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Pharmacokinetic of four probe drugs in adriamycin-induced nephropathy rat

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Abstract. – BACKGROUND AND AIM: Probe drugs have been widely used to assess the activities of various CYP450 (cytochromes P450) isoenzymes in many fields of drug metabolism and pharmacogenetics. The nephrotic syndrome characterized by massive proteinuria and hypoproteinemia, whether that would influence the pharmacokinetics of probe drugs or not is still unclear. The purpose of the study was to investigate the pharmacokinetic of four probe drugs in adriamycin (ADR)-induced nephropathy rat.

MATERIALS AND METHODS: The rats were randomly divided into Control-group (n = 10) and ADR-group (n = 10). Nephrotic syndrome was established by weekly injections of ADR for 2 weeks. After dynamic monitoring of 24-h total urinary protein for 4 weeks, we confirmed that nephrotic syndrome had developed. The rats were administered intragastrically with phenacetin, tolbutamide, omeprazole and bupropion (15, 5, 15, and 15 mg/kg, respectively). The blood samples were determined by LC-MS (Liquid Chromatography-Mass Spectrometry) method.

RESULTS: The pharmacokinetics parameter of tolbutamide in ADR-group and Control-group were $AUC_{(0-t)}$ 15.371 ± 4.107, 6.901 ± 5.738 (mg/L*h), $MRT_{(0-t)}$ 8.751 ± 0.754, 6.032 ± 0.63 (h), $t_{1/2}$ 3.88 ± 0.423, 3.602 ± 0.693 (h), T_{max} 6.2 ± 3.768, 1.95 ± 0.798 (h), CL/F 0.038 ± 0.005, 0.107 ± 0.037 (L/h/kg), V/F 0.212 ± 0.043, 0.567 ± 0.258 (L/kg), C_{max} 1.853 ± 0.384, 1.422 ± 1.312 (mg/L). There was statistical difference in AUC, MRT, CL, V and T_{max} of tolbutamide between two groups ($p < 0.05$), but no pharmacokinetics difference for phenacetin, bupropion and omeprazole.

CONCLUSIONS: The pharmacokinetics of tolbutamide was changed in ADR-induced nephropathy rat. It is not suitable for tolbutamide to evaluate the activity of CYP450 in nephrotic syndrome.

Key Words:
Pharmacokinetics, Probe drug, CYP450, Nephrotic syndrome, Rat.

Abbreviations

AUC = Area under the curve;
MRT = Mean residence time;
$t_{½}$ = Elimination half life;
T_{max} = Peak time;
CL/F = Apparent total clearance of the drug from plasma after oral administration;
V/F = Apparent volume of distribution after non-intravenous administration;
C_{max} = Maximum (peak) plasma drug concentration;
CL = Apparent total body clearance of the drug from plasma;
V = Volume distribution;
V2 = Apparent volume of distribution during terminal phase.

Introduction

Cytochromes P450 (CYPs) belong to the superfamily of proteins containing a heme cofactor. They are a large and diverse group of enzymes that metabolize thousands of endogenous and exogenous chemicals. The most common reaction catalyzed by CYP is a monooxygenase reaction, it plays an important role in Phase I metabolism. Among the CYP isoforms, families 1 through 4 are the major enzymes involved in drug metabolism, accounting for about 75% of the total number of different metabolic reactions[1]. More than 90%-marketed drugs are metabolized by the CYP1A2, 2D6, 2C9, 2C19 and 3A isoforms[2].

The expression of CYP enzymes can be influenced by liver disease, for instance, the expression of CYP 1A2, 2E1 and 3A isoenzymes was decreased in cirrhotic patients[3,4]. Genetic determinants, dietary components, environmental factors, and medications can also induce or inhibit enzyme activity[5]. Therefore, there is a wide degree of inter-individual variability, resulting in large differences among the population in the rate and extent of drug metabolism[6].

