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Prodrugs of scutellarin: Ethyl, benzyl and N,N-diethylglycolamide ester synthesis, physicochemical properties, intestinal metabolism and oral bioavailability in the rats

Feng Cao[a], *Jian-Xin Guo*[a], *Qi-Neng Ping*[a,*], *Zheng-Gen Liao*[b]

[a] *Department of Pharmaceutics, School of Pharmacy, China Pharmaceutical University, Nanjing 210009, China*
[b] *Key Laboratory of Modern Preparation of TCM, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China*

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ABSTRACT

In an effort to enhance the oral bioavailability of scutellarin, ethyl, benzyl and *N*,*N*-diethylglycolamide ester of scutellarin were synthesized. The hydrolysis of the prodrugs follows first-order kinetics in aqueous solution, and produced a V-shaped pH profile. The *N*,*N*-diethylglycolamide ester is highly susceptible to enzymatic hydrolysis in human plasma ($t_{1/2} \approx 7$ min) with a high stability in aqueous solution ($t_{1/2} \approx 16$ day, pH 4.2). Compared with the solubility of scutellarin, the solubility of glycolamide ester was about ten times in pH 4.0 buffer, and about thirty five times in water. Its apparent partition coefficient increased significantly from −2.56 to 1.48. Glycolamide ester of scutellarin was chosen to investigate the intestinal metabolism and *in vivo* bioavailability. Degradation studies in the intestinal tract content and homogenates indicated intestinal metabolism before absorption was a crucial obstacle for the prodrug. *N*,*N*-Diethylglycolamide ester can be protected from the degradation in the intestinal lumen by an emulsion. A significant increase in the plasma AUC and C_{max} of the prodrug emulsion was observed in rats, compared with that of the scutellarin–cyclodextrin complex ($P < 0.01$). The emulsion of *N*,*N*-diethylglycolamide ester produces a 1.58-fold enhancement in apparent bioavailability and 1.4-fold increase in the absolute bioavailability compared to the scutallarin–cyclodextrin complex.

1. Introduction

Scutellarin is the major flavonoid component of traditional Chinese medicine *Erigeron breviscapus*. It has been proved to be effective in dilating blood vessels, impoving homodynamics, decreasing the viscosity of blood, reducing the blood platelet count and preventing platelet conglomeration (Liu et al., 2003; Chen and He, 1998). Because of its poor aqueous solubility and low lipophilicity, the oral bioavailability was approximately 0.4% in beagle dogs (Ge et al., 2003). Its poor ability to penetrate cell membranes has long been a major impediment to its overall effectiveness as an oral drug.

Recently, considerable attention has been focused on the development of prodrugs, the bioreversible derivatives of the active compound, to mask polar or ionizable groups within a molecule. With respect to prodrugs of a higher lipophilicity derived from active compounds with carboxylic functions, ester formation represents a well-known approach. Strategies

* *Corresponding author*. Tel.: +86 25 8327 1098; fax: +86 25 8330 1606.
E-mail address: pingqn@cpu.edu.cn (Q.-N. Ping).
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to produce ester prodrugs include the esterification of ganciclovir with the amino acid valine (Sugawara et al., 2000), the formation of bisphosphonate prodrugs (Niemi et al., 2000) or the formation of ester prodrugs of β-lactam antibiotics (Mizen and Burton, 1998). This increases the overall lipophilicity of the molecule and promotes membrane permeability and oral absorption. However, the increase in lipophilicity produced by a prodrug approach does not always lead to major improvements in oral bioavailability. This is due to the multiple barriers towards oral delivery. It is well known that efflux transporters are present in the enterocyte membrane, which are capable of intercepting drugs during membrane passage and placing them back into the gut lumen. Enterocytes are metabolically competent cells that express a wide range of drug metabolising enzymes including esterases, cytochrome P450 isoforms and UDP-glucuronyl transferases (Prueksaritanont et al., 1996). Ester-type drugs and prodrugs are hydrolyzed by esterases present in the intestinal tract content (Crauste-Manciet et al., 1998) and mucosa (Cook et al., 1995). A few studies have paid much attention to the metabolism of ester prodrug in the intestinal tract content and mucosa (Hirayama et al., 2000; Annaert et al., 2000). Oil-in-water (o/w) submicron emulsion was previously found to be effective in protecting the ester prodrug from enzymatic attack in the intestinal lumen and improving its oral absorption (Nicolaos et al., 2003).

The present work was initiated to develop prodrugs of scutellarin, possessing a high enzymatic bioconversion rate and favorable physicochemical properties. Three ester prodrugs were synthesized and their physicochemical properties were evaluated. *N*,*N*-Diethylglycolamide ester of scutellarin was selected for the systemic study of intestinal degradation. Furthermore, the protective effect and oral bioavailability of microemulsion were investigated to gain insight of the efficiency of the ester prodrug.

