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平衡透析法考察豆腐果苷血浆蛋白结合率:种属差异研究

贾元威,沈　杰*,谢海棠,李相鸿,梁大虎,汪旻晖,胡　骅

安徽省药物临床评价中心 皖南医学院弋矶山医院,芜湖 241001

摘　要:糖苷类化合物豆腐果苷在人与大鼠体内处置过程存在种属差异。药物血浆蛋白结合率是决定药物整体分布、排泄、活性及毒性的重要决定过程。本文旨在应用平衡透析法评价蛋白结合率差异在豆腐果苷种属差异中的作用。研究采用 HPLC-ESI-MS 方法进行检测,并对试验中的一些影响因素进行了优化。在试验中的四个浓度下,大鼠血浆蛋白结合率在 10.33% ~11.03%,人血浆蛋白结合率在 10.60% ~10.98%。结果表明人与大鼠药代动力学参数的差异不是由蛋白结合率的差异所导致。这是有关豆腐果苷蛋白结合率相关研究的首次报道。

关键词:血浆蛋白结合率;豆腐果苷;HPLC-ESI-MS;种属差异

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Analysis of Plasma Protein Binding of Helicid Using Equilibrium Dialysis Method: An Investigation on Species Differences

JIA Yuan-wei, SHEN Jie*, XIE Hai-tang, LI Xiang-hong, LIANG Da-hu, WANG Min-hui, HU Hua

Anhui provincial Center for Drug Clinical Evaluation, Yijishan Hospital of Wannan Medical College, Anhui Wuhu 241001, China

Abstract: Previous studies showed species difference of helicid metabolic process between human and rat. Plasma protein binding rate of drugs is an important process for determining their overall distribution, excretion, activity and toxicity. The aim of this study was to assess the effects of helicid plasma protein binding rates in species difference using equilibrium dialysis. Liquid chromatography-mass spectrometry analysis was carried out and was optimized against some influencing factors during experimental procedures. The method developed in this research was validated to be specific, sensitive and accurate. The binding fractions were about 10.33%-11.03% for rats and 10.60%-10.98% for human within four concentration levels. According to our results, it was concluded that the PK parameters differences between human and rat were not due to plasma protein binding. This was the study of helicid plasma protein binding reported for the first time.

Key words: plasma protein binding; helicid; LC-ESI-MS; species differences

Introduction

Helicid nilgirica Bedd was considered as a "supernatural" effectiveness to cure headache and insomnia by people in some area of Yunnan province, China[1]. As one of the main constituents present in *H. nilgirica*, helicid is found to be with well-documented sedation and analgesic effects along with low side effects. Neurotoxicological teratology study on the offspring of rats revealed that even a high intragastric gavage dose of 350 mg/kg still did not affect the early development of nervous system, neurobehavioral function and brain histology of offspring[2]. Helicid together with a series of its analogs were prepared and evaluated *in vitro* to unclose their pharmacologic actions[3-10].

However, according to our and other's previously studies, we found that there were great differences of pharmacokinetics behaviors occurred between human and rats[11-15]. After administration of a single dose of 100 mg helicid in human, the C_{max} and T_{max} were 10.6 ±

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* Corresponding author Tel: 86-553-5738350; E-mail: article2071@sohu.com

3.11 ng/mL and 1.08 ± 0.47 h, respectively. Plasma concentration declined with the $t_{1/2}$ of 5.27 ± 0.89 h. The AUC_{0-24} and $AUC_{0-\infty}$ values obtained were 37.25 ± 5.33 and 40.34 ± 6.04 ng h/mL, respectively. Meanwhile, after p.o. administration 25, 50 and 100 mg/kg of helicid to rats, C_{max} were 746.17 ± 154.94, 1378.99 ± 222.73, and 5385.25 ± 2701.75 ng/mL, respectively; the terminal half-lives were 1.43 ± 0.81 h, 1.59 ± 0.80 h and 2.16 ± 0.80 h, respectively; the AUC_{0-t} were 2065.52 ± 425.85, 4115.96 ± 1131.96 and 10311.55 ± 3026.09 ng h/mL, respectively. Thus, we cannot directly extrapolate the behavior of helicid in rats to human, which means the results of preclinical research which were mainly carried out on rodents cannot be simply transferred to clinical research on mankind. Hence, it is of great importance to uncover the reasons behind these species differences.