In order to assess various individual cytochrome P450 (CYP) activities, probe drugs have been widely used in many clinical investigations in the field of drug metabolism and pharmacogenetics[7-9]. Probe drug is one kind of compounds specially catalyzed by CYP isoforms, and the ac-

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tivities of CYP isoforms can be reflected by the metabolic rate of probe drug. As several CYP isoforms involved in drug metabolism, the cocktail approach was developed. That is concurrent administration of multiple probe drugs in which multiple CYP enzymes can be tested at once. So far, several cocktail approachs have been established, such as the 'Pittsburgh cocktail'[10,11], the 'GW cocktail'[12], the 'Cooperstown cocktail'[13,14] and the 'Karolinska cocktail'[15,16]. These cocktail approachs contribute to the comprehensive analyses of metabolic pathways, and have successfully used to characterize the effects of liver disease on multiple CYP enzymes in human.

Serum albumin is the most abundant protein in circulatory system; it plays an important role in the transport and deposition of many drugs in blood. The nephrotic syndrome is one of the most common clinical disease characterized by massive proteinuria, hypoproteinemia, hyperlipidemia and edema[17]. Whether this kind of hypoproteinemia would influence the pharmacokinetics of probe drugs, and whether it would have clinical significance to evaluated the activities of CYP by probe drugs in hypoproteinemia are still unclear. So far, few studies have focused on the metabolic abilities of patient with nephrotic syndrome when its serum albumin is lower than normal level. Thus, the aim of this study was to investigate the pharmacokinetics of probe drugs in renal diseases. As adriamycin (ADR)-induced nephropathy rat is a widely used nephropathic model, its glomerular sclerosis and tubulointerstitial damage are similar to humans[18,19]. This investigation was conducted at ADR-induced nephropathy rat.

Materials and Methods

Animals and Chemicals

Adriamycin were obtained from Hisun Pharmaceutical Co Ltd (Zhejiang, China). Phenacetin, tolbutamide, omeprazole, bupropion (all > 98 %) and the internal standard carbamazepine (IS) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

A total of 20 male Sprague-Dawley (SD) rats weighing 228 ± 17 g were obtained from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China). All experimental procedures were conducted according to the Institutional Animal Care guidelines and approved ethically by the Administration Committee of Experimental Animals, Zhejiang Province, China.

Determination Method of Probe Drugs

Instrumentation and Analytical Conditions

Agilent 1200 Series liquid chromatograph equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment (Agilent Technologies, Santa Clara, CA, USA), and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source.

Chromatographic separation was achieved on a 150 mm x 2.1 mm, 5 μm particle, Agilent Zorbax SB-C18 column at 30 °C. A gradient elution programme was conducted with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0-3.0 min (10-85% B), 3.0-7.0 min (85-85% B), 7.0-8.0 min (85-10% B), 8.0-11.0 min (10-10% B). The flow rate was 0.4 mL/min.

The determination of target ions were performed in SIM (selected in monitoring) mode (m/z 180 for phenacetin, m/z 271 for tolbutamide, m/z 346 for omeprazole, m/z 240 for bupropion and m/z 237 for IS) and positive ion electrospray ionization interface (ESI). Drying gas flow was set to 7 L/min and temperature to 350 °C. Nebuliser pressure and capillary voltage of the system were adjusted to 25 psi and 3,500 V, respectively. Figure 1 showed LC-MS chromatograms and mass spectra of the probe drugs.

Sample Preparation

An aliquot of 10 μL of the internal standard (IS) working solution (1.0 μg/mL) was added into a 1.5 mL centrifuge tube containing 0.1 mL plasma sample, then followed by the addition of 0.2 mL of acetonitrile. After the tube was vortex-mixed for 1.0 min, the sample was centrifuged at 15000 rmp for 10 min. The supernatant (10 μL) was injected into the LC-MS system for analysis.

