



1999

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### Recommended Citation

Cheng, H.-H.; Lin, W.-C.; Kuo, S.-C.; Huang, T.-H.; and Tsai, S.-Y. (1999) "HPLC determination of 9,10-anthraquinone-2-carboxylic acid in serum and its application to pharmacokinetic study," *Journal of Food and Drug Analysis*: Vol. 7 : Iss. 3 , Article 3.  
Available at: <https://doi.org/10.38212/2224-6614.2864>

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## HPLC Determination of 9,10-Anthraquinone-2-Carboxylic Acid in Serum and Its Application to Pharmacokinetic Study

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### ABSTRACT

A simple and sensitive high-performance liquid chromatographic (HPLC) method involving UV detection was developed for the determination of 9,10-anthraquinone-2-carboxylic acid (AQCA) in rabbit serum. A reverse-phase column (Lichrospher<sup>®</sup> 100RP-18) was eluted with a mobile phase of 0.4% phosphoric acid: acetonitrile (7:3) / methanol = 45 : 55 at a flow rate of 1.2 mL/min. The UV absorbance was monitored at 256 nm. After a simple clean-up procedure, the limit of quantitation achieved was 0.6 µg/mL and the standard curve was found to be linear over the serum concentration of 0.6 ~ 18µg/mL. The intra-assay and inter-assay coefficient of variance in serum was less than 2%, and the recovery was 96.98%.

The established HPLC method was applied to the study of pharmacokinetics and bioavailability of 9,10-anthraquinone-2-carboxylic acid (AQCA). Eighteen healthy rabbits were divided into three groups and given intravenous (i.v.), oral and rectal administrations of different AQCA preparations with a single dose of 10 mg/kg. Blood samples were collected and AQCA concentrations in serum were analyzed. The results were performed with Winnolin programs and the ANOVA test ( $\alpha = 0.05$ ) was used to compare the pharmacokinetic parameters of AQCA in the three regimens. For the i.v. route, the  $t_{1/2}$  and AUC were  $4.43 \pm 0.13$  hrs and  $141.79 \pm 7.84$  µg\*hr/mL, respectively. Parameters for the oral route were :  $T_{max} = 7.17 \pm 0.41$  hrs ;  $t_{1/2} = 15.32 \pm 1.86$  hrs;  $MRT = 20.02 \pm 2.07$  hrs and  $AUC = 107.53 \pm 4.50$  µg\*hr/mL. The oral route indicated a slower absorption rate, a longer residence time and a lower extent of the AQCA absorption. For the rectal route,  $T_{max}$  occurred at  $1.58 \pm 0.20$  hrs, the  $t_{1/2}$  was  $5.67 \pm 0.82$  hrs and the AUC was  $121.18 \pm 6.19$  µg\*hr/mL. The results indicated that the rate and extent of AQCA rectal absorption were better compared to those of the oral route. At this dose, the absolute bioavailabilities of AQCA were 0.876 and 0.872 for oral and rectal administrations, respectively. The differences of these pharmacokinetic parameters might be due to the physicochemical properties of AQCA in different conditions.

**Key words:** 9,10-anthraquinone-2-carboxylic acid, pharmacokinetics, bioavailability, high performance liquid chromatography (HPLC).