As we all know that, a drug in blood exists in two forms: bound and unbound. The less bound a drug is, the more efficiently it can traverse cell membranes or diffuse. Thus, blood proteins' binding is an important process for determination of the overall distribution, excretion, activity and toxicity of a drug[16,17]. Thereby, in this study, in order to investigate the role of blood proteins' binding plays in helicid pharmacokinetics behavior species difference between human and rats, a method for drug-plasma protein binding study of helicid was developed using equilibrium dialysis for sample preparation and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) for quantitation. During method optimization, some influencing factors were investigated and necessary steps were taken to control for these factors.

Materials and Methods

Materials

Helicid (Batch No. 040801, molecular structure seen in Fig. 1a) was kindly provided by Kunming Baker Norton Co., Ltd. Bergeninum (Batch No. 1532-200202, internal standard, I. S. Fig. 1b) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities of all chemicals were above 99.9%. HPLC grade acetonitrile was obtained from Fisher Scientific (Toronto, Canada). HPLC grade methanol was supplied by Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). All other chemicals and solvents used were of analytical grade. Semipermeable dialysis membranes of cellulose acetate with a molecular weight cut-off (MWCO) >5000 Da were purchased from and enlaced as bags by Shanghai Reagents Corporation (China).

Fresh blank human plasma was purchased from Nanjing Blood Supplier Center of Red Cross. Rats (Certificate No. SCXK-2002-0011) were provided by the animal breeding Center of China Pharmaceutical University, and supplied for blood sampling from thigh vein. The study was approved by the Animal Ethics Committee of the China Pharmaceutical University. All blood was anticoagulated by sodium citrate and then centrifugated to get blank rat plasma. All blank plasma were stored at -20 ℃.

Instruments and chromatographic conditions

A Shimadzu 2010A LC-MS system with electrospray ionization (ESI) interface, and Shimadzu LCMS solution workstation software (Ver 2.02) for the data processing, were utilized to perform the analytical procedures. The LC system consisted of binary LC-10ADvp pumps, a Shimadzu SIL-HTc auto-sampler, a Shimadzu CTO-10Avp column oven and a Shimadzu DGU-14AM online degasser. The LC was coupled with a Q-array-Octapole-Quadrupole mass analyzer with ESI interface. The LC process was carried out on a Luna C_{18} column (150 × 2.00 mm, 5 μm, Phenomenex, Torrance, CA, USA) with a Security Guard C_{18} guard column (4 mm × 3.0 mm, Phenomenex). The column and autosampler tray temperatures were set at 40 ℃ and 4 ℃, respectively. The volume ratio of mobile phase, made up of acetonitrile (solvent A) and water containing ammonium chloride (solvent B, 26.75 mg: 1 L, w/v) at a flow rate of 0.2 mL/min from separate pumps, was 12 : 88. The following optimized MS parameters were selected: CDL (curved desolvation line) temperature 250 ℃, block temperature 200 ℃, probe temperature 200 ℃, probe temperature 250 ℃, detector gain 1.6 KV, probe voltage -4.5kV, CDL voltage -5 V, Q-array DC

(direct current) voltage 0 V, RF (radio frequency) voltage 150 V. Nitrogen (99.995 %, from Gas Supplier Center of Nanjing University, China.) used as nebulizer gas (flow rate 1.5 L/min) and curtain gas (pressure 1 MPa). Mass spectrometer was operated in negative ion mode. Mass spectra were obtained at a dwell time of 0.2 s in Selective ion monitoring (SIM) mode and 1 s in scan mode. SIM was used and chlorinated molecular ion adduct $[M + Cl]^-$ at *m/z* 319.00 and 363.05 were used to quantify helicid and bergeninum (internal standard), respectively.

Preparation of reagent, standards and quality control samples

Equilibrium dialysate was prepared by mixing well 0.067 mol/L Na_2HPO_4 and 0.067 mol/L NaH_2PO_4 water solutions (4∶1, v/v) and subsequent addition of NaCl to the final concentration of 0.15 mol/L. Its pH value was adjusted to 7.4 using HCl or NaOH water solutions. The standard stock solutions of helicid (10 mg/mL) was prepared in deionized water, while bergeninum (1 mg/mL) in methanol. A series of standard working solutions were obtained by further dilution of the standard stock solutions with the deionized water. Internal standard working solution (1 μg/mL) was prepared by diluting internal standard stock solution (1 mg/mL) with methanol. All solutions were stored at 4 ℃. Appropriate amounts of working solution were diluted with drug-free plasma or buffer dialysate to make standard or quality control (QC) samples.