Method Validation

Linearity of each probe drugs were constructed in the range 5-2000 ng/mL for plasma (5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL) by plotting the ratios of the peak area vs. concentrations. Three different plasma concentrations (10, 400, and 1600 ng/mL) for quality-control (QC) samples were prepared by the same way.

The selectivity, accuracy, precision, recovery and stability was validated according to the literature for validation of bioanalytical methods[20,21]. Validation runs were conducted on three consecutive days. Each validation run consisted of one

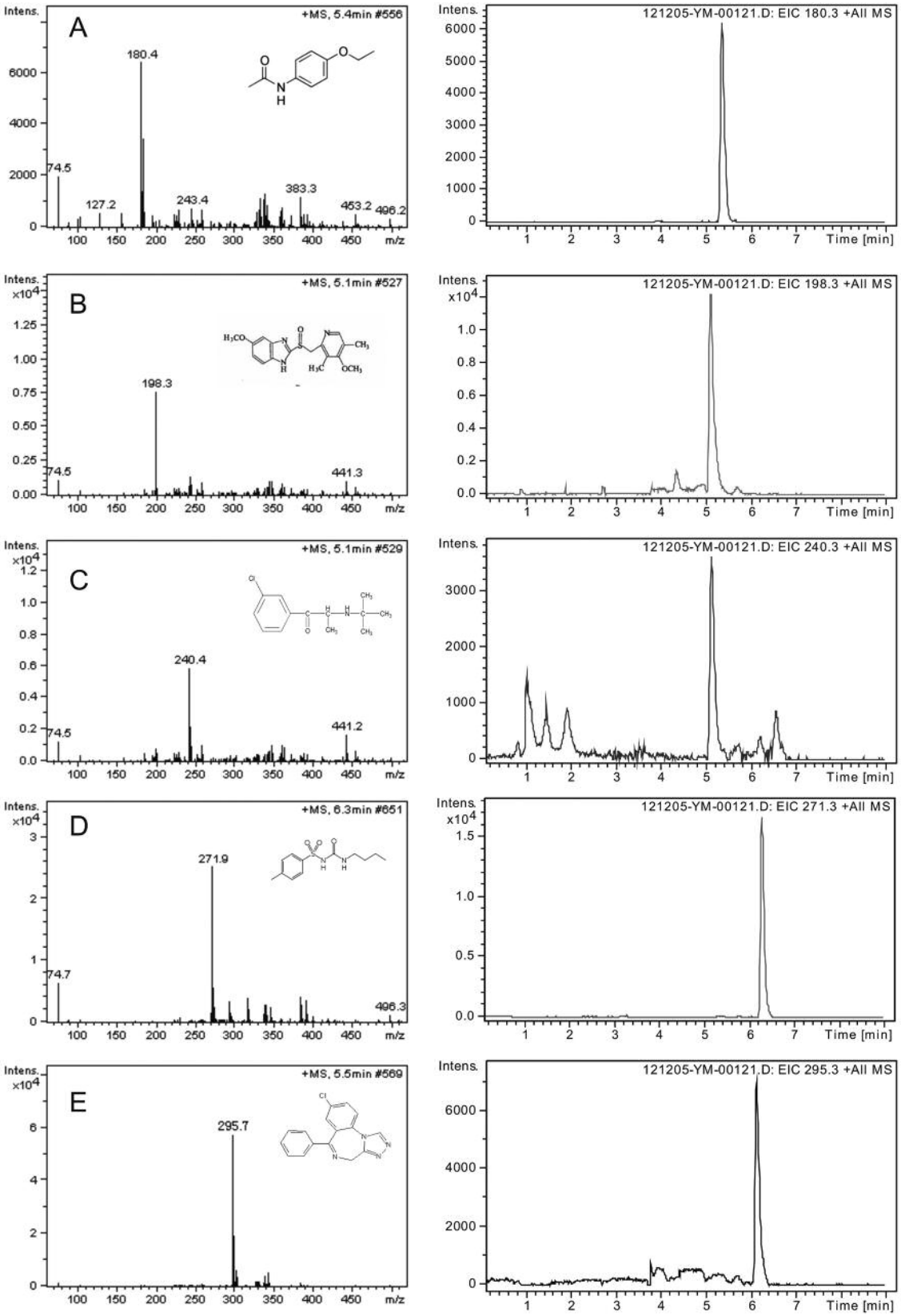

Figure 1. LC-MS chromatograms and mass spectra of phenacetin ***A***, omeprazole ***B***, bupropion ***C***, tolbutamide ***D***, and IS ***E***, in SIM mode with ESI (+) source.

set of calibration standards and six replicates of QC plasma samples.

Established ADR-induced Nephropathy Rat

20 healthy male rats were randomly divided into two groups: (1) the normal control group (Control-group, n = 10), intravenously injected an equal volume of normal saline through the caudal vein; (2) the ADR control group (ADR-group, n = 10), intravenously injected 4 mg/kg of ADR through the caudal vein at first week, 3.5 mg/kg of ADR at second week; animals of both groups drank tap water throughout the experiment. During the treatment period, body weights of the rats were measured every week, and the doses of ADR were adjusted according to the change in body weight.

Two weeks later, after administration of ADR, the rats were placed in metabolism coops and the 24-h urine were collected at every week until ADR-induced nephropathy rats were established. The concentration of 24-h total urinary protein was measured by Cobas Integra 400 Chemistry Analyzer (F. Hoffmann-La Roche, Basel, Switzerland).

When pharmacokinetic study of probe drugs was finished the rats were anesthetized by 10% chloral hydrate (3 mL/kg), and 5 mL of arterial blood was collected into tubes containing heparin from abdominal aorta. The samples were immediately centrifuged at 3000 rpm within 10 min. The plasma was separated and stored at –80 °C until further analysis. The biochemical parameters of hepatic and renal functions were measured by Hitachi 7180 Automatic Biochemical Analyzer (Hitachi High-Tech Science Systems Corporation, Tokyo, Japan).