2. Materials and methods

2.1. Materials

Scutellarin, 4′,5,6-trihydroxyflavone-7-glucuronide (purity >97%, lot no. HB 20031203) was purchased from Yunnan phytopharmaceutical Co. Ltd. (China). Soya lecithin was purchased from Shanghai Taiwei pharmaceutical Co. Ltd. (Shanghai, China, PC% >92%). Labrafac@CC (caprylic/capric triglycerides) and Labrasol (caprylocaproyl macrogolglycerides) were kindly presented by Gattefosse Co. (France). Transport medium consisted of Hanks' balanced salt solution (HBSS) containing 25 mM glucose and 10 mM Hepes. HBSS contained 0.40 g/l KCl, 0.06 g/l KH_2PO_4, 8.00 g/l NaCl, 0.047 g/l Na_2HPO_4 (anhydrous), 1.00 g/l D-glucose, 0.35 g/l $NaHCO_3$, 0.01 g/l phenol red and was adjusted to pH 7.0. Doubly distilled water (ddwater) was used.

2.2. Synthesis

Conventional procedures were used for the synthesis of the novel derivatives. Melting points were determined on a melting point apparatus and were uncorrected. Electrospray ionization (positive ion mode)–mass spectrometry [ESI (+)–MS] was recorded on a HP1100 LC/MSD instrument. Proton (^{1}H) nuclear magnetic resonance spectra (^{1}H NMR) were recorded on an ACF-500BRUK spectrometer. Samples were dissolved in an appropriated deuterated solvent, and chemical shifts were reported as parts per million (δ) relative to tetramethylsilane (0.00) which served as an internal standard. Elemental analyses were performed on an Elementar Vario EL III instrument. The progress of various reactions was followed by thin layer chromatography (TLC). TLC was performed on EM Reagents DC aluminum foil plates coated to a thickness of 0.2 mm with silica gel (60 mesh). All solvents and chemicals were of reagent grade.

2.2.1. *Sodium scutellarin*

A mixture of scutellarin (5.00 g, 0.011 mol), $NaHCO_3$ (0.89 g, 0.011 mol) and 50 ml water was heated with a water bath maintained at 50 °C for 1 h. Upon cooling, the brown-colored solution was filtered off and evaporated under reduced pressure. The product was dried to yield sodium scutellarin (5.12 g), yield: 96%.

2.2.2. *Ethyl-4′,5,6-trihydroxyflavone-7-glucuronide ester (D1)*

Sodium scutellarin (7.00 g, 0.015 mol) was suspended in 30 ml *N*,*N*-dimethylformamide. The suspension was admixed with ethyl bromine (3.37 ml, 0.045 mol) and catalytic amount of KI (0.1 g, 0.6 mmol). The reaction mixture was heated at 120 °C while monitoring by TLC (eluent: 6% water/0.1% glacial AcOH in methanol). After about 3 h, the reaction mixture was poured onto ice (50 g) and then extracted with ethyl acetate (4 × 50 ml). The combined extracts were washed with a 2% aqueous solution of sodium thiosulphate and water saturated with sodium chloride. After drying over anhydrous sodium sulphate, the ethyl acetate was removed under reduced pressure to give D1 (1.4 g) which was purified by recrystallization from methanol–ethyl acetate, yield: 19.8%; mp 257–259 °C; ^{1}H NMR (DMSO-d_6): δ 12.73 (s, 1H, 5-OH), 10.38 (s, 1H, 6-OH), 8.60 (s,1H, 4′-OH), 7.92–7.96 (m, 2H, 2′-H and 6′-H), 7.00 (s, 1H, 8-H), 6.86–6.95 (d, J = 7.4 Hz, 2H, 3′-H and 5′-H), 6.82 (s,1H, 3-H), 5.47–5.53 (m, 2H, 1″-H and 5″-H), 5.28 (m,2H, –CH_2–), 4.12–4.18 (m, 3H, 2″-H, 3″-H and 4″-H), 2.50–3.46 (m, 3H, other sugar protons), 1.21 (t, J = 4.4 Hz, 3H, –CH_3–); ESI(–)–MS (m/z): 489.1[$M-H$]$^-$, calculated molecular weight: 490.4. Anal. calcd. for $C_{23}H_{22}O_{12}$: C, 56.33; H, 4.52. Found: C, 55.88; H, 4.75. HPLC analysis showed a single major peak accounting for 98% of the total peak area.

2.2.3. *Benzyl-4′,5,6-trihydroxyflavone-7-glucuronide ester (D2)*

Sodium scutellarin (4.84 g, 0.01 mol) was suspended in 20 ml *N*,*N*-dimethylformamide. The mixture was added with benzyl bromide (1.20 ml, 0.01 mol) and then heated at 120 °C while monitoring by TLC (eluent: 25% acetone in petroleum ether). After about 1.5 h, the reaction mixture was poured onto ice (50 g) and then extracted with ethyl acetate (4 × 50 ml). The combined extracts were washed with water saturated with sodium chloride. After drying over anhydrous sodium sulphate and filtering, the ethyl acetate was removed under reduced pressure to give D2 (1.6 g), yield: 30.0%; mp 223–226 °C;

^{1}H NMR (DMSO-d_6): δ 12.73 (s, 1H, 5-OH), 10.36 (s, 1H, 6-OH), 8.59 (s, 1H, 4′-OH), 7.88–7.59 (m, 2H, 2′-H and 6′-H), 7.27–7.38 (m, 5H, -ph), 6.99 (s, 1H, 8-H), 6.91–6.94 (d, J = 8.8 Hz, 2H, 3′-H and 5′-H), 6.81 (s, 1H, 3-H), 5.52–5.54 (m, 2H, 1″-H and 5″-H), 5.21–5.30 (m, 3H, 2″-H, 3″-H and 4″-H), 4.25–5.17 (d, J = 9.6 Hz, 2H, –CH_2–), 3.33–3.46 (m, 3H, other sugar protons); ESI(−)–MS (m/z): 551.2$[M-H]^-$, calculated molecular weight: 552.4. Anal. calcd. for $C_{28}H_{24}O_{12}$: C, 60.87; H, 4.38. Found: C, 60.47; H, 4.42. HPLC analysis showed a single major peak accounting for 98% of the total peak area.