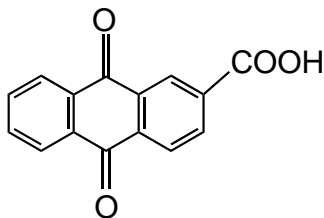
## INTRODUCTION

In studies of a series of anthraquinone derivatives for usage in the relief or prophylaxis of allergic conditions<sup>(1)</sup>, it was found that anthraquinone derivatives had an excellent activity<sup>(2)</sup>. Through the rat passive cutaneous anaphylaxis method study, an oral dose of 5~10 mg/kg indicated an obvious inhibition effect<sup>(3)</sup>. Its activity was about 2 to 4 times that of Anatomide<sup>R</sup> and 30 to 60 times that of Tranilast<sup>R</sup>, respectively. A 1mg/Kg i.v. dose of 9,10-anthraquinone-2-carboxylic acid (AQCA) showed a significantly stronger effect than Ketotifen<sup>R</sup>, a current clinical medicine, and was equivalent to that of disodium cromoglycate. Therefore, AQCA might be a good candidate to be developed for clinical use. The understanding of pharmacokinetics should enhance the likelihood of safe and effective drug therapy. However, since there is little AQCA pharmacokinetic information available, the study of AQCA absorption from various administration routes is of great interest. Also, no determination method for AQCA in serum had been previously described. Developing a validated determination method for pharmacokinetic study was seen as necessary. Thus, in this study, a good HPLC method for the determination of AQCA in serum was established, and the pharmacokinetic behavior of AQCA in rabbits after i.v., oral and rectal administrations was also investigated.

## MATERIALS AND METHODS

### I. Materials

AQCA (Lot no. FCZ01) was purchased from TOKYO KASEI Chemical Company (Tokyo,



**Scheme 1.** 9,10-Anthraquinone-2-carboxylic acid.

Japan). All other chemicals were analytical grade and used without further purification.

### II. Sample Preparations

The parental preparation was freshly prepared with 10% AQCA tetraglycol solution and was sterilized by 0.45  $\mu\text{m}$  pore size membrane filtration before use. The oral dosage form was prepared by levigating 10% AQCA with microcrystalline cellulose well and enveloping it into a gelatin capsule shell for use. The suppository was prepared by fusing 10% AQCA with pluronic F-68 in water bath and kneading it into an oviform after cooling for use.

### III. Animal Studies

Eighteen male New Zealand white rabbits weighing about 2 ~ 3 kg were divided randomly into three groups for use. AQCA were administered intravenously, orally and rectally with a single dose of 10 mg/kg to each group of rabbit after an overnight fasting during each treatment. Blood samples (1.0 mL) were periodically collected from the marginal vein of the ear for up to 30 hrs after AQCA ingestion. Those samples were immediately centrifuged at 3500 rpm for 5 min. Prior to HPLC analysis, serum proteins were precipitated by acetonitril and removed by centrifugation twice<sup>(4)(5)</sup>. The supernatant (380  $\mu\text{L}$ ) was assayed by a validated HPLC method with 20  $\mu\text{L}$  of p-toluic acid as an internal standard.

### IV. HPLC Conditions

Shimadzu LC-10AT HPLC with SPD-M10AVP detector was applied for the determination. AQCA concentration in serum was analyzed by HPLC method at UV wavelength 256 nm. A reverse-phase column (Lichrospher<sup>R</sup> 100RP-18, 5  $\mu\text{m}$ , 250-4 mm endcapped MERCK 50995) was eluted with a mobile phase of 0.4% phosphoric acid: acetonitrile (7:3) / methanol = 45:55 at a flow rate of 1.2 mL/min and p-toluic acid as an internal standard. Six standard concentrations of AQCA in the range of 0.6 ~ 18  $\mu\text{g}/\text{mL}$  were prepared, and peak area ratios indicated by chromatograms were used to find a calibration curve

for the determination. The treated samples were assayed under the same conditions, and the AQCA content in serums were then be calculated. The intra- and inter-day accuracy and precision of the HPLC method were validated, and the mean, standard deviation (S.D.) and coefficient of variance (C.V.%) were also determined.

#### V. Data Analysis

To compare the pharmacokinetic model fitting using the Winnolin Program -Version 1.1, the non-compartment model was selected because the compartmental model with one or two compartments yields a poor fitting. The peak serum concentration ( $C_{max}$ ), times for peak serum concentration ( $T_{max}$ ), terminal half-life ( $t_{1/2}$ ), total area under the concentration-time curve (AUC), total serum clearance (CL/F) and mean residence time (MRT) were obtained from the selected program. The  $C_{max}$  and  $T_{max}$  were obtained directly from the observation concentration-time data. The AUC was evaluated by linear trapezoidal method. The  $AUC_{0-\infty}$  was calculated by  $AUC_{0-t}$  added to the value of  $C_p$  at the last detection time divided by the terminal elimination rate constant. At this dose, the absolute bioavailability of AQCA after oral or rectal administration was calculated from the equation

$$F = \frac{(AUC_{0-\infty})_{po}}{(AUC_{0-\infty})_{i.v.}} \text{ or } F = \frac{(AUC_{0-\infty})_{rectal}}{(AUC_{0-\infty})_{i.v.}}$$

