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HPLC analyses and pharmacokinetic studies of baicalin and oxymatrine in rabbits

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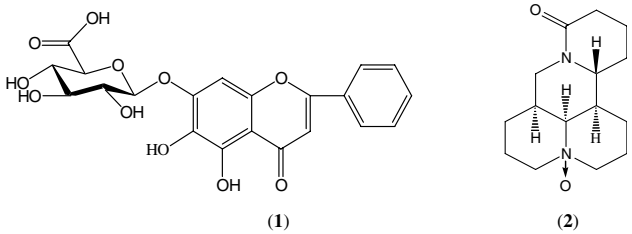
In the present study two simple RP-HPLC methods were developed to determine baicalin and oxymatrine in rabbit serum. Separation was performed on a Diamonsil™ C_{18} column (200 mm × 4.6 mm I.D., 5 μm) with UV detector at 277 nm for baicalin and 220 nm for oxymatrine. The mobile phase was methanol-water-phosphoric acid 50 : 50 : 0.2 v/v for baicalin and acetonitrile-water (20 : 80, v/v, 5 mmol/L sodium octanesulfonate was contained and pH was adjusted to 3.2 with phosphoric acid for oxymatrine. p-Nitrobenzoic acid and phenacetin were used as internal standards for baicalin and oxymatrine, respectively. The standard curves were linear from 0.5 to 200.0 mg/L for baicalin and from 0.5 to 100.0 mg/L for oxymatrine with correlation coefficients of 0.9994 and 0.9965, respectively. The intra-day and inter-day RSD were less than 5.4% and 7.2% for baicalin and 6.6% and 13.8% for oxymatrine. The mean recoveries were 100.1% for baicalin and 99.1% for oxymatrine. The methods were applied to a pharmacokinetic study of baicalin and oxymatrine in rabbits. The pharmacokinetic parameters were determined after intravenous injections of baicalin and oxymatrine (40 mg/kg) separately and together to rabbits. They all fit to the two-compartment open model. Student's t test shows that there is no significant difference in the main pharmacokinetic parameters including $AUC_{0-\infty}$, when α and β, when baicalin and oxymatrine were administered separately or together.

1. Introduction

Pharmacological studies of baicalin, which is the flavone isolated from baikal skullcap root, show that it has antibacterial, antiviral, anti-inflammatory, decompression and immunoloregulation activities [1]. Oxymatrine, an alkaloid isolated from light yellow sophora root, has anti-inflammatory, anti-tumor, anti-arrhythmia, and also immunoloregulation activities [2]. Both compunds have been used as excellent liver protecting drugs in Traditional Chinese Medicine (TCM). Their use in combination however, has never been described in the literature and pharmacokinetic studies after concomitant administration have not been performed before. It seems, therefore, to be useful to establish sensitive, specific and simple methods to determine the plasma concentration of baicalin and oxymatrine and to study their pharmacokinetics *in vivo*.

Varieties of HPLC methods have been reported in the literature to determine baicalin from biological fluids [3–5]. These methods require a special detector (electrochemical detector) and have low sensitivity. The methods for the determination of oxymatrine in biological fluids include colorimetric [6, 7], fluorescence quenching [8] and HPLC [9] methods. But the low sensitivity and complicated preparation procedures are disadvantages of these methods. So this report describes the development and validation of simple and sensitive RP-HPLC methods to determine baicalin and oxymatrine in rabbit plasma and their application to the analysis of biological samples.



(**1**) (**2**)

baicalin (**1**) and oxymatrine (**2**)

2. Investigations and results

2.1. Chromatographic separation

A baseline separation of baicalin and p-nitrobenzoic acid (internal standard) was obtained in plasma. The retention times of p-nitrobenzoic acid and baicalin were 11.7 min and 14.3 min, respectively (Fig. 1).

A baseline separation of oxymatrine and phenacetin (internal standard) was obtained with a retention time of 5.7 min for oxymatrine and 18.6 min for phenacetin (Fig. 2).