Pharmacokinetic study of Probe Drugs

At 6 week, after the nephrotic syndrome were successfully induced, all rats of both two groups were administered intragastrically with phenacetin, tolbutamide, omeprazole and bupropion (15, 5, 15, and 15 mg/kg, respectively). Then, the blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.08333, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 20, 32, and 44 h.

The samples were immediately centrifuged at 5000 rmp for 5 min. The plasma obtained (100 μL) was stored at –20 °C until analysis. Plasma concentration versus time data for each rat was analyzed by DAS software (Version 2.0, Wenzhou Medical College, China).

Statistical Analysis

A one-way repeated measures analysis of variance was used to analyze the changes in the excretion of 24-h urinary protein. Pharmacokinetic parameters were expressed as means ± SD and ANOVA with a post hoc test (differences between groups) were used. Statistical analyses were performed using SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Method Development

The typical chromatograms of blank plasma sample spiked with phenacetin, tolbutamide, omeprazole, bupropion, and blank plasma were shown in Figure 1. No interfering endogenous substances were observed at the retention times of the analytes.

The linearity of the probe drugs peak area versus the theoretical concentration was verified in plasma by using a 1/x weighted linear regression for all the probe drugs. Coefficient of determination (r 2) was > 0.99 for all the compounds in plasma. The calibration parameters for each probe drugs in plasma were shown in Table I.

Precision of the method was determined by calculating RSD (relative standard deviation) for QCs at three concentration levels (Table II). The RSD of intra-day and inter-day precision were both less than 15% at each QC level. The accuracy of the method was ranged from 87.40 to 114.07 % at each QC level. Extraction recoveries of analytes were between 82.84 and 99.34%. The

Table I. Regression equation, correlation coefficient for probe drugs (Mean ± SD, n = 3) (y = peak area ratio of probe drugs versus IS; c = concentration of probe drugs).

