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Original Research Article

Plasma Pharmacokinetic and Heart Distribution Studies of Z-GP-EPI, a Hypocardiotoxic Prodrug of Epirubicin

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Abstract

Purpose: *To explore the plasma pharmacokinetics and heart distribution of Z-GP-EPI, a low cardiotoxic prodrug of epirubicin (EPI).*
Methods: *The drugs were administered to 20 rats (11. 22, 44 μmol/kg) by intravenous injection and 70 mice (30 μmol/kg) by tail intravenous injection. The profiles of Z-GP-EPI and EPI in rat plasma or mice heart were determined by high performance liquid chromatography (HPLC) method, which employed the Octadecylsilyl (ODS) column with a mobile phase of acetonitrile : 0.1 % trifluoroacetic-water (42:58, v/v) at a flow rate of 1.0 mL/min and an ultraviolet (UV) detector at a wavelength of 495 nm. Pharmacokinetics parameters were calculated using a pharmacokinetic software.*
Results: *Relative standard deviation (RSD) of intra- and inter-day precision values was < 15 % in each case while method accuracy with recovery was between 85 and 110 % for plasma and heart samples. After administration of 22 umol/kg Z-GP-EPI or EPI, terminal elimination half-life (t1/2) of Z-GP-EPI (1.41 h) was smaller than that of EPI (12.24 h). Furthermore, the concentration of Z-GP-EPI in heart rapidly decreased from 17.3 μg/g (0.05 h) to undetectable levels (2 h) while EPI changed from 14.3 μg/g (0.05 h) to 9.5 μg/g (2 h).*
Conclusion: *The HPLC method established in this study is a feasible approach to detecting Z-GP-EPI and EPI in plasma and heart tissue. In addition, Z-GP-EPI is eliminated more rapidly from plasma and heart tissue than EPI, which probably contributes to the low cardiotoxicity of Z-GP-EPI.*

Keywords: *N-Benzyloxycarbony-prolinyl-glycinyl epirubicin (Z-GP-EPI), Epirubicin, Prodrug, Hypocardiotoxic, Pharmacokinetics, Heart distribution*

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INTRODUCTION

EPI possesses broad-spectrum antineoplastic activity [1]. However, the cardio- toxicity associated with EPI is cumulative and dose-limiting, thus hampering its use in the clinic [2]. There are considerable efforts to improve the selectivity of anthracycline antibiotics and reduce their toxicity using nontoxic prodrugs. Such prodrugs are preferentially converted into active anticancer agents at the tumor site, where drug-converting enzymes are expressed at significantly higher levels than in normal tissues [3-6]. Fibroblast activation protein α (FAPα), a serine protease [7], is expressed in over 90 % of the stroma of malignant epithelial tumors, but not in normal tissues [8-10].

Given this, we have designed an EPI prodrug using the FAP-specific dipeptides glutamine (Z), glycine (G), and proline (P), and named it Z-GP-

EPI. In previous studies we have demonstrated that Z-GP-EPI could show similar inhibition activity to the growth of tumor in tumor-bearing mice model compared with EPI. Meanwhile, it could reduce the myocardial toxicity, which was the main adverse side effect of EPI, and didn't show obvious toxicity to other tissues. In addition, it is generally believed that the pharmacokinetic properties of a drug are closely linked to its efficacy and toxicity [11-14].

Therefore, the objective of this study was to explore the plasma pharmacokinetics and heart distribution of Z-GP-EPI and compare them with those of EPI.

EXPERIMENTAL

Animals

Male, specific pathogen-free (SPF) Sprague-Dawley rats (weighing 250 ± 50 g) and female SPF Bal b/c mice (20 ± 2 g) were purchased from Medical Experimental Animal Center of Guangdong, China. Animal production license NO. SCXK (Yue) 2008 - 0002. All the animals were housed in an air-conditioned animal quarter at a temperature of 25 ± 2 °C and a relative humidity of 75 ± 5 %, and kept on 12 h light/12 h dark cycle with free access to food. Before the experiment, the animals were fasted overnight for 12 h and provided water *ad libitum*. All animal experiments were approved by the ethical committee of Institute of Laboratory Animal Science, Jinan University (License no. SYXK (Yue) 2012 - 0117). The animals were handled according to standard protocols for the use of laboratory animals [15].