2.2.4. *N,N-Diethyl-2-chloroacetamide*

A stirred solution of diethylamine (11.9 ml, 0.11 mol) and benzene (20 ml) was cooled to 0 °C with a drying tube over the condenser and chloracetyl chloride (7.9 ml, 0.11 mol) was added dropwise, maintaining the temperature below 10 °C. After 0.5 h at room temperature, 15 ml water was added twice. The combined organic extracts were dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure to give a brown oil, which was used direct in the following procedure.

2.2.5. *N,N-Diethylglycolamide-4′,5,6-trihydroxyflavone-7-glucuronide ester (D3)*

The procedure was based on the general method of Bundgaard and Nielsen (1988). In brief, to a solution of the scutellarin (5 g, 0.11 mol) in *N*,*N*-dimethylformamide (20 ml) was added triethylamine (1.2 ml), KI (0.15 g) and *N*,*N*-diethyl-2-chloroacetamide (1.79 g, 0.012 mol). The reaction mixture was heated to 120 °C while monitoring by TLC (eluent: 5% water/0.1% glacial AcOH in methanol). After about 3 h, the reaction mixture was poured onto ice (50 g) and then extracted with ethyl acetate (4 × 50 ml). The combined extracts were washed with a 2% aqueous solution of sodium thiosulphate, 2% sodium bicarbonate and water saturated with sodium chloride. After drying over anhydrous sodium sulphate, the ethyl acetate was removed under reduced pressure to give D3 (4.52 g), yield: 72.7%; mp 145–147 °C; ^{1}H NMR (DMSO-d_6): δ 12.73 (s, 1H, 5-OH), 10.34 (s, 1H, 6-OH), 8.58 (s, 1H, 4′-OH), 7.93–7.98 (m, 2H, 2′-H and 6′-H), 7.02 (s, 1H, 8-H), 6.86–6.96 (d, J = 7.4 Hz, 2H, 3′-H and 5′-H), 6.81 (s, 1H, 3-H), 5.54 (s, 2H, O–CH_2–CO), 5.27–5.30 (m, 2H, 1″-H and 5″-H), 4.33–5.02 (m, 3H, 2″-H, 3″-H and 4″-H), 3.43 (m, 3H, other sugar protons), 3.25–3.30 (m, 4H, –CH_2– and –CH_2–), 0.98–1.14 (t, J = 7.1 Hz, 6H, –CH_3– and –CH_3–); ESI(+)–MS (m/z): 576.1$[M+H]^+$, calculated molecular weight: 575.5. Anal. calcd. for $C_{27}H_{29}NO_{13}$: C, 56.35; H, 5.08. Found: C, 56.15; H, 5.12. HPLC analysis showed a single major peak accounting for 98% of the total peak area.

2.3. *Physicochemical properties studies*

2.3.1. *Degradation kinetics in aqueous solutions*

The degradation of prodrugs was investigated in different buffer solutions (0.02 M, μ = 0.5) in the pH range 1.2–9.0 following standard procedures (Dias et al., 2001).

2.3.2. *Degradation studies in human plasma*

The hydrolysis of the esters was studied in human plasma diluted to 80% with pH 7.4 isotonic phosphate buffer solution (IPBS) following the method described by Bundgaard and Nielsen (1988).

2.3.3. *Solubility determinations*

Solubility determinations were carried out in phthalate buffer (pH 4.2) where the prodrugs may exhibit maximal stability, and deionized water as the method described by Dias et al. (2001). Parallel experiments were performed to determine the solubility of scutellarin in the same media.

2.3.4. *Apparent partition coefficient determination*

Partitioning studies were performed with *n*-octanol and IPBS following standard procedures (Dias et al., 2001). Parallel experiments were performed to determine the apparent partition coefficient of scutellarin.

2.4. *Degradation in intestinal tract*

2.4.1. *Preparation of intestinal tract contents and intestinal homogenates of rat*

All the animal studies reported here adhere to the Principles of Laboratory Animal Care. According to the method of Crauste-Manciet et al. (1997) intestinal tract contents (pH 6.8) were prepared. The intestinal homogenates (pH 7.0) were prepared as the method described by Augustijns et al. (1998). Protein content of the intestinal tract contents and intestinal homogenates were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.4.2. *Metabolism studies in intestinal tract contents and intestinal homogenates*

After optimization of the incubation conditions in preliminary experiments, protein content for each sample was adapted to balance between sensitivity of the analytical method and linearity of the esterase activity of the preparations. Protein contents of the intestinal tract content and intestinal homogenates were adapted to 0.6 and 0.1 mg protein ml incubation solution^{-1}, respectively. The enzymatic preparations (2 ml) were pre-incubated at 37 °C for 5 min. The reactions were started by the addition of 15 μl of the D3 stock solution in DMSO to make a final concentration of 13 μmol. At predetermined time, the reactions were stopped by adding 100 μl of the incubation solution to 200 μl of ice-cold acetonitrile. The samples were centrifuged at 12,000 rpm for 10 min and the amount of D3 in the supernatant was measured by HPLC.