Probe Drugs	Linear rane	Regression Equation	R2 (ng/mL)
Bupropion	5-2000 ng/mL	y = (0.013375 ± 0.002689)c + (0.073925 ± 0.029046)	0.9906 ± 0.0114
Omeprazole	5-2000 ng/mL	y = (0.00395 ± 0.000569)c + (0.06663 ± 0.028635)	0.9980 ± 0.0018
Phenacetin	5-2000 ng/mL	y = (0.00335 ± 0.000342)c + (0.027775 ± 0.016827)	0.9978 ± 0.0014
Tolbutamide	5-2000 ng/mL	y = (0.0035 ± 0.000432)c + (0.11405 ± 0.179651)	0.9962 ± 0.0026

Table II. Precision and recovery of probe drugs in rat plasma (Mean ± SD, n = 5).

Probe drugs	Concentration (ng/mL)	Intra-day precision	Inter-day precision	Recovery (%)
Bupropion	10	10.76 ± 0.58	10.98 ± 0.49	84.34 ± 3.21
	400	405.31 ± 14.81	400.73 ± 35.99	80.65 ± 3.12
	1600	1658.51 ± 95.88	1646.45 ± 88.12	99.01 ± 3.01
Omeprazole	10	10.43 ± 1.01	11.09 ± 0.56	89.87 ± 9.02
	400	380.26 ± 25.52	385.59 ± 15.15	90.42 ± 2.89
	1600	1506.25 ± 100.84	1585.28 ± 100.24	99.34 ± 0.03
Phenacetin	10	11.16 ± 0.85	11.26 ± 1.02	93.23 ± 3.33
	400	405.09 ± 11.28	415.09 ± 20.81	85.13 ± 2.08
	1600	1632.29 ± 92.69	1638.64 ± 108.39	93.67 ± 1.02
Tolbutamide	10	10.17 ± 0.76	10.32 ± 0.44	85.95 ± 3.84
	400	420.62 ± 24.51	430.99 ± 15.42	82.84 ± 7.56
	1600	1658.51 ± 91.88	1608.54 ± 106.58	83.74 ± 1.12

Table III. 24-hour urinary protein excretion of two groups (mean ± SD), Control-group n = 10; ADR-group n = 8.

	week 3	week 4	week 5	week 6
Control-group	6.4653 ± 1.5809	6.7138 ± 4.5945	7.3338 ± 2.6537	6.8648 ± 3.0254
ADR-group	6.2623 ± 4.4980	13.3270 ± 5.9654	18.1777 ± 4.5554	29.4345 ± 8.7736

RSD of the mean test responses was within 10% in all stability tests. The results showed that all the phenacetin, tolbutamide, omeprazole and bupropion spiked into rat plasma were stable for 2 h at room temperature, for 30 days at –20 °C. Therefore, the method was accurate and precise and can be applied to pharmacokinetic study.

Established ADR-induced Nephropathy Rat

Excretion of 24-h urinary protein at week 3, 4, 5, and 6 was increased significantly in the ADR-group as compared with that in the Control-group (Table III). All the data were meet Spherically Symmetric Test ($p = 0.081$), and repeated measures analysis of variance was used to analyze the relationship between two groups ($p < 0.01$). The results indicated that ADR-induced nephropathy rat was successfully established.

The biochemical parameters of hepatic and renal functions are shown in Table IV. There are no significantly difference in alanine aminotransferase (ALT), aspartate aminotransferase (AST), high density lipoprotein (HDL), low density lipoprotein (LDL), serum creatinine (sCr) and blood urea nitrogen (BUN). The serum albumin (ALB) was significantly decreased in ADR-group compared with that in Control-group ($p < 0.05$). Total Cholesterol (TC) and Glycerin (TG) were increased in ADR group; however, there no statistically difference between two groups.