Chemicals and reagents

The preparation of Z-GP-EPI (purity > 99.0 %) was accomplished by the College of Pharmacy, SunYat-sen University (Guangzhou, China). EPI hydrochloride (EPI - HCl) was purchased from Hisun Pharmaceutical (purity > 98 %, Zhejiang, China). Daunorubicin (DNR; purity > 91.0 %), used as the internal standard in the study, was provided by the National Institute for Food and Drug Control, China. High-performance liquid chromatography (HPLC) - grade methanol and acetonitrile were provided by Jiangsu Hanbon (Jiangsu, China). HPLC-grade trifluoroacetic acid was purchased from Guangzhou Dongzheng Chemical Glass Instrument (Guangdong, China). All other chemicals and reagents at the highest grade available were obtained from Guangzhou Dongzheng Chemical Glass Instrument (Guangdong, China). Distilled water was produced in the Laboratory of Pharmacy.

Chromatography

The chromatographic analysis was performed by a 1260 Agilent chromatographic system (USA) with UV detector. The detection wavelength was 495 nm. Ultimate XB-C18 (4.6 × 250 mm, 5 µm, Welch materials) was used as analytical column with elution mobile phase was acetonitrile: 0.1 % trifluoroacetic-water (42:58, v/v) at a flow rate of 1.0 mL/min, column temperature 25 °C.

Pretreatment of plasma samples and heart tissues

A plasma sample (200 µL) was mixed with 20 µL of DNR (5 µg/mL for EPI analyses and 8 µg/mL for Z-GP-EPI analyses); the heart tissues were homogenized in lysis buffer [16]. Then 400µL of homogenate were mixed with 40 µL of the internal standard DNR (10 µg/mL). These samples were treated as the following liquid-liquid extraction procedure: the samples were mixed with 3 mL of the extraction solution (methanol : acetonitrile : dichloromethane (1:2:8, v/v/v)) and then centrifuged for 15 min at 3000 rpm and room temperature (25 °C). The supernatant was transferred to a clean tube and dried by 55 °C nitrogen stream. The residue was reconstituted in 100 µL of methanol; 20 µL of the final sample was injected for HPLC analysis.

Method validation

To quantify the Z-GP-EPI and EPI concentrations in plasma and heart tissue samples, the aforementioned HPLC method was validated in accordance with the United States Food and Drug Administration guidelines for specificity, extraction recovery, linearity, intra- and inter-day precision and accuracy.

Plasma pharmacokinetic studies

Sprague-Dawley rats were randomly divided into 4 groups of 5 rats each and treated as follows: Group A: 22 µmol/kg EPI; Group B: 11 µmol/kg Z-GP-EPI; Group C: 22 µmol/kg Z-GP-EPI; Group D: 44 µmol/kg Z-GP-EPI. Rats in Group A-D were treated by intravenous injection at a dose equivalent to various µmol drugs per kilogram body weight. Before 24 h of the experiment, jugular vein intubation was performed on each rat. In the experiment, blood samples (0.5 mL), which were centrifuged for 5 min at 3000 rpm to obtain plasma samples, were drawn from the jugular vein at 0, 0.05, 0.1, 0.17, 0.34, 0.76, 1, 2, 4, 6, and 8 h after drug administration. Simultaneously, an identical volume of saline was administered to maintain total body fluid balance. Then the blood samples

were pretreated as the method mentioned above. After determination of EPI and Z-GP-EPI concentration, their main pharmacokinetic parameters were calculated by pharmacokinetic software DAS 3.0.