An aliquot of 200 μL of various concentrations (0.5, 2.5, 5.0, 25.0, 50.0, 100.0 and 200.0 mg/L) of baicalin and 200 μL p-nitrobenzoic acid was added to 200 μL blank rabbit plasma. Then the samples were prepared as described in chapter 4.3. The standard curve for baicalin was $Y = 0.12596 + 0.18715X$ ($r = 0.9994$), where Y is the

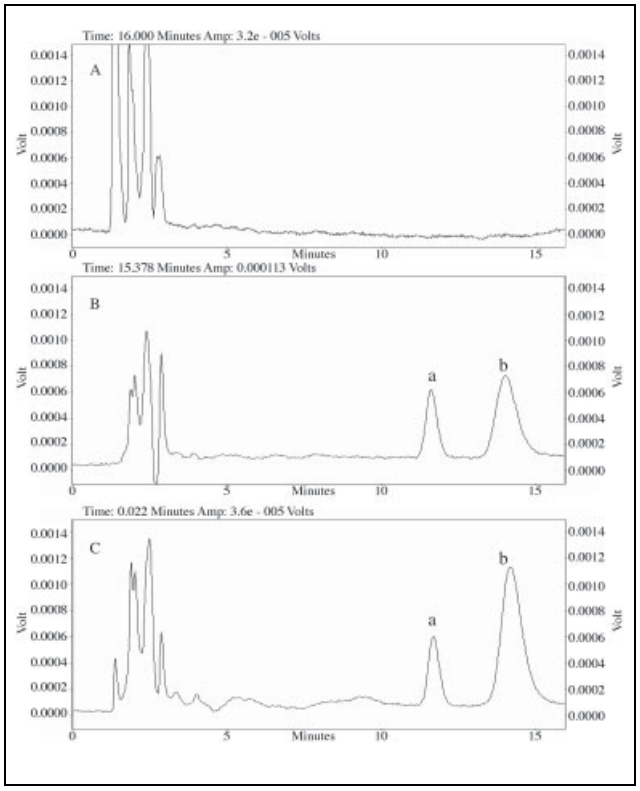


Fig. 1: Representative chromatograms of baicalin and p-nitrobenzoic acid in rabbit plasma
A – blank rabbit plasma; B – blank rabbit plasma spiked with 20 mg/L baicalin and 5 mg/L p-nitrobenzoic acid; C – a plasma sample 30 min after an iv dose of 40 mg/kg baicalin.
a – p-Nitrobenzoic acid b – Baicalin

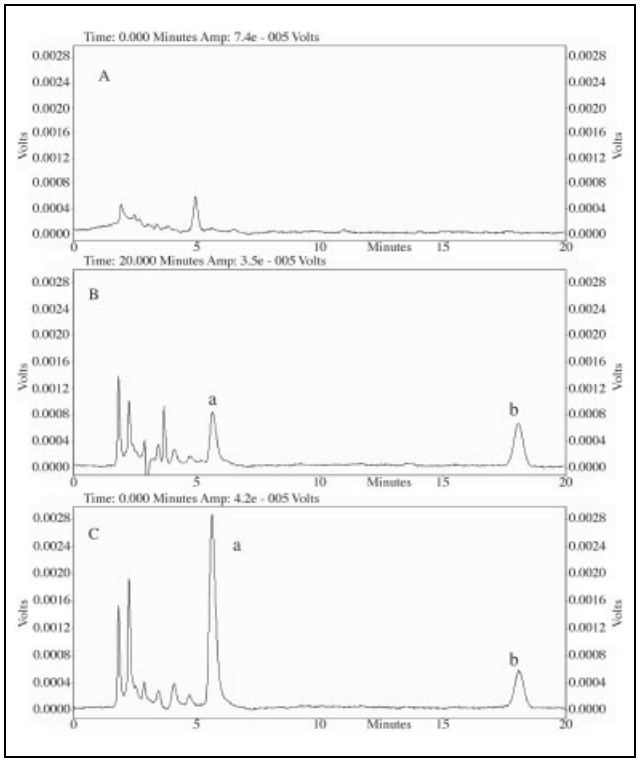


Fig. 2: Representative chromatograms of oxymatrine and phenacetin in rabbit plasma
A – blank rabbit plasma; B – blank rabbit plasma spiked with 20 mg/L oxymatrine and 5 mg/L phenacetin; C – a sample 30 min after an iv dose of 40 mg/kg oxymatrine.
a – Oxymatrine b – Phenacetin

Table 1: Accuracy and precision for the determination of baicalin in rabbit plasma (n = 6)

Concentration (mg/L)		RSD (%)		RE (%)
Added	Found	Intra-day	Inter-day	
0.50	0.50	4.6	7.2	0
25.00	25.45	5.4	6.7	1.8
200.00	197.20	3.7	5.3	−1.4

Table 2: Accuracy and precision for the determination of oxymatrine in rabbit plasma (n = 6)

Concentration (mg/L)		RSD (%)		RE (%)
Added	Found	Inter-day	Intra-day	
0.50	0.52	6.6	10.2	4.0
10.00	9.59	4.9	13.8	−4.1
100.00	97.37	3.7	8.4	−2.6

Table 3: Recoveries of oxymatrine and phenacetin (n = 6)

Compound	Added conc. (mg/L)	Recovery (%)	RSD (%)
Phenacetin	5	80.7	3.0
	0.5	80.6	5.2
Oxymatrine	10	82.2	3.7
	100	79.4	1.2

peak area ratio of baicalin to p-nitrobenzoic acid, and X is the concentration of baicalin. The linear range of baicalin is $0.5 \sim 200.0$ mg/L and the lower limit of quantitation (LLOQ) is 0.5 mg/L.