Pharmacokinetics of Probe Drugs

The phenacetin, tolbutamide, omeprazole and bupropion concentration vs. time profiles of rats were presented in Figure 2. The pharmacokinetic parameters were given in Tables V, VI. The phar-

Table IV. Biochemical parameters in two groups (mean ± SD), Control-group n =10; ADR-group n = 8.

	ALT (U/L)	AST	ALB (g/L)	RUN (mmol/L)	sCr (μmol/L)	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Control-group	58.00 ± 12.33	16.28 ± 2.71	26.38 ± 5.94	10.93 ± 7.28	5.80 ± 3.70	2.15 ± 1.37	0.70 ± 0.44	1.31 ± 0.88	0.29 ± 0.11
ADR-group	51.86 ± 10.99	11.57 ± 3.43	15.75 ± 5.89*	8.82 ± 4.78	11.14 ± 5.15	5.06 ± 2.27	2.47 ± 1.23*	2.17 ± 0.97	0.67 ± 0.49

*$p < 0.05$; ADR-group vs. Control-groups.

Table V. Pharmacokinetic parameters of Bupropion and Omeprazole (mean ± SD), Control-group n =10; ADR-group n = 8.

Parameters	Bupropion ADR-group	Control-group	Omeprazole ADR-group	Control-group
$AUC_{(0-t)}$ mg/L*h	2.049 ± 2.132	2.303 ± 2.456	0.876 ± 0.281	0.935 ± 0.453
$AUC_{(0-\infty)}$ mg/L*h	2.105 ± 2.109	2.715 ± 2.454	1.169 ± 0.444	1.009 ± 0.517
$MRT_{(0-t)}$ h	2.545 ± 0.734	2.772 ± 0.536	2.661 ± 0.541	2.39 ± 0.483
$MRT_{(0-\infty)}$ h	2.976 ± 1.064	2.81 ± 0.518	6.01 ± 3.365	3.622 ± 1.158
t1/2z h	2.198 ± 1.056	1.151 ± 0.319	5.263 ± 3.43	3.904 ± 2.268
Tmax h	0.75 ± 0.25	1.2 ± 1.037	0.5 ± 0	0.6 ± 0.224
CLz/F L/h/kg	11.449 ± 6.111	5.633 ± 4.001	14.581 ± 5.96	8.602 ± 2.911
Vz/F L/kg	38.279 ± 20.364	10.103 ± 7.875	121.459 ± 104.906	43.71 ± 24.814
C_{max} mg/L	0.686 ± 0.494	1.211 ± 0.634	0.763 ± 0.271	1.015 ± 0.396

macokinetic parameters were computed by Drug and Statistics Software (DAS) 2.0. The pharmacokinetic profiles of phenacetin, tolbutamide, omeprazole and bupropion were best described by an open two compartment model.

Discussion

In order to determine four probe drugs in rat plasma, a sensitive, simple and reliable LC-MS method must be developed. As identify the optimal mobile phase is important for achieving good chromatographic behavior, various combinations of acetonitrile, methanol and water with changed content of each component were investigated. Finally, acetonitrile was chosen as the organic solvent and gradient elution was adopted because of its suitable sharper peak shape. As for pretreatment of rat plasma samples, acetonitrile was proved to be the best precipitation reagent when compared with methanol and 10% trichloroacetic acid in water (w/v). It was rapid and had a high recovery.

Subsequently, ADR-induced nephropathy rat must be established. After two different dose of ADR were injected for two consecutive weeks, the parameters of serum albumin, total triglyceride and 24-h urinary protein excretion were dramatically changed in ADR-group compared with that in Control-group ($p < 0.05$). Therefore, ADR successfully induced a severe nephrotic syndrome with massive albuminuria, proteinuria, hyperlipidemia, the ADR-induced nephropathy rat model was established.