Drug distribution in the heart

The Bal b/c mice were randomly divided into 2 groups of 35 mice each and treated as follows: Group A' : 30 μmol/kg EPI and Group B' : 30 μmol/kg Z-GP-EPI. Mice of Group A' and B' were treated by tail intravenous injection at a dose of 30 μmol EPI or Z-GP-EPI per kilogram body weight, which was equivalent to 22 μmol EPI or Z-GP-EPI per kilogram weight when administered into rats. After intravenous injection of EPI or Z-GP-EPI, 5 mice were euthanized for each time points at 0.05, 0.167, 0.5, 2, 4, 8, 12 h in each group. The heart was removed, washed with physiological saline, and blotted onto filter papers. Then the heart tissue samples were pretreated as the method mentioned above. After determination of EPI and Z-GP-EPI concentration by HPLC, the comparison of concentration between EPI and Z-GP-EPI was carried out to describe their different distribution in heart tissue.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). The data for drug distribution in heart were evaluated by one-way ANOVA followed by Dunnett Multiple Comparison test between different groups. Statistical significance of differences was analyzed using SPSS software (SPSS for Windows 15.0, SPSS Inc, USA) at a significance level of $p < 0.05$.

RESULTS

Validation results

Fig 1 and 2 illustrated the representative HPLC chromatograms of Z-GP-EPI, EPI, and the internal standard DNR in plasma samples and heart tissues. The results showed good resolution with no interfering peaks among the plasma samples and heart tissue of the Z-GP-EPI, EPI, and DNR (internal standard) groups. The extraction recoveries of Z-GP-EPI, EPI, and DNR were listed in Tab 1, which indicated that they were all more than 85 % at various concentrations in plasma and heart tissues. Tab 2 illustrated the calibration curves of Z-GP-EPI and EPI in plasma samples (0.1-100 μg/mL, R^2 = 0.999, 0.01-20 μg/mL, R^2 = 0.998) and heart tissue (0.5 - 50 μg/mL, R^2 = 0.998, 0.5 - 20 μg/mL, R^2 = 0.999). Moreover, the precision and accuracy results obtained were presented in Tab 3. The results showed that RSD % of intra- and inter-day precision values were less than 15 % (RSD) and method accuracy with recoveries were between 85 % - 110 % for the plasma and heart samples. The Tab 4 and 5 showed the results of stability for Z-GP-EPI or EPI in plasma and heart tissues. The results illustrated the recoveries were more than 85 % at 4 °C and 90 % at - 80°C but less than 80 % at 25 °C.

Z-GP-EPI is eliminated more rapidly than EPI in plasma

The main pharmacokinetic parameters of Z-GP-EPI and EPI in plasma were summarized in Tab 6. As shown, the mean $AUC_{0-\infty}$ of Z-GP-EPI was 3.93 $\mu g \cdot mL^{-1} \cdot h^{-1}$ and the $t_{1/2}$ was 1.41 h with $MRT_{0-\infty}$ of 0.97 h, while the mean $AUC_{0-\infty}$ of EPI was 2.84 $\mu g \cdot mL^{-1} \cdot h^{-1}$ and the $t_{1/2}$ was 12.24 h with $MRT_{0-\infty}$ of 7.9 h. The CL of Z-GP-EPI was 6.24 $L \cdot kg^{-1} \cdot h^{-1}$ with V_d of 5.46 L/kg, while that of EPI was 6.18 $L \cdot kg^{-1} \cdot h^{-1}$ with V_d of 115.48 L/kg.

Z-GP-EPI eliminated more rapidly than EPI in heart

Fig 3 illustrates the concentration of Z-GP-EPI and EPI in heart tissues at different time after intravenous administration of Z-GP-EPI and EPI. The results showed that the concentration of Z-GP-EPI quickly decrease from 17.3 μg/g at 0.05 h to undetectable concentration at 2 h. However, the concentrations of EPI change from 14.3 μg/g at 0.05 h to 9.5 μg/g at 2 h. Even at 12h after administration, the concentration of EPI was still 2.4 μg/g.

DISCUSSION

The low cardiotoxicity of Z-GP-EPI was vilified by our previous *in vivo* experiment. However, the bioanalytic method of Z-GP-EPI *in vivo* was still unclear. In view of the relationship between toxicity and pharmacokinetic, the HPLC method for detecting Z-GP-EPI *in vivo* was established in this study. Moreover, the plasma pharmacokinetic and heart distribution of Z-GP-EPI was calculated to support the result of low cardiotoxicity from *in vivo* experiment.