The ANOVA test ( $\alpha = 0.05$ ) was used to compare the pharmacokinetic parameters obtained from various regimens.

**Table 2.** The inter-day and intra-day precision for analysis of AQCA in rabbit serum

Spiked Conc. ( $\mu\text{g/mL}$ )	Interday			Intraday		
	Mean <sup>a</sup>	Std dev	CV.(%)	Mean <sup>a</sup>	Std dev	CV.(%)
0.6	0.61	0.004	0.75	0.61	0.001	0.16
1.2	1.18	0.020	1.75	1.18	0.006	0.51
3.0	2.99	0.055	1.89	3.09	0.056	1.81
4.8	4.77	0.003	0.06	4.81	0.045	0.94
6	5.98	0.030	0.50	6.09	0.039	0.64
18	17.88	0.134	0.76	18.08	0.144	0.80
$R^2 =$		0.9999			0.9999	

<sup>a</sup> N=3.

## RESULTS AND DISCUSSION

Under the chromatographic conditions described in Experimental section, the retention times for the internal standard, p-toluic acid, and AQCA were 3.71 minutes and 7.42 minutes, respectively. AQCA was eluted without any interference peaks from the blank serum (Fig. 1). The calibration curve was linear over the working range of 0.6 ~ 18  $\mu\text{g/mL}$  with a correlation coefficient greater than 0.9998 (Fig. 2). The lowest limit of detection of AQCA in serum was 0.6  $\mu\text{g/mL}$ . The recovery of the method was greater than 96%. Both of the intra-assay and inter-assay coefficient of variance (C.V.) in serum were less than 2%. Resulting data are shown in Tables 1 and 2. The

**Table 1.** Extraction recoveries (%) of AQCA at different concentration from rabbit serum

Spiked Conc. ( $\mu\text{g/mL}$ )	A <sup>a</sup>	B <sup>a</sup>	Recovery <sup>b</sup> (%)
0.6	0.563	0.580	96.981
1.2	1.113	1.125	98.974
3.0	2.767	2.774	99.721
4.8	4.613	4.645	99.307
6	5.403	5.491	98.400
18	16.214	16.238	99.852

Notes: A: when blank serum was as the solvent.

B: when an aqueous solution was as the solvent.