The protective effect of D3 formulation was evaluated by replacing the stock solution of D3 in DMSO with the o/w emulsion (which was prepared as the following section) and following the above-mentioned methodology. Controlled experiments were made by directly adding 15 μl of D3 stock solution to 2 ml of transport medium. All experiments were conducted in triplicate.

2.5. *Bioavailability studies in the rats*

2.5.1. *Formulation preparation*

2.5.1.1. *Parenteral solution*. Scutellarin (15 mg, 0.032 mol) and diethanolamine (3.4 mg, 0.032 mol) were dissolved in 10 ml of

an aqueous solution containing 0.9% of sodium chloride. The solution was sterilized though 0.22 μm filter.

2.5.1.2. *Microemulsions*. The emulsion was prepared according to a standard procedure (Benita and Levy, 1993) and the method of Nicolaos (2003). D3 (50 mg) was dissolved in a mixture of Labrafac@CC (4.0 g, 11.9%, w/w), Labrasol (1.0 g, 3.0%, w/w) and soya lecithin (0.8 g, 2.4%, w/w) at 80 °C under nitrogen gas protection (the solubility of D3 in the oil phase was 15 mg/ml at 80 °C.). The aqueous phase was made by a mixture of polysorbate 20 (0.2 g, 0.6%, w/w) and water (27.5 g). Both phases were then mixed at 80 °C by a phase inversion method using a high shear mixer and a coarse o/w emulsion was made. The coarse emulsion was rapidly cooled and homogenized using high-pressure homogenizer (EmulsiFlex-C5, Avestin Inc., Ottawa, Canada). The pH of the emulsion was then adjusted to pH 4.5. The finished emulsion was purged with nitrogen gas and stoppered. The mean droplet size and ξ-potential value of submicron emulsion were determined by a laser diffraction particle size analyzer (Zetasizer3000HS$_A$, Malvern, Orsay, France). Each emulsion sample was diluted in ddwater (1:200) to an appropriate concentration before measurement at 25 °C. The mean droplet size and ξ-potential value of the emulsion were 189.1 ± 20.1 nm and −23.7 ± 4.3 mV, respectively. These results were mean ± S.D. error bar of three samples. D3 in the emulsion preparation process was stable, which was checked by quantitative analysis of D3 in the emulsion. Results from the study of the stability of the emulsion revealed that D3 in the emulsion was stable at 4 °C for at least 1 year.

2.5.1.3. *Scutellarin complex*. Scutellarin–cyclodextrin tetra-component complex was prepared as the described in our paper (Cao et al., 2005). Oral solution was prepared by dissolving 44.7 mg of scutellarin–cyclodextrin complex in 4 ml of ddwater to make a concentration 1.5 mg/ml of the scutellarin.

2.5.2. *Rat experiment*

Male Sprague–Dawley rats (weight range 280–300 g) were fasted for 24 h prior to experiment. A random experimental design was used. Animals (six rats for each formulation) were randomly assigned to one of the three groups. The oral solution of scutellarin complex and the emulsion of D3 were performed by means of a steel probe with a length of 6 cm, whereas the i.v. dosing was carried out by injecting the parenteral solution through the tail vein. According to the clinical scutellarin dosage (120 mg/day 60 kg for adult), the dosage of 10.8 mg/day kg for rat was used whatever the administration route. The dosage of prodrug D3 was 13.5 mg/day kg for rat, which was equivalent to 10.8 mg/day kg of scutellarin (on a molar basis). Before blood sampling, the animals were anesthetized with diethyl ether. Blood samples of 0.4 ml were taken from the ophthalmic venus plexus and put into heparinized tubes at 5, 10, 15, 30, 45, 60, 120, 240, 360 and 420 min for oral formulations. For parenteral solution, blood was collected at 0.5, 2, 5, 10, 15, 30, 60, 240, 360, 720 min. The blood was immediately centrifuged for 10 min at 4000 rpm and 200 μl of plasma was then removed and stored at −20 °C until assay for scutellarin.

2.5.3. *Treatment of plasma samples*

Fifty microliters of 1% phosphoric acid solution was added to 200 μl of plasma thawed. The mixture was vortexed for 3 min and kept for 5 min. Methanol (0.4 ml) was added and the mixture was vortexed for 3 min prior to centrifugation for 10 min at 12,000 rpm. The organic phase was transferred into new tubes and the contents evaporated to dryness under a stream of air at approximately 40 °C. The dried extracts were reconstituted with 150 μl of mobile phase solution, vortexed at high speed for 3 min, and centrifuged again for 10 min at 12,000 rpm. The entire volume of the reconstituted material (150 μl) was transferred to autosampler vials and 50 μl injected onto the HPLC.