After that, the pharmacokinetics of phenacetin, tolbutamide, omeprazole and bupropion in adriamycin-induced nephropathy rat was conducted based on the LC-MS method and rat renal model. The pharmacokinetic results indicated the AUC, MRT, T_{max}, $t_{1/2}$, CL, C_{max} of three probe bupropion, omeprazole and phenacetin were not decreased or increased when the appearance of nepropathy in the rat. However, there was statistical difference in pharmacokinetics of tolbutamide between two groups, The results of pharmacokinetics showed that AUC, MRT and T_{max} increased, CL and Vz decreased in ADR-group compared with Control-group.

Table VI. Pharmacokinetic parameters of Phenacetin and Tolbutamide (mean ± SD), Control-group n = 10; ADR-group n = 8

Parameters	Phenacetin ADR-group	Control-group	Tolbutamide ADR-group	Control-group
$AUC_{(0-t)}$ mg/L*h	2.919 ± 0.739	3.516 ± 2.473	15.371 ± 4.107*	6.901 ± 5.738
$AUC_{(0-\infty)}$ mg/L*h	2.922 ± 0.740	3.523 ± 2.473	15.462 ± 4.081*	6.919 ± 5.739
$MRT_{(0-t)}$ h	1.181 ± 0.217	1.416 ± 0.416	8.751 ± 0.754*	6.032 ± 0.63
$MRT_{(0-\infty)}$ h	1.187 ± 0.215	1.438 ± 0.396	8.78 ± 0.746*	6.049 ± 0.645
t1/2z h	0.56 ± 0.064	0.66 ± 0.195	3.88 ± 0.423	3.602 ± 0.693
Tmax h	0.6 ± 0.137	0.65 ± 0.224	6.2 ± 3.768*	1.95 ± 0.798
CLz/F L/h/kg	5.528 ± 1.975	8.274 ± 7.533	0.038 ± 0.005*	0.107 ± 0.037
Vz/F L/kg	4.529 ± 1.889	9.335 ± 10.339	0.212 ± 0.043*	0.567 ± 0.258
C_{max} mg/L	1.686 ± 0.587	1.332 ± 0.947	1.853 ± 0.384	1.422 ± 1.312