According to criteria of United States Food and Drug Administration guidelines, a suitable bioanalytic method should be specified, linear, precise and accurate. The HPLC method established in this study show good resolution between Z-GP-EPI, EPI and DNR as well as the

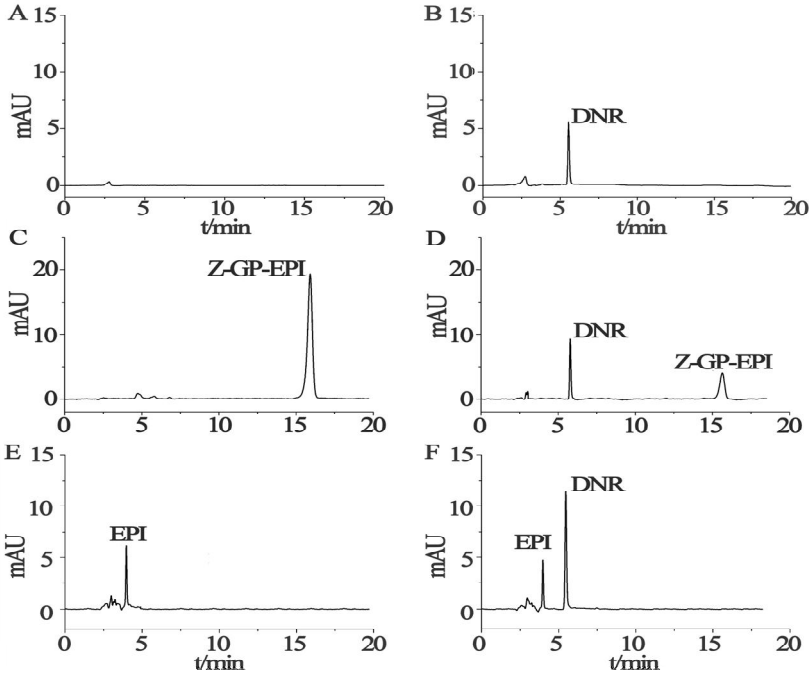

Figure 1: The representative HPLC chromatograms of Z-GP-EPI, EPI, and the internal standard DNR in plasma samples. (A. blank plasma; B. blank plasma spiked with DNR; C. blank plasma spiked with Z-GP-EPI; D. plasma sample(taken 1 h after jugular vein administration of Z-GP-EPI) spiked with DNR; E. blank plasma spiked with EPI; F. plasma sample(1 h after jugular vein administration of EPI) spiked with DNR)