2.5.4. *Pharmacokinetics and statistical analysis*

The plasma concentration–time data of scutellarin were fitted by 3P87 Pharmacokinetics Program (The Section of Mathematical Pharmacology of Chinese Mathematical Pharmacological Society) and the pharmacokinetic parameters were calculated. The area under the concentration–time curve (AUC_{0-t}) was determined with trapezium method. $AUC_{t-\infty}$ was calculated by dividing the last plasma concentration over the elimination rate constant. C_{max} and T_{max} were determined through the observation of individual animal concentration versus time curses. According to the definition of Krise et al. (1999), the apparent bioavailability (F_{app}) of the scutellarin following administration of the prodrug was calculated by dividing the scutellarin AUC following prodrug dosing by that from scutellarin dosing. The absolute bioavailability (F_{abs}) of scutellarin was estimated as the $AUC_{0-\infty}$ ratio of the oral preparations to the parenteral solution.

To compare the main parameters of different dosages, a two-sided unpaired *t*-test was conducted with Microsoft Excel. Statistical significance was indicated with $P < 0.01$.

2.6. Analytical procedures

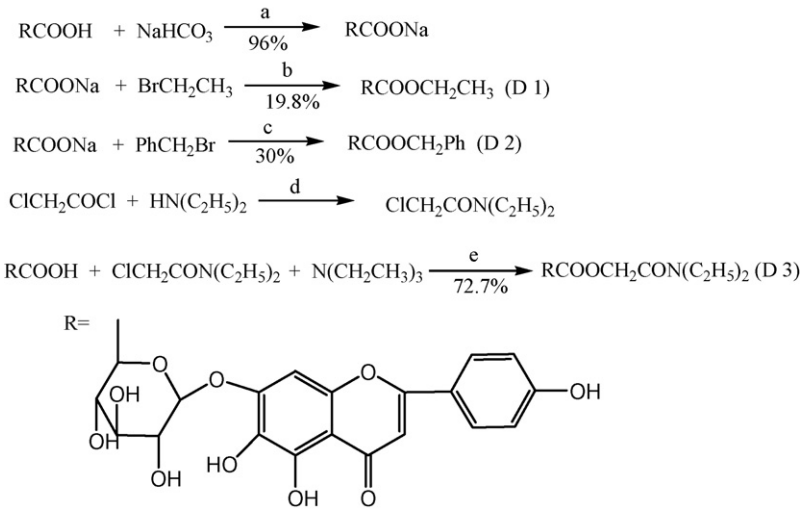
All samples obtained from the various studies were assayed by using HPLC. The HPLC system consisted of a pump (Model LC-10A, Shimadzu, Japan), a shim-pack CLC-ODS column (150 mm × 6 mm i.d., Shimadzu) maintained at 30 °C, an UV detector (Model SPD-10A, Shimadzu) at 334 nm and a data station (Model SCL-10A, Shimadzu).

2.6.1. *HPLC assay I*

This method was used to analyze the samples obtained from the studies *in vitro*. The composition of the mobile phase was methanol–water–phosphoric acid–triethylamine (53:47:0.1:0.1, v:v:v:v). The mobile phase was delivered at a flow rate of 1 ml/min. The injection volume was 20 μl and the relative retention time was found to be about 10.0 min. The correlation coefficients of the standard curve for the prodrugs were all above 0.9998. Precision assay showed that the averages of the relative standard deviations (R.S.D.) within 1 day were all below 2.4% and among every other day were all below 4.0%.

2.6.2. *HPLC assay II*

This method was used to analyze plasma samples of scutellarin. The composition of the mobile phase was methanol–water–phosphoric acid (40:60:0.1, v:v:v). The mobile phase was delivered at a flow rate of 1 ml/min. The



Scheme 1 – Synthesis of the prodrugs. (a) H_2O, 50 °C, 1 h; (b) DMF, KI, 120 °C, 3 h; (c) DMF, 120 °C, 1.5 h; (d) benzene, 0 °C, 0.5 h; (e) TEA, KI, 120 °C, 3 h.

injection volume was 50 μl and the relative retention time was found to be 8.0 min. The regression equation for scutellarin content (ng/ml) in the plasma ranging from 20 to 400 ng/ml was $A = 114.62C - 554.97$ ($r^2 = 0.9992$). The plasma assay was validated by analysis of $n = 3$ quality control samples containing 20, 100, and 400 ng/ml scutellarin in blank plasma. The recoveries were found to be accurate to 89.4%, 84.2%, and 80.7% and precise to ±6.0%, 3.1%, and 5.1% of 20, 100, and 400 ng/ml, respectively. Precision assay showed that the average of the R.S.D. within 1 day was 3.4% and among every other day was 5.0%.

3. Results and discussion

3.1. *Synthesis of the prodrugs*

The general procedures for the synthesis of scutellarin prodrugs are described as Scheme 1. Various methods have been used to synthesis ester prodrug from carboxylic compounds (Yano et al., 2002; Leisen et al., 2003). Here a mild synthesis condition was found to protect from the instabilization of flavonoid and sodium scutellarin successfully reacted with the corresponding bromide in DMF. To making the process more smoothly, ethyl bromine and sodium scutellarin should be separately excessive for the preparation of D1 and D2. According to the procedure of Bundgaard and Nielsen (1988), D3 was prepared in high yield.