Experiments and sample preparation

Dialysis membrane bags were refluxed first in ethanol for 2 h and then in 0.01 mol/L $NaHCO_3$ water solutions for 0.5 h, followed by multiple rinses with water and then the equilibrium dialysate immediately prior to dialysis. Forty-milliliter glass tubes were prepared for placement of the dialysis bags and making two "chambers." Thirty milliliters of dialysates spiked with different amount of helicid to the concentration of 0.1, 1, 5 and 10 μg/mL respectively were transferred into the tubes accurately. 1.0 mL blank human or rat plasma was injected into the dialysis bag. Thereafter, the bag was enlaced air tightly and immersed thoroughly in the dialysis buffer. Tubes were stoppered well. Dialysis was carried out at 37 ℃ while rotating at 10 rpm in temperature controlled water bath. After 24 h of dialysis, which was determined by evaluating times between 4 to 48 h (see Results and Discussion), 50 (L of plasma and dialysate were sampled from the two "chambers" respectively into silianized polypropylene vials and stored at -20 ℃ till drug concentration analysis (within one week). Perchloric acid solution (0.1%, w/w) was used to estimate the leak of the plasma into the buffer outside. If so, the sample would be abandoned. Silianized pipet tips and polypropylen vials were used during the whole procedure to prevent loss of drug through adsorption.

An aliquot (50 μL) of the plasma or buffer dialysate, spiked with internal standard working solution (10 μL), was vortex-mixed for 30 seconds and extracted with n-butanol (1000 μL) using a vortex mixer (Scientific Industries, Inc. USA) for 3 min. Then the tubes were centrifuged at 20000 rpm at 4 ℃ for 10min (Micromax RF, Thermo Electron Corporation, USA). The upper organic phase (800 μL) of each was transferred into a clean tube and evaporated to dryness in the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was immediately reconstituted in 200 μL water and centrifuged at 20000 rpm at 4 ℃ for 10 min. The supernatant (80 μL) was pipetted to an autosampler vial, and 10 μL was injected into column for analysis.

Quality control (QC) samples (5, 100, and 2500 ng/mL) were made by spiking blank human or rat plasma with appropriate standard solutions to requisite plasma concentrations, followed by the same operation mentioned above.

Method validation

The method was validated according to FDA guidelines on specificity, sensitivity, precision, recovery, matrix effects, over-curve dilution and stability. To evaluate the assay specificity, drug-free matrices (plasma and dialysate) without adding internal standard were analyzed. The linearity of the method was determined by plotting the peak area ratios of the analyte to the IS against the concentrations of helicid in different matrices respectively. Intra-day and inter-day precisions were

investigated by reanalyzing LC/MS QC samples at low, medium and high concentrations, which were determined accurately by calibration curves prepared on the same day, for five times on a single day and once on five consecutive days. The precision should be equal to or less than 20% and accuracy between 80% and 120% of nominal concentrations. The extraction recovery was determined by calculating the peak areas obtained from blank plasma/dialysate samples spiked with analytes before extraction, to which analytes were added after extraction. According to the guidance of FDA, recovery experiments should be performed at three concentrations (low, medium, and high). So this procedure was repeated for five replicates at three concentrations of 5, 100 and 2500 ng/mL. Matrix effects were investigated by comparing the peak-area for a known amount of helicid added to the extracted blank plasma/dialysat, with the peak-area of the same amount of the test compounds in the mobile phase (B). The ratio (A/B × 100) is defined as the matrix factor. Over-curve dilution was determined by diluting 2500ng/mL QC samples by a factor of 10. The diluted samples were processed and analyzed as described in the method. The stabilities of helicid in human plasma were evaluated by analyzing replicates (n = 5) of plasma samples at three levels, which were exposed to different conditions (time and temperature).

Data processing

Drug concentrations were determined by the calibration curves constructed on the same day. The drug protein binding fraction was calculated as: binding fraction (%) = (1-out-of-bag drug concentration/in-bag drug concentration) × 100%.

Results and Discussion

Method optimization

Factors affecting dialysis effects, such as dialysis times, pH, and adsorption of helicid to the dialysis membrane were studied. Time to dialysis equilibrium was determined using drug-free dialysate buffer substituting blank plasma inside the dialysis bag. Dialysis was performed at a nominal helicid concentration of 1 μg/mL for 4, 8, 12, 24, 36, and 48 h. Time to dialysis equilibrium was determined by plotting the ratio of drug concentration in-bag to out-of-bag *versus* incubation time (Fig. 2), which showed the in-bag drug concentration almost reached a maximal plateau from 12 h. Therefore, the time to dialysis equilibrium in subsequent experiments was defined as 24 h, longer to ensure the equilibrium conditions.

For pH experiments, concentrated HCl or NaOH were used to adjust the dialysate pH range between 6.0 and 8.0. Then the pH-adjusted solutions were spiked. The dialysis was performed at a nominal helicid concentration of 1 μg/mL for 24h in silanized and unsilanized glass tubes, in order to check silanization effects at the same time. In the pH and silanization evaluation, silanized glass with pH 7.4 exhibited a relative better and stable recovery of helicid, meanwhile, this pH value approached to normal physiological condition.