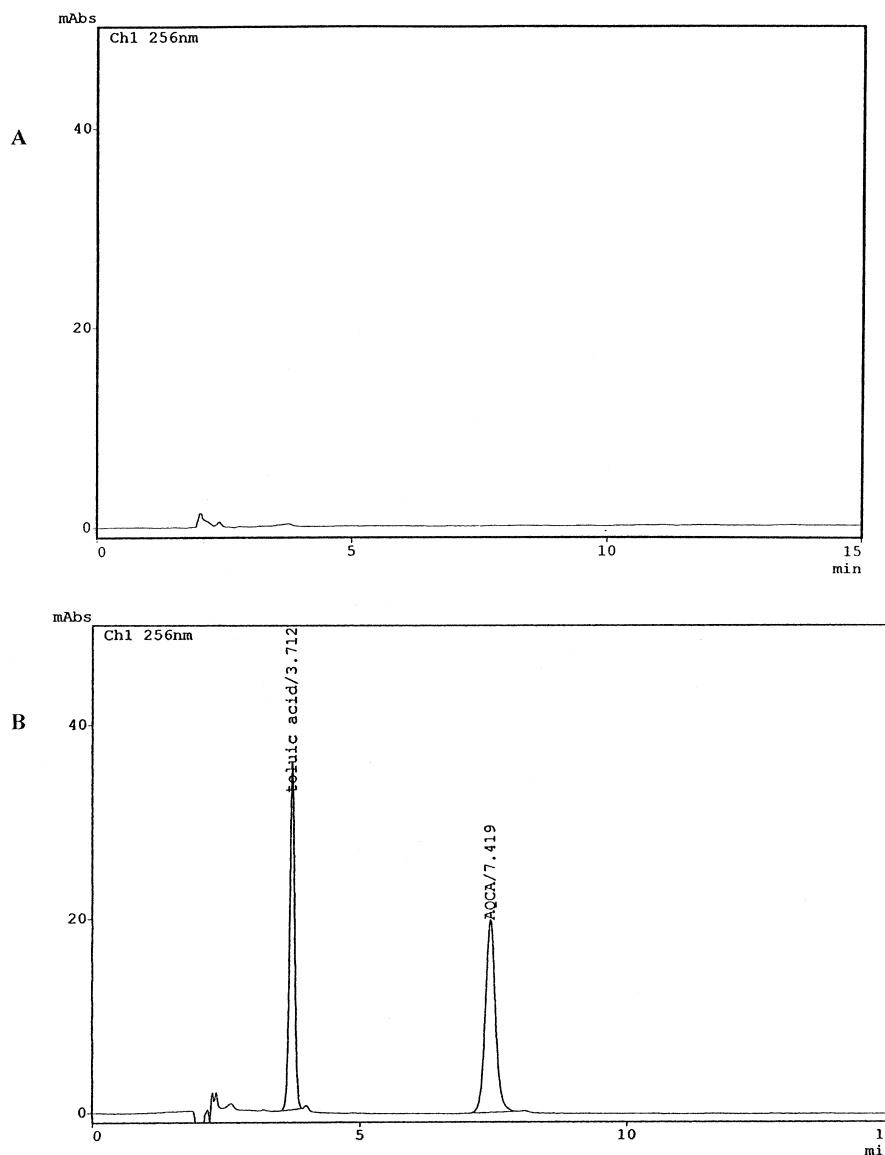
<sup>a</sup> mean of AQCA/p-toluic acid response ratio (N=3).

<sup>b</sup> (A/B) x 100%.

established HPLC method was qualified.

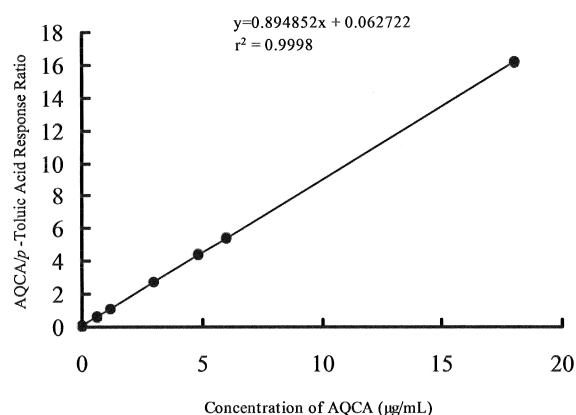
The mean serum levels of AQCA in six rabbits after i.v., oral and rectal administrations of AQCA preparations are shown in Fig. 3. Some of pharmacokinetic parameters under the Winnolin Program treatment are listed in Table 3. Most of listed parameters showed a significant difference ( $P < 0.05$ ) from various regimens by ANOVA testing. The  $t_{1/2}$  of i.v. and oral administrations were  $4.43 \pm 0.13$  hrs and  $15.32 \pm 1.86$  hrs, respectively. The AUC of i.v. was  $141.79 \pm 7.84$   $\mu\text{g}\cdot\text{hr}/\text{mL}$  while of the oral route was  $107.53 \pm$