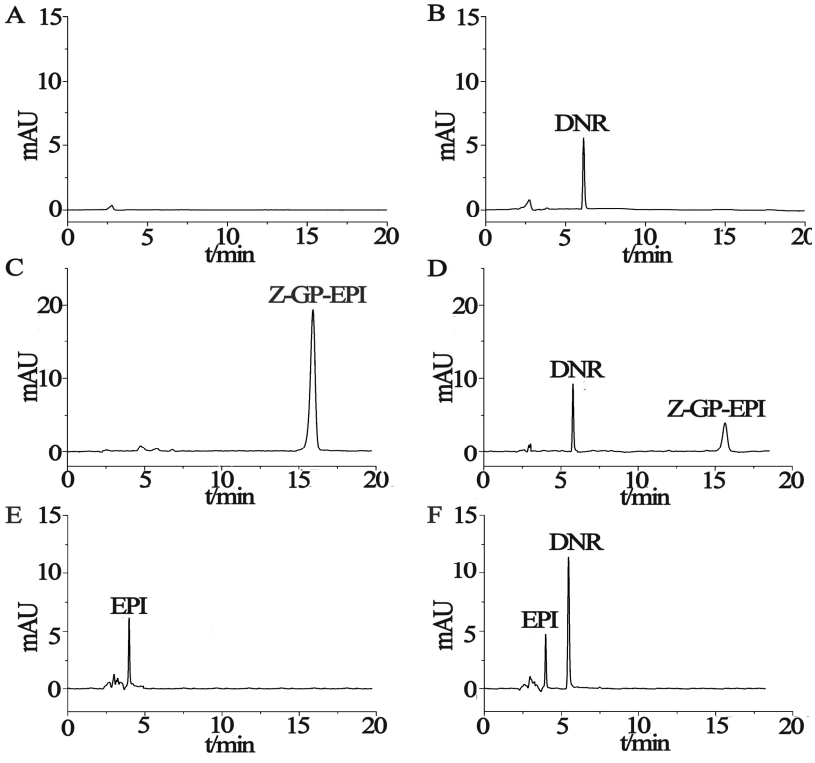

Figure 2: The representative HPLC chromatograms of Z-GP-EPI, EPI, and the internal standard DNR in heart tissue samples. (A. blank heart tissue; B. blank heart tissue spiked with DNR; C. blank heart tissue spiked with Z-GP-EPI; D. heart tissue sample(taken 0.5 h after jugular vein administration of Z-GP-EPI) spiked with DNR; E. blank heart tissue spiked with EPI; F. heart tissue sample(12 h after jugular vein administration of EPI) spiked with DNR)

Table 1: Extraction recovery of Z-GP-EPI, EPI, and DNR (n = 5)

Variable	Administered(µg/mL)		Extraction recovery, mean ± SD %	
	Plasma	***Heart***	***Plasma***	***Heart***
Z-GP-EPI	0.2	0.8	103.62 ± 3.50	85.60 ± 4.24
	5	5	86.95 ± 0.35	89.78 ± 2.91
	80	45	106.83 ± 1.15	94.19 ± 2.43
EPI	0.02	0.8	95.71 ± 0.91	92.61 ± 1.91
	5	5	85.45 ± 0.29	95.35 ± 2.36
	15	15	91.89 ± 0.90	105.32 ± 3.60
DNR	0.8	1	85.65 ± 0.77	87.76 ± 0.57

RSD = relative standard deviation

Table 2: Linear regression equations for Z-GP-EPI and EPI (n=7)

Variable		Regression equation	R^2	Range, µg/mL
Z-GP-EPI	Plasma	$y = 0.921\ x + 0.043$	0.999	0.1 - 100
	Heart	$y = 0.731\ x - 0.057$	0.998	0.5 - 50
EPI	Plasma	$y = 1.351\ x + 0.002$	0.998	0.01 - 20
	Heart	$y = 0.492\ x - 0.002$	0.999	0.5 - 20

Table 3: Precision and accuracy for Z-GP-EPI and EPI (n=5)

Variable		Administered (µg/mL)	Precision/RSD %		Recovery (%, mean ± SD)
			Intra-	Inter-	
Plasma	Z-GP-EPI	0.2	8.13	12.08	102.68 ± 10.47
		5	5.92	5.49	98.01 ± 1.00
		80	8.74	5.63	93.40 ± 5.32
	EPI	0.02	6.95	6.41	107.46 ± 0.95
		5	7.71	2.18	111.82 ± 0.72
		15	2.31	5.78	104.53 ± 1.22
Heart	Z-GP-EPI	0.8	7.57	11.67	93.17 ± 0.89
		5	6.41	5.32	98.36 ± 1.54
		45	2.45	3.34	89.76 ± 3.87
	EPI	0.8	9.59	8.75	88.36 ± 2.67
		5	4.51	6.07	94.62 ± 1.05
		15	8.06	5.54	95.36 ± 0.97

Table 4: Stability for Z-GP-EPI and EPI in plasma (n = 5)