Adsorption of helicid to the dialysis membrane was investigated by adding 1mL (defined as V_1) blank dialysate buffer into dialysis bags, with nominal helicid concentration of 1 μg/mL in 30 mL (defined as V_2) dialysate buffer outside, and dialyzing for 24 h (n = 4). The actual out-of-bag concentrations were analyzed at 0 h (defined as C_{start}) and 24 h (defined as C_{end}). The percentage of helicid adsorbed to the membrane (defined as X) was calculated as: $X(\%) = (C_{start} \times V_2 - C_{end} \times (V_1 + V_2))/(C_{start} \times V_2) \times 100\%$. The mean percentage of membrane binding were 2.30%, which indicate us the adsorption effects of dialysis membrane as to helicid appeared negligible in this study.

Method validation

Under the acquisition of negative SIM (selective-ion monitoring) mode, blank plasma yielded relative clean chromatograms without co-eluting interference peaks at the retention of helicid and I.S. Typical chromatograms of the blank and spiked plasma are given in Fig. 3. The representative peaks had the same m/z values from standard samples. The retention time of helicid and I.S. were about 4.28 ± 0.05 and 4.86 ± 0.05 min respectively. Five sets of calibration curves were constructed in the range 5-2500 ng/mL for helicid in different matrices respectively. Best fit for the calibration curve could be achieved by a linear equation of y

$=0.0014x+0.0031$ ($R^2=0.9991$) for rat plasma, y $=0.0014x+0.0038$ ($R^2=0.9982$) for human plasma, and y $=0.0014x-0.0005$ ($R^2=0.9993$) for dialysate buffer, with $1/x^2$ weighting factor (where, y is the peak-area ratio and x the concentration). Recovery, accuracy and precision data were shown in Table 1. The recoveries were in the range of 96.20-104.80%. Intra-day and inter-day precision values, expressed as CV, were less than 15% at all concentrations within the standard. The matrix factors in three matrixes were 97.51% (rat plasma), 98.77% (human plasma) and 97.97% (dialysate). Results showed endogenous substances did not significantly influence the ionization of the analyte. Besides, diluting samples 10-fold with blank matrices did not show any effects on the assay values, which allowed analysis after dilution for the samples which show values greater than the quantifiable limits. Stability results showed in Table 2 illuminated helicid was stable for at least 24 h at the Equilibrium dialysis condition, at least 48 h at the autosampler's conditions (4 ℃) after extraction. Meanwhile, storing at -20 ℃ for a whole week followed by three freeze-thaw cycles did not affect its stability markedly.

Table 1 Recovery, accuracy and precision of the method for analysis of helicid in different matrices ($n=5$)

Matrices	Spiked concentration (ng/mL)	Recovery			Intra-day			Inter-day		
		Measured concentration (Mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (Mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (Mean ± SD, ng/mL)	CV (%)	Accuracy (%)
Human plasma	5	4.81 ± 0.61	1.27	96.2	4.85 ± 0.24	4.95	97	4.85 ± 0.38	7.84	97
	100	100.54 ± 2.36	2.35	100.54	91.34 ± 5.22	5.71	91.34	95.25 ± 4.56	4.82	95.25
	2500	2610.23 ± 42.21	1.62	104.41	2387.99 ± 30.26	1.27	95.52	2425.56 ± 40.38	1.66	97.02
Rat plasma	5	5.14 ± 0.47	9.14	102.8	4.54 ± 0.21	4.63	90.8	4.56 ± 0.17	3.73	91.2
	100	98.88 ± 5.28	5.34	98.88	95.56 ± 3.28	3.43	95.56	92.12 ± 6.46	7.01	92.12
	2500	2512.25 ± 53.29	2.12	100.49	2512.59 ± 20.49	0.82	100.5	2399 ± 49.33	2.06	95.96
Dialysate	5	5.24 ± 0.30	5.73	104.8	4.75 ± 0.30	6.32	95	5.11 ± 0.24	4.7	102.2
	100	100.17 ± 6.11	6.1	100.17	105.24 ± 7.31	6.95	105.24	103.51 ± 3.38	3.27	103.51
	2500	2521.91 ± 44.32	1.76	100.88	2398.31 ± 58.54	2.44	95.93	2492.54 ± 57.52	2.31	99.7

Accuracy (%) = measured concentration/spiked concentration × 100%.