200 μL of various concentrations (1.0, 2.0, 4.0, 10.0, 40.0, 100.0 and 200.0 mg/L) of oxymatrine and 200 μL phenacetin solution were added to 400 μL blank plasma. The samples were prepared as described in chapter 4.3. The standard curve for oxymatrine was $Y = -0.01358 + 0.18728X$ ($r = 0.9965$), where Y is the peak area ratio of oxymatrine to phenacetin, and X is the concentration of oxymatrine. The linear range of oxymatrine is $0.5 \sim 100.0$ mg/L and the lower limit of quantitation (LLOQ) is 0.5 mg/L.

Both methods were evaluated in terms of recovery, accuracy and precision. The mean recovery was 100.1% for baicalin and 99.1% for oxymatrine. Intra- and inter-day precision and accuracy for the determination of baicalin and oxymatrine (Table 1 and Table 2, respectively) fell well within the limits of acceptability. All values were <15%. The intra-day and inter-day RSD were <5.4% and <7.2% for baicalin and <6.6% and <13.8% for oxymatrine, respectively. The extraction recoveries of oxymatrine and phenacetin in plasma are shown in Table 3.

2.2. Pharmacokinetics

Both plasma baicalin concentration-time curves after separate administration and in combination with oxymatrine fit to the open two-compartment model with a weight factor 1/C. Figure 3 shows the baicalin concentration-time curves in rabbit plasma after i.v. dose of 40 mg/kg ($n = 9$). Table 4 summarizes the main pharmacokinetic data of baicalin administered alone or in combination with oxymatrine in rabbit plasma. Student's t test shows that there is no significant difference in $AUC_{0\sim\infty}$, α and β (Table 5).

Both plasma oxymatrine concentration-time curves fit to the open two-compartment model with a weight factor

Table 4: Main pharmacokinetic data of baicalin administered separately or combined after an i.v. dose of 40 mg/kg baicalin to 9 rabbits (mean ± SD, n = 9)

Parameters	Unit	Separate	Combined
A	$\mu g \cdot mL^{-1}$	774.0 ± 1054.7	1447.7 ± 2067.2
B	$\mu g \cdot mL^{-1}$	21.5 ± 13.8	24.0 ± 53.3
α	min^{-1}	0.29 ± 0.18	0.37 ± 0.27
β	min^{-1}	0.049 ± 0.011	0.064 ± 0.027
Vc	$L \cdot kg^{-1}$	0.096 ± 0.061	0.095 ± 0.084
$t_{1/2\alpha}$	min	2.9 ± 1.1	2.8 ± 1.7
$t_{1/2\beta}$	min	14.6 ± 2.9	12.7 ± 5.5
$AUC_{0\sim240\,min}$	$\mu g \cdot min \cdot mL^{-1}$	2071.68 ± 555.9	1770.2 ± 460.6
$AUC_{0\sim\infty}$	$\mu g \cdot min \cdot mL^{-1}$	2077.9 ± 556.4	1781.2 ± 462.4
CL	$L \cdot kg^{-1} \cdot min^{-1}$	0.018 ± 0.006	0.019 ± 0.011

Table 5: Main pharmacokinetic parameters of oxymatrine administered separately or combined after an i.v. dose of 40 mg/kg oxymatrine to 9 rabbits (mean ± SD, n = 9)

Parameters	Unit	Separate	Combined
A	$\mu g \cdot mL^{-1}$	54.8 ± 20.5	80.6 ± 41.2
B	$\mu g \cdot mL^{-1}$	8.8 ± 9.2	5.6 ± 3.5
α	min^{-1}	0.092 ± 0.040	0.10 ± 0.057
β	min^{-1}	0.011 ± 0.008	0.008 ± 0.005
Vc	$L \cdot kg^{-1}$	0.76 ± 0.45	0.57 ± 0.25
$t_{1/2\alpha}$	min	9.5 ± 4.8	8.1 ± 3.8
$t_{1/2\beta}$	min	143.6 ± 71.8	95.9 ± 52.9
$AUC_{0\sim240\,min}$	$\mu g \cdot min \cdot mL^{-1}$	1353.4 ± 326.4	1488.6 ± 271.7
$AUC_{0\sim\infty}$	$\mu g \cdot min \cdot mL^{-1}$	1409.0 ± 377.4	1790.6 ± 306.4
CL	$L \cdot kg^{-1} \cdot min^{-1}$	0.035 ± 0.014	0.026 ± 0.009

$1/C^2$. Figure 4 shows the oxymatrine concentration-time curves in rabbit plasma after i.v. dose of 40 mg/kg (n = 9). Table 6 summarizes the main pharmacokinetic data of oxymatrine administered separately and together with baicalin. Student's t test shows that there is no significant difference in $AUC_{0\sim\infty}$, α and β (Table 7).