Variable	Administered (µg/mL)	Time	25 °C/ mean ± SD %	4 °C/ mean ± SD %	- 80 °C/ mean ± SD %
Z-GP-EPI	0.2	12 h or 30 d	32.75 ± 0.22	90.27 ± 0.74	95.17 ± 0.76 (30 d)
		24 h or 90 d	25.39 ± 0.46	91.43 ± 0.91	91.84 ± 0.51 (90 d)
	5	12 h or 30 d	40.21 ± 0.42	95.48 ± 0.12	89.43 ± 0.25 (30 d)
		24 h or 90 d	31.90 ± 0.48	87.15 ± 0.33	98.52 ± 0.14 (90 d)
	80	12 h or 30 d	52.94 ± 0.64	112.68 ± 0.92	103.58 ± 0.79 (30 d)
		24 h or 90 d	42.63 ± 0.77	83.25 ± 1.27	88.79 ± 0.47 (90 d)
EPI	0.02	12 h or 30 d	53.49 ± 0.32	100.61 ± 0.21	93.48 ± 1.01 (30 d)
		24 h or 90 d	23.38 ± 0.57	93.43 ± 0.63	93.69 ± 2.07 (90 d)
	1	12 h or 30 d	64.99 ± 1.01	80.94 ± 0.32	93.69 ± 0.98 (30 d)
		24 h or 90 d	41.98 ± 0.99	81.38 ± 0.55	91.74 ± 0.70 (90 d)
	15	12 h or 30 d	79.42 ± 0.71	94.30 ± 0.70	91.74 ± 1.42 (30 d)
		24 h or 90 d	54.12 ± 0.52	87.02 ± 0.45	105.20 ± 1.49 (90 d)

Table 5: Stability for Z-GP-EPI and EPI in heart (n = 5)

Variable	Administered (μg/mL)	Time	25 °C/ (%, mean±SD)	4 °C/ (%, mean±SD)	– 80 °C/ (%, mean±SD)
Z-GP-EPI	0.8	12 h or 30 d	25.13 ± 0.79	89.55 ± 2.04	94.33 ± 0.46 (30 d)
		24 h or 90 d	21.37 ± 0.62	85.43 ± 1.09	92.59 ± 0.31 (90 d)
	5	12 h or 30 d	41.39 ± 0.73	93.05 ± 1.11	87.22 ± 0.98 (30 d)
		24 h or 90 d	37.11 ± 0.92	86.33 ± 0.33	97.44 ± 0.73 (90 d)
	45	12 h or 30 d	26.41 ± 0.72	105.68 ± 1.86	101.39 ± 1.91 (30 d)
		24 h or 90 d	21.64 ± 0.86	93.14 ± 2.75	95.62 ± 0.81 (90 d)
EPI	0.8	12 h or 30 d	49.55 ± 0.13	104.54 ± 0.77	95.38 ± 0.51 (30 d)
		24 h or 90 d	31.89 ± 0.08	90.80 ± 0.46	93.11 ± 0.43 (90 d)
	5	12 h or 30 d	70.65 ± 0.09	86.32 ± 1.01	98.72 ± 1.18 (30 d)
		24 h or 90 d	50.77 ± 2.06	84.24 ± 1.03	90.51 ± 0.95 (90 d)
	15	12 h or 30 d	73.21 ± 0.55	89.62 ± 0.51	91.74 ± 1.02 (30 d)
		24 h or 90 d	51.44 ± 0.69	86.02 ± 0.93	105.20 ± 1.49 (90 d)

Table 6: Plasma pharmacokinetic parameters for Z-GP-EPI and EPI

Parameter	Z-GP-EPI (mean ± SD)*	EPI (mean ± SD)*
AUC_{0-8} ($\mu g \cdot mL^{-1} \cdot h^{-1}$)	3.90 ± 1.97	2.43 ± 0.30
$AUC_{0-\infty}$ ($\mu g \cdot mL^{-1} \cdot h^{-1}$)	3.93 ± 2.17	2.84 ± 0.44
MRT_{0-8} (h)	0.74 ± 0.21	1.77 ± 0.30
$MRT_{0-\infty}$ (h)	0.97 ± 0.52	7.90 ± 1.58
CL ($L \cdot kg^{-1} \cdot h^{-1}$)	6.24 ± 1.56	6.18 ± 1.68
V_d (L/kg)	5.46 ± 2.80	115.48 ± 11.43
$t_{1/2}$ (h)	1.41 ± 0.53	12.24 ± 2.57

**22 μmol/kg dose; AUC = area under the concentration-time curve; CL = clearance; MRT = mean residence time; $t_{1/2}$ = terminal elimination half-life; V_d = apparent volume of distribution*

