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Pharmacokinetics, excretion, and distribution of combretastatin A4 phosphate in rats

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In order to characterize the pharmacokinetics, excretion, and distribution of combretastatin A4 phosphate (CA4P) and its active metabolite, combretastatin A4 (CA4), in rats, a reliable gradient HPLC-based method has been developed and validated. The pharmacokinetic profiles of CA4P and CA4 in rats after CA4P intravenous injection at doses of 0.7, 1 and 4 mg•kg^{-1} were best described by a two-compartment model. The terminal half-lives of CA4P or CA4 were similar at different CA4P dose levels, 5∼9 min for CA4P and 39∼60 min for CA4, while $t_{1/2\alpha}$ and V_d of CA4P or CA4 were very different. CA4 was largely distributed to the heart, intestine, lung, spleen and liver during 15 to 40 min after intravenous injection of CA4P. CA4P was predominantly excreted into urine (10.72%) and feces (9.703%) and to a lesser extent into bile (0.897%), whereas a greater portion of CA4 were excreted into feces (6.235%) and to a lesser extent into urine (0.782%) and bile (0.496%) during 0–28 h after intravenous injection of 1 mg•kg^{-1} to rats. This is the first study to characterize the distribution of the active CA4P metabolite, CA4, in rat.

1. Introduction

Combretastin A4 phosphate (*cis*-α-[3,4,5-trimethoxyphenyl]-β-[3-hydroxy-4-methoxyphenyl] ethane, CA4P) is the water-soluble prodrug of combretastatin A4 (CA4), which is one of the most potent combretastatins, and is the lead compound of vascular disrupting agents without destroying normal vascular tissue (Pettit et al. 1989; Galbraith et al. 2003; Chaplin et al. 2006; Nagaiah and Remick 2010). CA4P can decrease tumor blood flow and induce necrosis in the central core of tumors (Wang et al. 2009; Greene et al. 2010). CA4P does not appear to be active by itself, but may be more efficacious when combined with cytotoxic agents and radiation therapy. It has been approved for patients with thyroid, ovarian, oesophageal, small-cell lung cancer, and melanoma in clinical trials to evaluate the safety and efficacy of CA4P when given in combination therapy (Galbraith et al. 2003; Chaplin et al. 2006; Meyer et al. 2009; Rustin et al. 2010).

The metabolic profile of CA4P was previously studied in mice, dogs, and human beings (Dowlati et al. 2002; Kirwan et al. 2004; Wang et al. 2009; Rustin et al. 2010). CA4P is rapidly dephosphorylated to form the biologically active CA4 with a half-life of a few minutes and CA4 was further metabolized at a slower rate to the glucuronide (CA4G) which is a major metabolite in the plasma (Rustin, Galbraith et al. 2003). The half-life of CA4P was 0.28 h in beagle dogs and 0.22–0.36 h in human-beings, while the half-life of CA4 was 2.75 h in beagle dogs (Wang et al. 2009) and 1.8–4.2 h in human-beings (Dowlati et al. 2002; He et al. 2011). Although the metabolism and pharmacokinetics of CA4P were well characterized, its distribution and excretion were not well recognized. The study was aimed to develop a simple HPLC-based method to characterize the pharmacokinetics, excretion, and distribution of CA4P and CA4 in rats.

2. Investigations and results

2.1. Assay specificity

Typical chromatograms of CA4P, CA4 and internal standard (IS) in rat plasma were presented in Fig. 1. No interfering endogenous compound peak was observed. The retention times of CA4P, CA4 and IS were about 12, 23 and 19 min, respectively.

2.2. Limit of quantitation

The limits of quantitation (LOQ) of CA4P and CA4 in rat plasma, feces, bile and urine were calculated as the minimum concentration that could be quantified with no more than 15% of relative standard deviation (RSD). LOQs of CA4P and CA4 were 9.90 ng•ml^{-1} and 9.85 ng•ml^{-1}, respectively.