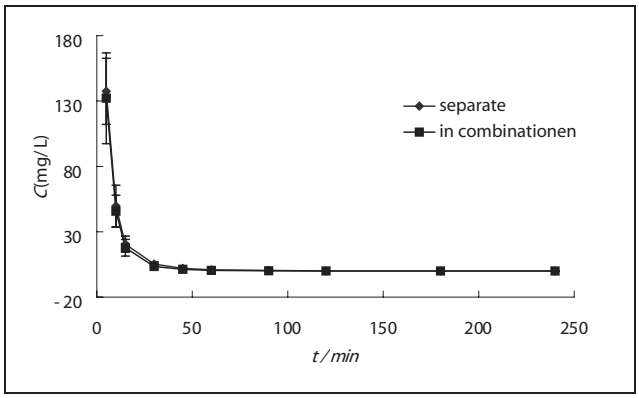


Fig. 3: Mean plasma concentration-time curve of baicalin after an i.v. dose of 40 mg/kg baicalin to 9 rabbits (mean ± SD)

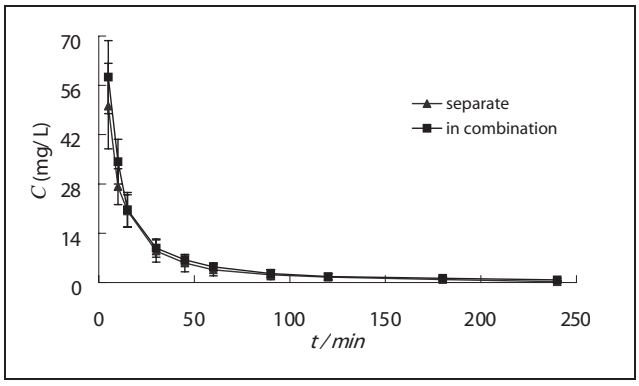


Fig. 4: Mean plasma concentration-time curve of oxymatrine after an i.v. dose of 40 mg/kg oxymatrine to 9 rabbits (mean ± SD)

3. Discussion

Our strategy was first to develop a simple HPLC method for the simultaneous determination of baicalin and oxymatrine. However, this was not possible because of very different polarity of these two drugs. So, we used two methods to determine each drug separately.

In the beginning we used methanol to precipitate protein [5], but the recovery of baicalin in plasma was found to be low as the compound was not stable in methanol. So we used acetonitrile to precipitate plasma protein. Then the recoveries of high, medium and low concentrations of baicalin were 93.9 ± 4.5%, 99.2 ± 3.1% and 89.0 ± 4.3% (n = 6), respectively.

Table 6: Comparison of pharmacokinetic parameters of baicalin administed separately or combined

Parameters	Separate (mean ± SD)	Combined (mean ± SD)	t test
$AUC_{0\sim\infty}$	2077.9 ± 556.4	1781.2 ± 462.4	$P > 0.05$
α	0.29 ± 0.18	0.37 ± 0.27	$P > 0.05$
β	0.049 ± 0.011	0.064 ± 0.027	$P > 0.05$

Table 7: Comparison of pharmacokinetic parameters of oxymatrine administered separately or combined

Parameters	Separate (mean ± SD)	Combined (mean ± SD)	t test
$AUC_{0\sim\infty}$	1409.0 ± 377.4	1790.6 ± 306.4	$P > 0.05$
α	0.092 ± 0.040	0.10 ± 0.057	$P > 0.05$
β	0.011 ± 0.008	0.008 ± 0.005	$P > 0.05$

The chromatographic conditions used to determine oxymatrine have not been reported before. Under these conditions, oxymatrine ($t_R = 5.7$ min) was separated well from endogeneous compounds.

It was not easy to extract oxymatrine with organic solvents due to its high polarity. We found that chloroform was a good solvent to extract oxymatine and we adopted a reported method [3] with slight modifications: Oxymatrine solution was prepared by precipitation of protein with 6% $HClO_4$ and basification with 20% NaOH, and extraction with chloroform : n-butanol (98 : 2, v/v). By this method of preparation, the endogenous substances did not interfere with the determination of oxymatrine.