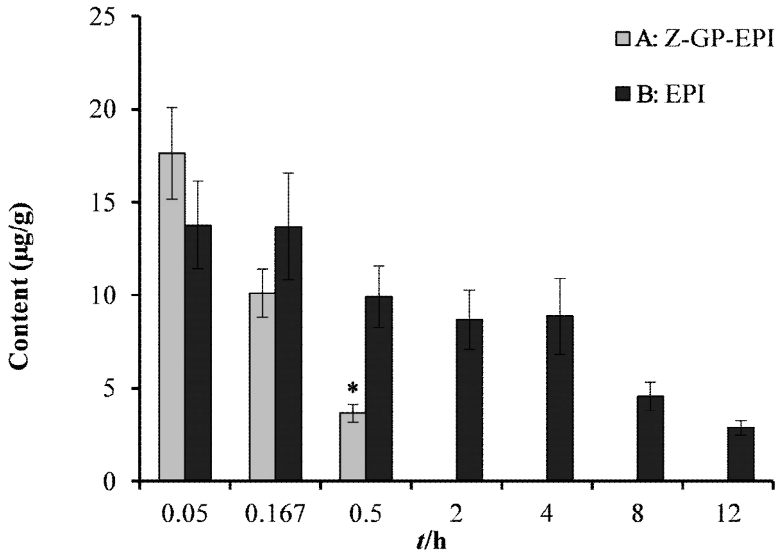

Figure 3: Concentrations of Z-GP-EPI and EPI in heart tissues after intravenous administration (*:P < 0.05 *vs.* EPI). A: The mice in this group were injected with Z-GP-EPI (30 μmol/kg). B: The mice in this group were injected with EPI (30 μmol/kg)

linear correlation between concentration and peak area at range 0.01 - 20 μg/mL in plasma and 0.5 - 20 μg/mL in heart tissue for EPI, 0.1 - 100 μg/mL in plasma and 0.5 - 50 μg/mL in heart tissue for Z-GP-EPI, respectively. Meanwhile, intra- and inter-day precision, extraction recovery and accuracy are all match the criteria. Thus the bioanalytic method was considered to be a feasible method to determine content of Z-GP-EPI and EPI in plasma and heart tissues. Then the stability of Z-GP-EPI and EPI were also evaluated by the established method in this study, result claimed that Z-GP-EPI and EPI were not stable at room temperature for the mean recoveries from the nominal concentrations declined to less than 80 %. This indicates that the plasma and heart samples

should be stored in 4 °C or - 80 °C immediately after collection.

After confirmation of the bioanalytic method of Z-GP-EPI and EPI, the plasma pharmacokinetic of Z-GP-EPI and EPI were calculated and compared. No significant difference was observed in AUC and CL between Z-GP-EPI and EPI. Smaller $MRT_{0-\infty}$, $t_{1/2}$ and V_d of Z-GP-EPI suggest that it might be distributed into organization faster or a quicker elimination process. Then we further determined the heart distribution of Z-GP-EPI and EPI, the result show Z-GP-EPI obviously eliminate more quickly than EPI in heart tissues. Z-GP-EPI could not be detected at even 2 h after administration. Meanwhile, we didn't detect free EPI enzymolysised from Z-GP-EPI in heart tissues at 2 h. However, EPI could still be detected at 12 h after administration of EPI (30 μmol/kg). The difference of elimination between Z-GP-EPI and EPI in heart tissues might support the low cardiotoxicity of Z-GP-EPI concluded from our previous *in vivo* studies. Next stage, PBPK model of Z-GP-EPI would be constructed for plasma and main organizations to clearly describe the ADMET of Z-GP-EPI.

CONCLUSION

The results obtained in this work are noteworthy as they indicate that the developed HPLC assay method for Z-GP-EPI and EPI is feasible. The results also demonstrate that the cardiotoxicity of Z-GP-EPI is lower than that of EPI. These findings provide a basis for the future study of Z-GP-EPI and its inclusion in cancer therapeutic regimens.

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