2.3. Recovery, matrix effect and stability

Recovery or extraction efficiency of CA4P and CA4 from rat plasma, feces, bile and urine was determined by comparison of peak areas of extracted QC samples with those of corresponding standard solutions (n = 5). The recovery of CA4P from plasma and excretions ranged from 97.0% to 101.1%, and the recovery of CA4 from plasma and excretions ranged from 96.4% to 97.7%. Matrix effects for analytes were evaluated by comparing

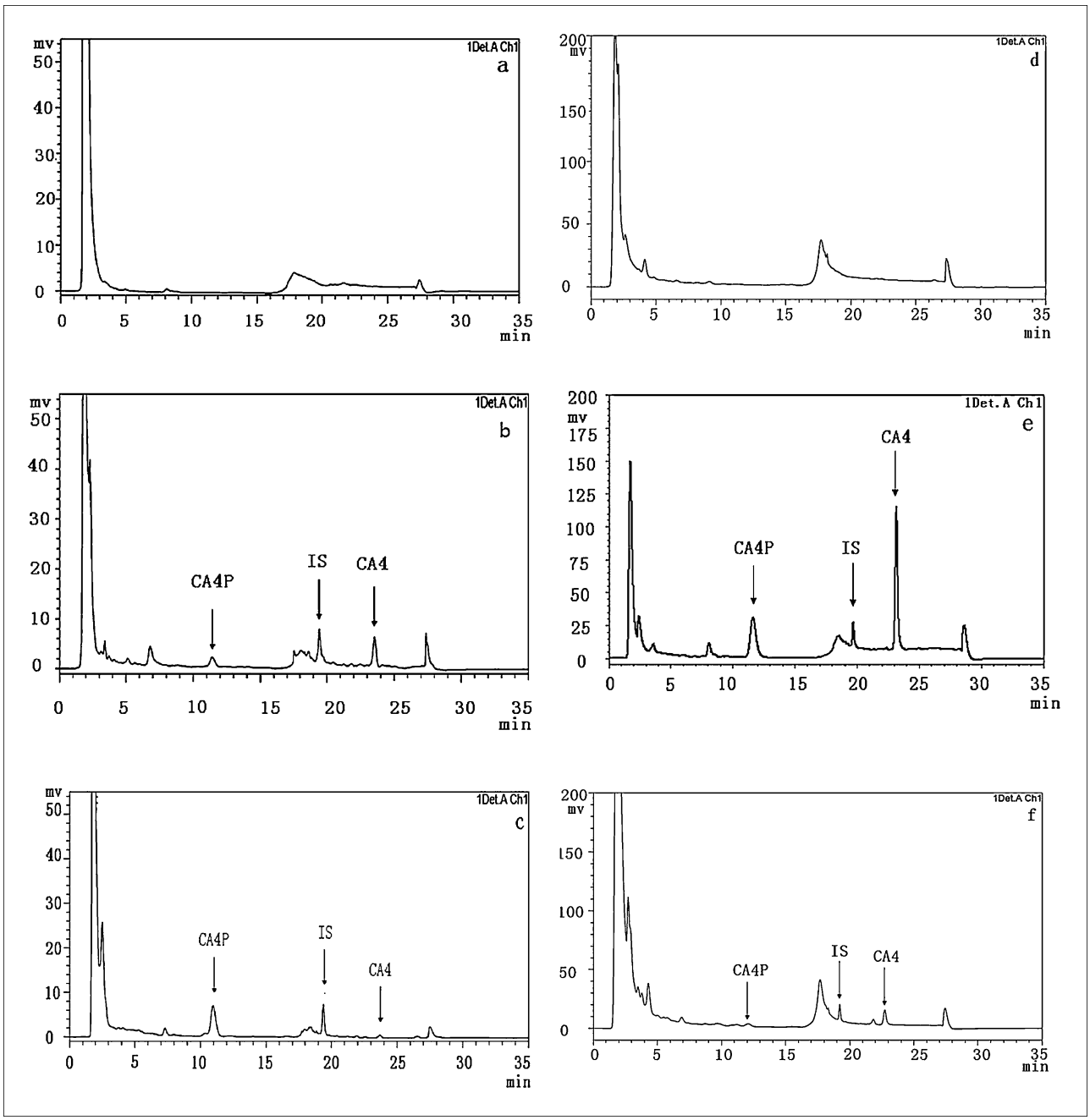


Fig. 1: Chromatograms of CA4P, CA4 and IS in rat plasma and urine, a) Blank plasma sample; b) Blank plasma with CA4P, CA4 and IS with 1.0 $\mu g \bullet ml^{-1}$; c) Plasma sample 30 min after intravenous injection administration of CA4P 1.0 $mg \bullet kg^{-1}$ to a rat; d) Blank sample; e) Blank urine with CA4P, CA4 and IS with 1.0 $\mu g \bullet ml^{-1}$; f) Urine sample 60 min after intravenous injection administration of CA4P 1.0 $mg \bullet kg^{-1}$ to a rat

the peak area of analytes resolved in blank plasma reconstituted solution (the final solution of blank plasma after extraction and reconstitution) with that resolved in the mobile phase. No significant matrix effect for analytes and IS was observed in five different lots of plasma. Thus, the ion suppression and enhancement from plasma matrix was negligible for this method.