As many compounds like caffeine, diphenhydramine, atropine, or ephedrine were not suitable as internal standard due to low recoveries or instability (matrine) we chose phenacetin despite its long retention time. Its extraction recovery was 90.7% (RSD 3.0%).

In summary, two rapid and simple HPLC methods have been developed for determination of baicalin and oxymatrine in rabbits. The methods show no interference with plasma compounds and have sufficient sensitivity to quantify baicalin and oxymatrine in plasma. The methods were then applied to a pharmacokinetic study of baicalin and oxymatrine in rabbits. There was no significant difference in the main pharmacokinetic parameters including $AUC_{0\sim\infty}$, α and β after seperate and combined administration of baicalin and oxymatrine. The result suggests that there is no significant interaction of these two drugs *in vivo* when they are used together.

4. Experimental

4.1. Animals

Nine healthy rabbits were obtained from the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, Liaoning). Their weight ranged from 2.1 to 2.5 kg.

4.2. Study design

The study was conducted in a randomized, three-period crossover fashion with a one-week washout interval between doses. The rabbits were fasted overnight but had free access to water. The rabbits were divided into three groups, and each group received the same intravenous dosage (40 mg/kg) of the following drugs: (1) baicalin (separate formulation); (2) oxymatrine (separate formulation); (3) baicalin and oxymatrine (combination). Then blood samples (1.5 ml) were collected into heparinized glass tubes at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min after medication. The blood samples were immediately centrifuged at 4000 rpm for 10 min to obtain plasma. The plasma samples were stored at −20 °C until assay.

4.3. Analytical determinations

An aliquot of 200 μL p-nitrobenzoic acid (5 mg/L), 200 μL water and 400 μL acetonitrile were added to 200 μL plasma. Then the mixture was vigorously vibrated for 30 s, and centrifuged at 15000 rpm for 10 min. The supernatant of 10 μL was injected into the HPLC system. Separation was carried out on a Diamonsil™ C_{18} column (200 mm × 4.6 mm I.D., 5 μm). The mobile phase consisted of methanol, water and phosphoric acid (50 : 50 : 0.2, v/v). The flow rate was 1.0 ml/min and the UV detector was set at 277 nm. Column temperature was set at 40 °C. p-Nitrobenzoic acid was used as the internal standard.

An aliquot of 100 μL phenacetin solution (5 mg/L), 200 μL of water and 400 μL of 6% $HClO_4$ was added to 400 μL of plasma. Then the mixture was vigorously vibrated for 30 s and centrifuged at 10000 rpm for 5 min. The supernatant was transferred to another tube and 400 μL of 20% NaOH and 4 ml chloroform : n-butanol (98 : 2, v/v) were added. The mixture was vigorously vibrated for 1 min, and centrifuged at 4000 rpm for 10 min. The separated organic phase was evaporated to dryness at 40 °C under N_2 stream. The residue was reconstituted in 200 μL of the mobile phase. Finally an aliquot of 10 μL was injected into the HPLC system. Separation was carried out on a Diamonsil™ C_{18} column (200 mm × 4.6 mm I.D., 5 μm). The mobile phase consisted of acetonitrile and water (20 : 80, v/v) containing 5 mmol/L sodium octanesulfonate. The pH of the mobile phase was adjusted to 3.2 with phosphoric acid. The flow rate was 1.0 ml/min and the UV detector was set at 220 nm. The column temperature was set at 30 °C. Phenacetin was used as the internal standard.

4.4. Method validation

All calibration curves of baicalin and oxymatrine were constructed prior to the experiments with correlation values of at least 0.995. The intra-day and inter-day variabilities of baicalin and oxymatrine were assayed (six replicates) at low, medium and high concentrations on the same day and on six sequential days, respectively. Accuracy and precision values of within 15% covering the range of actual experimental concentrations were considered acceptable [10].

4.5. Pharmacokinetic study

Pharmacokinetic parameters were determined from the plasma concentration-time data. The distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$) were calculated with a TOPFIT program on a personal computer. The area under the plasma concentration-time curve from time zero to the last measurable plasma concentration point ($AUC_{0\sim240\,min}$) was calculated by the linear trapezoidal rule. Extrapolation to time infinity ($AUC_{0\sim\infty}$) was calculated as follows: $AUC_{0\sim\infty} = AUC_{0\sim240\,min} + C_{240}/\beta$, where C_{240} is the last measurable plasma concentration and β is the elimination rate constant. The compartment models were also determined with TOPFIT 2.0 program on a personal computer.

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