3.2. *Physicochemical properties of the prodrugs*

3.2.1. *Aqueous stability*

In most cases the degradation was followed for several half-lives. However, in cases where the degradation was very slow, the half-lives of degradation were determined using the initial rate method. The influence of pH on the overall rate of hydrolysis of the three prodrugs was investigated at 37 °C (Fig. 1). From the figure is seen that the compounds are subjected to specific acid- and base-catalyzed hydrolysis together with a water-catalyzed hydrolysis. Lipophilicity of the prodrug (Table 1) does not appear to correlate with the stability of the prodrug. These esters exhibit maximum stability between pH 4.0 and 4.5 and moderate stability at neutral pH. Considering this fact the solubility studies were carried out at pH 4.2. With pH in that range and at 37 °C the half-lives of approximately 13, 8 and 16 days were calculated for D1, D2 and D3 in an aqueous solution, respectively. It is possible that the benzyl group in the ester D2 can stabilize the carbonium ion intermediate in the course of hydrolysis and increased the rate of hydrolysis. The half-lives of D3 in the buffer (0.02 M, $\mu = 0.5$) with pH 2.5, 5.6, 7.4, 9.0 are 3.37 days, 19.9 days, 1.9 h and 1.14 h, respectively.

3.2.2. *Degradation in human plasma*

The susceptibility of the prodrugs to enzymatic hydrolysis was studied in 80% human plasma in IPBS solution. The degradation equation of D1 and D2 in the human plasma was $\log C = -0.0011t + 0.5189$ ($r^2 = 0.9732$) and $\log C = -0.0036t + 0.9957$ ($r^2 = 0.9030$), respectively. The observed degradation rate was higher than that in the buffer solution. $t_{1/2}$ value

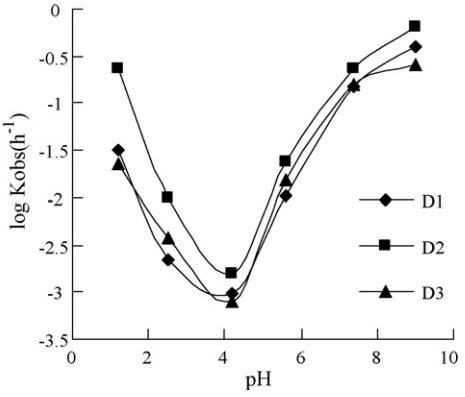


Fig. 1 – pH-rate profiles for the degradation of the prodrugs at 37 °C. Results are mean of three determinations.

Table 1 – The physio-chemical properties for scutellarin and its prodrugs

Compound	Solubility in pH 4.2 buffer (μg/ml)	Solubility in ddwater (μg/ml)	log P_{app}
Scutallarin	42.66 ± 0.80	14.44 ± 0.67	−2.56 ± 0.04
D1	12.40 ± 0.56	13.06 ± 0.74	2.37 ± 0.10
D2	10.30 ± 0.45	ND	ND
D3	420 ± 30	500 ± 20	1.48 ± 0.09

Values are the mean ± S.D. of three determinations. ND: not determined. Apparent partition coefficient values (P_{app}) were carried out in *n*-octanol/phosphate buffer (pH 7.4) at 37 °C.

of D1 was 273 min and D2 88 min. From the time course for the disappearance of D3 in 80% human plasma at 37 °C, the apparent half-life of hydrolysis of D3 is estimated to be 10 min. These results strongly suggest that these prodrugs are susceptible to serum esterase and the liberation of scutallarin from the prodrugs satisfies with the prerequisite in prodrug design. Furthermore, in this case, simple alkyl ester, D1, has the longest half-life, aryl ester, D2, has a moderate half-life and *N*,*N*-disubstituted glycolamide ester shows to be cleared with remarkable speed in human plasma. The result of the lowest half-life for the glycolamide esters is in agreement with Bundgaard (1991).

3.2.3. *Solubility and partition coefficient*

Aqueous solubility of the prodrugs and parent drug was determined at pH 4.2 (phthalate buffer) and ddwater after 24 h equilibration period. The values of solubility and apparent partition coefficient are provided in Table 1. The solubility of D2 in ddwater and its log P_{app} could not be found due to the instability. As the substitute group changes from aliphatic group to aromatic group, the solubility of D1 and D2 in the buffer decreases and the lipophilicity of the prodrugs increases to a larger extent, compared with the parent drug. In comparison, the solubility of D3 increases about 10-fold in buffer, and about thirty five times in ddwater. Partition coefficient values increases from −2.56 of scutellarin to 1.48 upon glycolamide substitution. It seems that not only the solubility but also the lipophilicity are significantly enhanced by D3. The dual enhancement was also shown for morpholinoalkyl ester prodrugs of indomethacin and naproxen by Tammara et al. (1993). It was, therefore, expected that the enhanced lipophilicity and solubility may increase oral bioavailability. Combining with the characteristics of high susceptibility to enzymatic hydrolysis in human plasma and the high stability in aqueous solution, D3 was selected as the candidate for further studies.