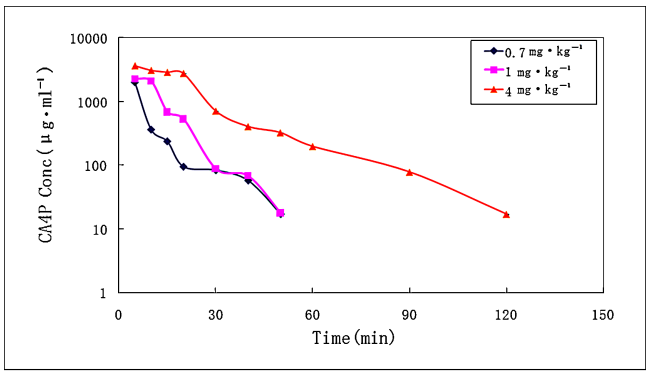
The stability of CA4P and CA4 was investigated under various storage and process conditions. CA4P and CA4 were stable in rat plasma, feces, bile and urine samples (relative errors (RE) within $\pm$ 15%) under conditions of room temperature for 24 h, 4 freeze-thaw cycles, auto-sampler for 10 h, and $-20\,^{\circ}C$ for 15 days.

2.4. Standard curve, precision and accuracy

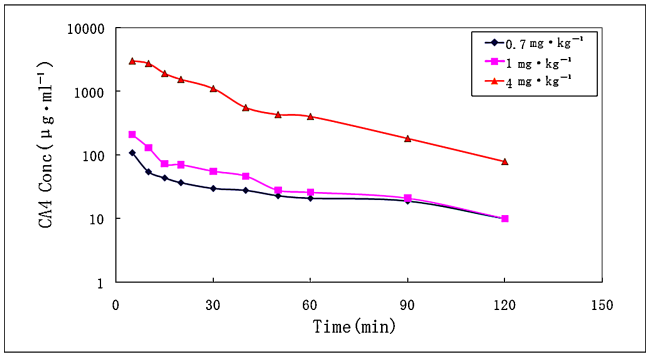
Calibration standards exhibited excellent linearity over ranges of 0.0099–19.8 $\mu g \bullet ml^{-1}$ for CA4P and 0.00985–19.7 $\mu g \bullet ml^{-1}$ for CA4 in rat plasma, feces, bile and urine (all $r > 0.995$). The intra-day and inter-day precision and accuracy were assessed by analyzing six aliquots of low, medium and high concentration samples. The intra-day precision of CA4P in different biosamples ranged between 1.42–3.82 % with RE of 1.12–3.67% and the inter-day precision ranged between 0.26- 1.60 % with RE of 0.19%- 2.45%. The intra-day precision of CA4 in different biosamples ranged between 1.03–2.42% with RE of 0.88–1.93% and the inter-day precision ranged between 0.24–2.37% with RE of 0.16%- 4.54%.

2.5. Pharmacokinetics study

The plasma concentration–time curves of CA4P and CA4 after intravenous injection of CA4P with 0.7, 1 and 4 $mg \bullet kg^{-1}$ to rats were illustrated in Fig. 2 A and B. The pharmacokinetic parameters of CA4P and CA4 were summarized in Table 1. The terminal half-lives of CA4P or CA4 were similar at different



(A)



(B)

Fig. 2: Plasma CA4P (A) and CA4 (B) concentration-time curves after intravenous injection of CA4P to rats at 0.7, 1 and 4 mg•kg^{-1}

CA4P dose levels, while $t_{1/2\alpha}$ and V_d of CA4P or CA4 were very different. The distribution of CA4P and CA4 were faster at lower CA4P dose than at higher CA4P dose. CA4P was distributed wider at higher CA4P dose, while CA4 was distributed wider at lower CA4P dose according to the volume of distribution (V_d). When the dose of CA4P increased, the $AUC_{0–120}$ values of both CA4P and CA4 increased in a linear manner.