*$p < 0.05$, ADR-group vs. Control-groups

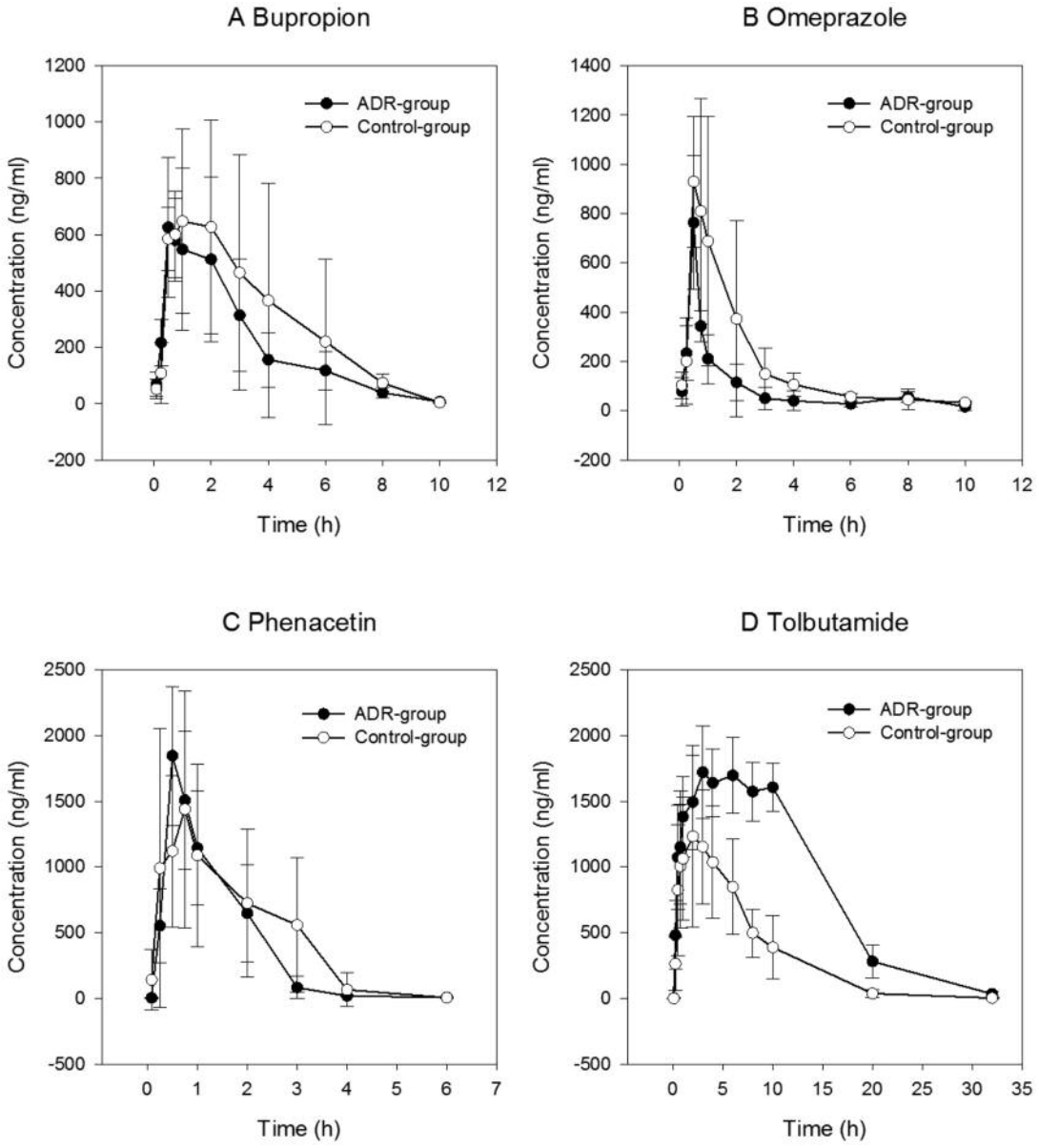

Figure 2. The pharmacokinetics profiles of bupropion ***A,*** omeprazole ***B,*** phenacetin ***C,*** and tolbutamide ***D***.

CYP450 are the major enzymes involved in drug metabolism, accounting for about 75% of the total number of different metabolic reactions, they plays an important role in metabolism of probe drugs. Tolbutamide is known to bind highly to serum proteins, and undergo a single pathway of primary metabolism catalyzed by CYP2C11 in rats[22]. This conversion of phenytoin (PHT) to p-hydroxy phenytoin is cytochrome P450 (P450)-dependent. Albumin presence in the incubation system may effect the tertiary and quater quaternary structure of the P450 system, resulting in an altered affinity to a substrate[23]. Kidney is the major excretory organs, its function is one of the determinant of the pharmacokinetics of drugs. Probe drugs are excreted through the kidney after metabolized by liver CYP450. Decreased glomerular filtration rate (GFR) can prolong probe drugs duration *in vivo* and change their pharmacokinetic characteristics.

Since there are no differences in $t_{1/2}$ (ADR-group vs Control-group, 3.88±0.423 vs 3.602 ± 0.693), the results presented herein indicated that serum albumin at low concentrations (15.75 ±

5.89 g/L) may decreased the conversion of TLB (tolbutamide) in liver microsome. Low serum albumin also decreased the distribution of TLB *in vivo* because of its highly binding rate (88-99%) with protein[24].

Conclusions

Based on the results described above, it is concluded that the pharmacokinetics of probe drugs tolbutamide was changed when serum albumin is less than normal. It is not suitable for tolbutamide to evaluate the activity of CYP450 in adriamycin-induced nephropathy rat. However, more data are required to investigate the interaction of low serum protein with probe drugs and various enzymes.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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