprantal elimination half-life ($T_{1/2}$) were 5.78±7.08 hours and 5.04±3.39 hours, respectively. The arithmetic mean ± standard deviation of AUC_{0-t} were 1108.11± 321.28ng·h/mL and 803.41±159.52ng·h/mL, respectively. The arithmetic mean ± standard deviation of $AUC_{0-\infty}$ were 1140.28±332.37ng·h/mL and 833.75± 163.68ng·h/mL and the median and range of T_{max} peak time were 2.00 (1~4.5) h and 3.84 (1~6) h, respectively. The arithmetic mean ± standard deviation of peak concentration C_{max} were 254.97± 72.45 and 178.79±66.99ng/mL, respectively.

DISCUSSION

The LC-MS method developed and validated in this study successfully quantified the content of rebamipide in human heparin plasma in 3min. The method was highly specific, the CV% of precision and accuracy was less than 15%, the limit of quantification was 1ng/mL, and the linear range was 1-800ng/mL. The precision of extraction recovery was less than 15%. The results of matrix effect showed that the matrix had no interference with the tested substance, and the verification results all met the standards. Thirty-six healthy subjects were enrolled and completed the study. The pharmacokinetic parameters of rebamipide were calculated. Fasting and postprandial elimination half-life ($T_{½}$) were 5.78±7.08 and 5.04±3.39 hours, respectively. The arithmetic mean ± standard deviation of AUC0-t was 1108.11±321.28 ng•h/mL and 803.41±159.52ng•h/mL, respectively. The arithmetic mean ± standard deviation of $AUC0_{-\infty}$ was 1140.28± 332.37ng•h/mL and 833.75±163.68ng•h/mL, respectively. The median and range of T_{max} were 2.00 (1-4.5) h and 3.84 (1-6) h, respectively. The arithmetic mean (± SD) of C_{max} were 254.97±72.45ng/mL and 178.79±66.99ng/mL, respectively. The results were consistent with those reported in the literature (Cho *et al*., 2009).

In this paper, the relationship between the blood concentration curve of rebamipide before and after meals was also studied. It can be seen that the blood concentration of rebamipide before meals was higher than that after meals, indicating that its oral absorption effect before meals was better. One of the reasons for this effect may be that the drug itself has different absorption ability at different locations in the intestine, so that the systemic clearance of drug is greater than the absorption of drug or the absorption is greater than the clearance of drug, thus forming a double peak. Another reason may be that the drug returns to the bloodstream through the enterohepatic circulation. These exemplify the complex pharma-cokinetics of rebamipide itself, so the bimodal effect should be fully taken into account in future studies.

CONCLUSION

The validated lcms analysis method presented in this paper has the advantages of rapidity, sensitivity, specificity, precision and accuracy. The concentration of rebamipide measured by this method was within the limit of the method. The calculated pharmacokinetic parameters were consistent with those reported in the literature. Therefore, we believe that this assay can be applied to the detection of rebamipide in human plasma. The analytical method can be applied to the bioequivalence study of rebamipide in the future.

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