3.3. *Metabolism in intestinal contents and intestinal homogenates*

Chen et al. (2003) indicated that the intestinal disposition contributed more to the poor bioavailabilities than hepatic disposition for flavonoids. Beaumont et al. (2003) also suggested that an ideal ester prodrug for oral delivery should exhibit the property of resistance to hydrolysis during the absorption phase. It is most important to study the degradation properties of the ester prodrug in the intestine, so as to protect the prodrug from the metabolism in the intestine. For this purpose, an oil-in water (o/w) submicron emulsion was prepared according to the method described by Nicolaos et al. (2003). The degradation of D3 in intestinal lumen is shown in Fig. 2. At 90 min, the remaining percentage of D3 in the incubation mixture of the emulsion, stock solution and control was 79.35 ± 1.2%, 44.96 ± 4.9% and 61.44 ± 6.5%, respectively. The stock solution had a significantly ($P < 0.01$) lower D3 remaining than that of the control solution. The amount of D3 remaining was significantly ($P < 0.01$) higher in the emulsion than in the stock solution and the control. The half-life of the prodrug D3 with pH 6.8 was estimated from Fig. 1 to be about 2.03 h, which was very similar to the half-life of 2.0 h of the prodrug D3 in intestinal contents with pH 6.8. These results indicated that emulsion exhibited a protective effect against enzyme attack and chemical degradation in rat intestinal lumen. The protective effect is consistent with previous finding with other esters (Nicolaos et al., 2003). On the other side, polysorbate 20 in the emulsion as emulsifying agent may show the esterase intrinsic inhibitory effect (Crauste-Manciet et al., 1998). However, the protective effect of the prodrug emulsion would be decreased because the prodrug D3 improves both aqueous solubility and lipophilicity of the parent drug, which could also be located in the aqueous phase or in the interfacial film and attacked easily by enzyme.

As esterase-mediated degradation of an ester prodrug may be a limiting factor for its *trans*-epithelial transport. The esterase activity in homogenates from various intestinal segments was investigated in order to identify a possible absorption window with low esterase activity and hence increased ester prodrug absorption. The time-dependent degradation of D3 in homogenates from several segments of rat intestine is represented in Fig. 3. The *in vitro* hydrolysis rate of D3 in duodenum mucosa was the fastest and D3 remaining after 30 min incubation was 32.26 ± 7.70%. The mean remain-

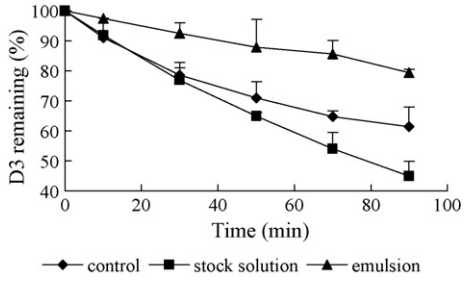


Fig. 2 – Time-dependent degradation of D3 in rat intestinal lumen, represented as the percentage of the initial concentration of 13 μmol. The amount of protein in the incubation mixture was 0.6 mg/ml. Results are mean + S.D. error bar of three determinations.

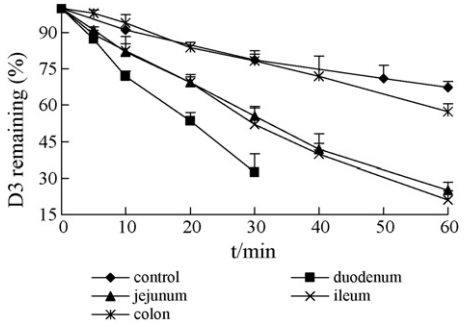


Fig. 3 – Time-dependent degradation of D3 in homogenates from several segments of rat intestinal, represented as percentage of initial concentration of 26 μmol. The amount of protein in the reaction mixture was 0.1 mg/ml. Results are mean + S.D. error bar of three determinations.

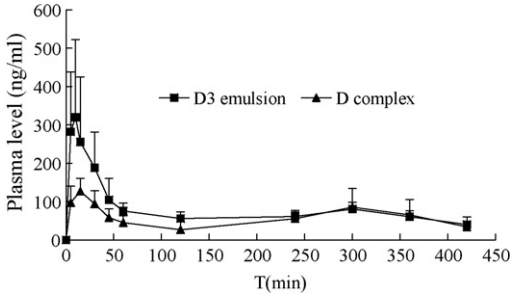


Fig. 4 – Scutellarin plasma concentration after administration of emulsion (■) ($n = 6$), and scutellarin–β-CD complex (▲) ($n = 6$).

ing percentage of D3 after 60 min incubation in jejunum, ileum and colon mucosa was 24.95 ± 3.4%, 21.02 ± 3.80% and 57.25 ± 3.40%, respectively. A significant difference ($P < 0.01$) was observed among the activity of duodenum mucosa with that of other mucosa. The degradation rates of D3 was in the order duodenum > ileum ≥ jejunum > colon. The rank is similar to those obtained by Gelder et al. (2000) and Cook et al. (1995). This order might be due to the lipid composition in the intestinal membrane, resulting in different cellular responses and enzyme activities (Ungell et al., 1998). From the site-specific degradation findings, a strategy of colon targeting may reduce esterase-mediated degradation of D3 and possibly increase its oral absorption.