2.6. Excretion of CA4P and CA4

The excretions of CA4P and CA4 in urine, bile and feces after intravenous injection of CA4P of 1 mg•kg^{-1} to rats were summarized in Table 2. These data indicated that CA4P was predominantly excreted into urine and feces and to a lesser extent into bile, whereas a greater portion of CA4 was excreted into feces and to a lesser extent into urine and bile. The total recoveries of CA4P were about 10.72%, 9.703%, and 0.897% in urine, feces and bile, respectively, while the total recoveries of CA4 were 0.782%, 6.235%, and 0.496% in urine, feces and bile during 0–28 h after intravenous injection of 1 mg•kg^{-1} to rats. The total recoveries of CA4P and CA4 were lower in urine, feces and bile, together accounting for less than 30% of total CA4P administered. The reason for the low recovery may be explained by CA4 conjugation with glucuronic acid before excretion. It has been reported that urinary excretion of CA4 was exclusively as the glucuronide and its recovery was about 60% of drug administered in patients with cancer (Rustin et al. 2003).

2.7. Distribution of CA4

The distribution of CA4 in tissues after intravenous injection of CA4P of 1 mg•kg^{-1} to rats was summarized in Table 3. The data indicated that CA4 was widely distributed and largely distributed to the heart, intestine, lung, spleen and liver during 15 to 40 min after intravenous injection of CA4P. CA4 concentration in different tissues decreased dramatically within 90 min except in brain, uterine and testicular, in which CA4 concentration increased slightly from 15 min to 90 min. CA4 concentration remained relative higher in intestine, brain, lung, and stomach than in other tissues.

2.8. Conclusion

A gradient high performance liquid chromatographic method with a good linearity, precision, accuracy, and a sufficient lower detection limit was developed to investigate the pharmacokinetics, distribution, and excretion of CA4P and its metabolite CA4 in rat biosamples. To the best of our knowledge, this is the first study to characterize the distribution of the active CA4P metabolite, CA4, in rat. CA4P was quickly eliminated and hydrolyzed to active CA4, which was widely distributed and may undergo conjugation with glucuronic acid before excretion.

3. Experimental

3.1. Chemicals and reagents

CA4P (MW = 440.30, purity > 99.6%), CA4 (MW = 316.35 purity > 99.6%) and the IS called intermediate II (*Trans* -α-(3,4,5-trimethoxyphenyl)-β-(4-methoxyphenyl)-acrylic acid) (MW = 344.36, purity > 99.6 %) were provided by Southwest Synthetic Pharmaceutical Corp (Chongqing, China). Chemical structures of CA4P, CA4 and IS were depicted in Fig. 3.
HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific Inc (Fair Lawn, NJ, USA). Other reagents were of analytical grade without further purification. Distilled water, prepared from deionized water, was used throughout the research.

3.2. Animals

Purebred SD rats weighing 170 ± 20 g were purchased from the Experimental Animal Center of Sichuan Academy of Medical Sciences (Chengdu, China). The rats were housed under the standard 12 h light and 12 h dark cycle with room temperature of 20 ± 1 °C and relative humidity of 55 ± 15%. The standard animal feeds and water were supplied to rats during the whole studies.

3.3. Study design and sample collection

All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China. After fasted for 12 h, rats were intravenously injected with 0.7, 1.0 and 4.0 mg•kg^{-1} of CA4P. Blood samples (5 ml) were collected in EDTA-coated tubes at 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min after administration, with five animals used at each time point. Samples were immediately placed on ice and protected from light. Plasma was then separated by centrifugation at 3000 g for 10 min and stored at −20 °C. Urine and feces samples were continuously collected during 0–1, 1–5, 5–13, 13–16, 16–20, 20–24, and 24–28 h, and bile samples were continuously collected during 0–1, 1–4, 4–6, 6–8, 8–10, 10–16, and 16–28 h after administration, with five animals used at each time point.
For the distribution study, tissue samples, including heart, liver, spleen, lung, kidney, stomach, intestine, brain, muscle, fat, uterus, testis, and bone marrow, were collected at 15, 40 and 90 min after being injected with 1 mg•kg^{-1} of CA4P, with five animals used at each time point.

3.4. Drug extraction

Fifty μl plasma, urine, bile, or extracting solution of homogenized feces or tissues were deproteinized by adding 140 μl methanol, followed by centrifugation at 12000 g for 10 min. The supernatant was dried in a rotary vacuum evaporator (protected from light), and the samples were resuspended in 50 μl of mobile phase A and then centrifuged at 12000 g for 10 min before injection onto the high-performance liquid chromatography system.