Table 2 Stability of helicid in different matrices under different conditions ($n=5$)

Matrices	Spiked concentration (ng/mL)	Equilibrium dialysis condition stability (37 ℃ for 24 h)			Within-run (kept in autosampler 4 ℃ for 48 h)			Long-term and freeze-thaw (stored at -20 ℃ for a whole week and followed by 3 freeze-thaw cycles)		
		Measured concentration (Mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (Mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (Mean ± SD, ng/mL)	CV (%)	Accuracy (%)
Human plasma	5	4.89 ± 0.51	10.43	97.80	5.17 ± 0.55	10.64	103.40	4.81 ± 0.38	7.90	96.20
	100	90.25 ± 5.74	6.36	90.25	100.24 ± 3.68	3.67	100.24	95.67 ± 6.41	6.70	95.67
	2500	2521.32 ± 25.77	1.02	100.85	2419.99 ± 26.35	1.09	96.80	2385.34 ± 25.14	1.05	95.41
Rat plasma	5	4.67 ± 0.41	8.78	93.40	5.37 ± 0.31	5.77	107.40	5.21 ± 0.46	8.83	104.20
	100	100.33 ± 5.32	5.30	100.33	106.21 ± 2.65	2.50	106.21	105.34 ± 4.47	4.24	105.34
	2500	2415.69 ± 16.78	0.69	96.63	2431.61 ± 38.97	1.60	97.26	2451.16 ± 24.44	1.00	98.05
Dialysate	5	5.09 ± 0.17	3.34	101.80	5.46 ± 0.19	3.48	109.20	4.52 ± 0.28	6.19	90.40
	100	108.87 ± 2.81	2.58	108.87	100.58 ± 6.51	6.47	100.58	111.15 ± 2.41	2.17	111.15
	2500	2469.13 ± 31.54	1.28	98.77	2398.82 ± 60.48	2.52	95.95	2413.24 ± 25.22	1.05	96.53

Plasma protein binding of helicid

Protein binding of helicid in human and rat plasma were studied at four concentration levels using equilibrium dialysis method, as shown in Table 3. Student's *t*-test proved no significant difference within four concentration groups of human and rat plasma respectively ($P>0.05$).

Table 3 Human and rat plasma protein binding fraction at four helicid concentration levels after dialysis for 24 h ($n=4$)

Species		Nominal concentration level(μg/mL)			
		0.1	1	5	10
Human	$C_{dialysate}$ (out-of-bag, μg/mL)	0.12 ± 0.0039	1.17 ± 0.058	5.69 ± 0.17	11.75 ± 0.35
	$C_{dialysate}$ (in-bag, μg/mL)	0.11 ± 0.0036	1.05 ± 0.041	5.06 ± 0.14	10.47 ± 0.30
	Mean binding fraction (Mean ± SD, %)	10.69 ± 0.004	10.33 ± 0.01	11.03 ± 0.007	10.89 ± 0.003
Rat	$C_{dialysate}$ (out-of-bag, μg/mL)	0.13 ± 0.0042	1.18 ± 0.060	6.05 ± 0.22	12.05 ± 0.52
	$C_{dialysate}$ (in-bag, μg/mL)	0.11 ± 0.0029	1.05 ± 0.058	5.41 ± 0.19	10.78 ± 0.49
	Mean binding fraction (Mean ± SD, %)	10.98 ± 0.0098	10.88 ± 0.005	10.60 ± 0.003	10.67 ± 0.003

Conclusion

Equilibrium Dialysis is a specific application of dialysis that is important for the study of the binding of small molecules and ions by proteins [17-19]. It is one of several methods available for this purpose, and its attractive feature continues to be its physical simplicity. This method has been widely used in plasma protein binding studies, such as (20*R*)-Ginsenoside Rh_2, Rg_3, *etc* [20]. Theoretically, drugs with high plasma protein binding have a relative less clinical safety, because if any factor that affects the binding changes, the plasma free-drug concentration would increase extremely, which always leads to serious side-effects or even toxicity. Our research indicates the plasma protein binding were about ten percent, and showed no significant different between human and rat. However, reported literatures about pharmacokinetics behaviors of helicid in humans and rats exhibiting great differences [11-15]. What in *vivo* disposition procedures generate these differences? A series of studies are under their way to uncover the mechanism of these phenomenons.

In summary, by using this proposed reliable and reproducible equilibrium dialysis method, the plasma protein binding of helicid was assessed *in vitro* for the first time. This LC-ESI-MS method was validated to be specific, sensitive, accurate and precise for analysis the drug concentrations in different matrices. Within four concentration levels, the plasma protein binding results 10.33%-11.03% for rats and 10.60%-10.98% for human showed no significant species difference between human and rats.

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