3.4. *Bioavailability studies in the rats*

Because of the low lipophilicity of scutellarin, it was difficult to make a stable emulsion (o/w) of scutellarin and scutellarin–cyclodextrin tetracomponent complex was used as control, which had a 1.25 ± 0.05-fold enhancement in the apparent bioavailability of the rats orally compared to the scutellarin suspension (data not shown). In all studies, equimolar doses of drug and prodrug were administered and plasma concentrations of parent drug were recorded as a function of time. As the half-life of D3 in the blood of rats was less than 0.50 min, the concentration of D3 was lower than detection limit at 5 min after oral administration (data not shown).

The plasma concentration–time profiles of scutellarin are shown in Fig. 4. The mean pharmacokinetic parameters derived from a non-compartmental analysis are presented in Table 2. Pharmacokinetic parameters obtained after parenteral administration of scutellarin ($n = 6$) show a constant of C_{max} of 175.07 ± 13.12 μg/ml, T_{max} of 0.50 min, a MRT of 40.16 ± 0.56 min and a $AUC_{0-\infty}$ of 1622.69 μg min/ml. C_{max} of the emulsion (335.29 ng/ml) is higher than that of the complex (139.29 ng/ml) ($P < 0.01$). This difference may be due to fast dissolution and absorption of the prodrug in the emulsion, a rapid and quantitative breakdown to yield high circulating concentrations once absorbed. On the other hand, T_{max} is significantly lower for the emulsions (9.17 min) in comparison to the complex (15 min) ($P < 0.05$). There is some difference between the AUC of D3 emulsion (32,941.84 ng min/ml) and the complex (20,858.89 ng min/ml) ($P < 0.01$). The apparent bioavailability of scutellarin following D3 administration was found to be 158%. Furthermore, the absolute bioavailability of D3 emulsion increased by 1.4-fold, compared to the complex.

A double-peak phenomenon is observed in plasma concentration–time profiles of scutellarin via oral administration. Double peaks have also been observed with other drugs (Wang, 1999), including flavonoid compound (Lesser et al., 2004). An "intraintestinal" recycling process proposed by Liu and Hu (2002) may explain the second peak. Because scutellarin is a drug of glucuronidated isoflavone and poorly permeable, it is expected to be reconverted by the intestinal microflora into their aglycone forms in the colon, which could then be easier reabsorbed and re-metabolized to scutellarin.

Probably due to the esterase-mediated and chemical degradation, the oral aborption of scutellarin prodrug is only little bit better than that of scutellarin–cyclodextrin complex. It is expected that a prodrug of scutellarin with higher log P value may exhibit higher membrane permeability and bioavailability, but more important in the case, to slow down the rate of presystematic metabolism may much more enhance the

Table 2 – Pharmacokinetic parameters and bioavailability of oral formulations

	C_{max} (ng/ml)	T_{max} (min)	AUC_{0-420} (ng min/ml)[a]	MRT_{0-420} (min)	F_{app} (%)	F_{abs} (%)
Scutellarin (complex)	139.77 ± 27.46	15.00	20858.89 ± 3498.91	207.08 ± 33.10	100	1.9
D3 (emulsion)	335.29 ± 186.22**	9.17 ± 2.04*	32941.84 ± 9113.03**	172.29 ± 24.74	158	2.6

Results are mean ± S.D. of six determinations.

[a] AUC is for scutellarin.

* Significantly different ($P < 0.05$) from scutellarin complex.

** Significantly different ($P < 0.01$) from scutellarin complex.

bioavailability. The species differences in esterase activity of intestinal were observed by Gelder et al. (2000), the general order of decreasing activity being: rat > man > pig. Yoshigae et al. (1998) observed that the hydrolase activities of rat intestinal mucosa for an ester prodrug were significantly high than those of dog (50–260-fold). Esterase activity in rat whole blood was approximately 100 and 400 times higher than that in dog and human whole blood, respectively (Minagawa et al., 1995) and the similar results were also observed in this paper. Other animals with lower esterase activity in the intestine and blood are expected to have a better enhancement effect on absorption. The assumption needs to be validated in our further studies of D3 in other animals.

4. Conclusions

In conclusion, the glycolamide ester prodrug of scutellarin has the highest susceptibility to enzymatic hydrolysis in human plasma ($t_{1/2} \approx 7$ min), a higher stability in aqueous solution, a good enhancement of apparent partition coefficient, compared with ethyl and benzyl ester prodrugs. The study showed that the esterase degradation of the prodrug in the intestinal lumen could be protected to some extent by making its o/w emulsion. However, comparing with the scutellarin–cyclodextrin complex, the absorption enhancement of the glycolamide ester prodrug was not as high as expected. These results suggested that the stability of the ester prodrug along the absorption in the intestinal tract would be improved further besides the emulsion. It is crucial to find prodrugs with lower metabolism or a formulation with much better protection effect in the intestinal lumen and mucosa. In general, this work emphasizes the need to study the presystematic degradation for any other oral prodrugs. In other hand, the choice of animal models is also important and the oral absorption of the glycolamide ester prodrug of scutellarin in other animals is in process and will be the subject of further studies.

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