3.5. Assay validation

Standard stock solutions of CA4P and CA4 with final concentrations at 1.98 mg•ml^{-1} and 1.97 mg•ml^{-1} were serially diluted with methanol to obtain standard working solutions over a concentration range of 0.0099–19.8 μg•ml^{-1} for CA4P and 0.00985–19.7 μg•ml^{-1} for CA4. Quality-control (QC) solutions of 1.98, 0.99, 0.0198 μg•ml^{-1} for CA4P and of 1.97, 0.985, 0.0197 μg•ml^{-1} for CA4 for different biosamples were

Table 1: Pharmacokinetic parameters of CA4P and CA4 after intravenous injection of CA4P at 0.7, 1 and 4 mg•kg^{-1} to rats

Parameters	CA4P			CA4		
	0.7	1	4	0.7	1	4
A	37.252	1.425	1.543	0.281	0.347	3.589
B	0.620	4.284	4.574	0.044	0.085	0.558
$t_{1/2\alpha}$(min)	1.091	4.980	18.992	2.456	3.677	12.301
$t_{1/2\beta}$(min)	9.388	4.980	7.633	60.503	38.888	44.426
k_{10}(1•min^{-1})	0.626	0.088	0.077	0.245	0.155	0.051
k_{12}(1•min^{-1})	0.008	0.051	0.007	0.035	0.030	0.004
k_{21}(1•min^{-1})	0.075	0.139	0.043	0.013	0.022	0.017
V_d (L•kg^{-1})	0.157	0.175	2.776	46.147	20.079	15.723
CL (L•min^{-1}•kg^{-1})	0.010	0.015	0.216	0.144	0.151	0.201
AUC $_{0–120}$(mg•min• L^{-1})	30.897	46.872	92.204	3.878	6.068	93.683

Table 2: Excretions of CA4P and CA4 in rat urine, bile and feces after intravenous injection of CA4P to rats at dose of 1 mg•kg^{-1} (n = 5, mean ± SD)

Time (h)	Urine (μg•ml^{-1})		Feces (μg•g^{-1})		Time (h)	Bile (μg•ml^{-1})	
	CA4P	CA4	CA4P	CA4		CA4P	CA4
0–1	0.377 ± 0.444	0.066 ± 0.088	2.544 ± 3.690	0.856 ± 0.759	0–1	0.508 ± 0.705	0.172 ± 0.235
1–5	0.683 ± 0.775	0.026 ± 0.006	1.029 ± 0.600	0.630 ± 0.918	1–4	0.049 ± 0.060	0.069 ± 0.109
5–13	1.231 ± 1.558	0.044 ± 0.032	0.952 ± 0.533	0.576 ± 0.451	4–6	0.058 ± 0.056	0.065 ± 0.064
13–16	1.113 ± 0.556	0.059 ± 0.056	3.763 ± 4.431	4.550 ± 3.250	6–8	0.707 ± 0.778	0.173 ± 0.214
16–20	0.506 ± 0.512	0.039 ± 0.013	2.990 ± 1.670	0.522 ± 0.340	8–10	0.598 ± 0.397	0.190 ± 0.199
20–24	0.151 ± 0.055	0.045 ± 0.009	2.166 ± 2.712	0.641 ± 0.550	10–16	0.063 ± 0.071	0.312 ± 0.084
24–28	0.277 ± 0.223	0.050 ± 0.044	1.762 ± 1.344	1.021 ± 0.875	16–28	0.175 ± 0.361	0.378 ± 0.270
0–28h (%)	10.72 ± 1.045	0.782 ± 0.221	9.703 ± 2.876	6.235 ± 1.94	0–28h (%)	0.897 ± 0.063	0.496 ± 0.196

Table 3: Concentration of CA4 in rat tissues after intravenous injection of CA4P to rats at dose of 1 mg•kg^{-1} (n=5, mean ± SD)

Tissue	CA4 concentration (μg•g^{-1} tissue, mean ± SD)					
	15 min		40 min		90 min	
	Male	Female	Male	Female	Male	Female
Heart	1.245 ± 1.545	0.231 ± 0.081	0.228 ± 0.003	0.087 ± 0.015	0.177 ± 0.054	0.108 ± 0.051
Liver	0.409 ± 0.003	0.261 ± 0.169	0.333 ± 0.134	0.123 ± 0.018	0.101 ± 0.028	0.090 ± 0.019
Spleen	0.473 ± 0.261	0.337 ± 0.091	0.328 ± 0.099	0.232 ± 0.234	0.074 ± 0.022	0.073 ± 0.016
Lung	0.427 ± 0.106	0.366 ± 0.115	0.441 ± 0.019	0.296 ± 0.024	0.253 ± 0.035	0.110 ± 0.097
Kidney	0.228 ± 0.110	0.286 ± 0.041	0.149 ± 0.030	0.146 ± 0.028	0.076 ± 0.013	0.097 ± 0.025
Stomach	0.240 ± 0.013	0.260 ± 0.023	0.176 ± 0.016	0.178 ± 0.029	0.186 ± 0.045	0.174 ± 0.099
Intestine	0.732 ± 0.002	0.440 ± 0.235	0.401 ± 0.141	0.358 ± 0.211	0.224 ± 0.129	0.234 ± 0.064
Brain	0.175 ± 0.002	0.173 ± 0.004	0.144 ± 0.065	0.083 ± 0.052	0.232 ± 0.146	0.129 ± 0.062
Uterus	–	0.077 ± 0.042	–	0.094 ± 0.005	–	0.100 ± 0.108
Testis	0.044 ± 0.009	–	0.109 ± 0.069	–	0.048 ± 0.046	–
Muscle	0.056 ± 0.003	0.046 ± 0.037	0.074 ± 0.005	0.045 ± 0.017	0.032 ± 0.010	0.014 ± 0.003
Fat	0.094 ± 0.108	0.059 ± 0.020	0.121 ± 0.031	0.113 ± 0.050	0.022 ± 0.023	0.021 ± 0.003

prepared. The IS solution with the concentration of 1.03 μg•ml^{-1} was prepared with methanol. All solutions were stored at 4 °C and brought to room temperature before using.
The assay was validated for specificity, recovery, stability, precision, and accuracy.

3.6. HPLC system and chromatographic conditions

The HPLC system consisted of Shimadzu LC-2010 and LC-solution ChemStation. HPLC separations were performed on a Gemini ODS column (4.6 × 150 mm i.d, 5 μm particle size, phenomenex Inc). The flow-rate was 1.0 mL/min and chromatography was performed at 40 °C. Mobile phases A, B and C were 0.05 M KH_2PO_4 (pH:2.2), methanol and acetonitrile, respectively. Solvent gradients were used to determine CA4P and CA4 concentrations in plasma, urine, bile, feces and tissue samples. The proportion of A/B/C was first maintained as 54/45/1 for 13 min. In the following 2 min, the proportion was changed to 54/0/46 and maintained for 10 min. In the following 0.01 min, the proportion was changed back to 54/45/1 and maintained for 10 min.

3.7. Data analysis

Pharmacokinetic parameters were estimated by Drug and Statistics version 2.0 program (Anhui Provincial Center for Drug Clinical Evaluation, China). A two-compartment model was selected to estimate pharmacokinetic parameters for CA4P and CA4 at the 0.7, 1.0 and 4.0 mg•kg^{-1} dose level of CA4P according to Akaike's information criterion (AIC). $AUC_{0–120}$ of CA4P and CA4 were estimated by the linear trapezoidal rule. The linear regression model was used to evaluate dose proportionality of the $AUC_{0–120}$. Statistical differences of the pharmacokinetic parameters were determined by analy-

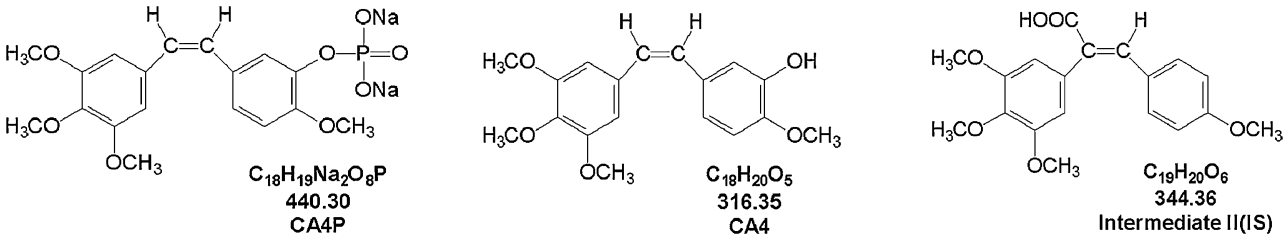


Fig. 3: Chemical structures of CA4P, CA4 and IS

sis of variance (ANOVA). Statistical analysis was performed in compliance with statistical program SPSS (SPSS, Chicago, IL).

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