$4.50$   $\mu\text{g}\cdot\text{hr}/\text{mL}$ . There was a significantly slower rate and a lower extent of AQCA absorption from the oral than those of the i.v. administration. When comparing the rectal to the oral route, the  $T_{\text{max}}$ ,  $t_{1/2}$  and MRT ( $1.58 \pm 0.20$  hrs,  $5.67 \pm 0.82$  hrs and  $7.01 \pm 0.92$  hrs, respectively) for the rectal route were shorter than those ( $7.17 \pm 0.41$  hrs,  $15.32 \pm 1.86$  hrs and  $20.02 \pm 2.07$  hrs, respectively) of the oral administration. The  $C_{\text{max}}$  and AUC ( $23.56 \pm 0.65$   $\mu\text{g}/\text{mL}$  and  $121.18 \pm 6.19$   $\mu\text{g}\cdot\text{hr}/\text{mL}$ , respectively) for the rectal route were higher than those ( $10.50 \pm 0.73$   $\mu\text{g}/\text{mL}$  and



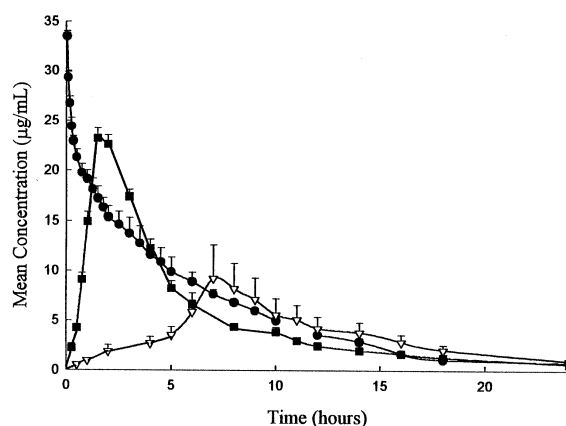
**Figure 1.** Representative chromatograms from (A) blank serum, (B) serum sample after 10 mg /kg AQCA was administrated. Toluic acid was used as an internal standard.

107.53± 4.50 µg\*hr/mL, respectively) of the oral administration. It was found that AQCA is a weak acid (pKa ~4.3), practically insoluble in water, very slightly soluble in organic solvent and the equilibrium solubility of AQCA increased at pH value > 4.0<sup>(6)</sup>. These properties imply that AQCA might be precipitated after oral administration in the acidic environment of the stomach and slowly



**Figure 2.** A typical calibration curve (0.6~18 µg/mL) for the analysis of AQCA in rabbit serum using toluic acid as an internal standard.

dissolved when it enters the intestinal track, resulting in the lower but longer levels shown in serum. The pH of the rectal area was between about 7.4 ~ 8.0, and would increase the solubility, thus resulting in a higher absorption rate of AQCA than that of the oral administration<sup>(6)</sup>.



**Figure 3.** Mean serum AQCA concentrations (µg/mL) v.s. time after i.v., oral and rectal administration in rabbits. Each point represents the mean ± SD of 6 samples.

Key: — i.v. — p.o. — rectal

**Table 3.** The pharmacokinetic parameters of AQCA in rabbits after i.v., oral and rectal administrations with a single dose of 10.0 mg/kg

Parameter	<u>Intravenous</u>		<u>Oral</u>		<u>Rectal</u>		ANOVA
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
T <sub>max</sub> <sup>a</sup> (hours)	---	---	7.17	0.41	1.58	0.20	P < 0.05
C <sub>max</sub> <sup>b</sup> (µg/mL)	33.5	0.57	10.50	0.73	23.56	0.65	P < 0.05
Lambda <sub>z</sub> <sup>c</sup>	0.156	0.008	0.050	0.006	0.120	0.018	P < 0.05
T <sub>1/2</sub> <sup>d</sup> (hours)	4.43	0.13	15.32	1.86	5.67	0.82	P < 0.05
AUC <sup>e</sup> (µg*hr/mL)	141.79	7.84	107.53	4.50	121.18	6.19	P < 0.05
Vz <sup>f</sup>	0.444	0.039	1.703	0.171	0.644	0.063	P < 0.05
CL/F <sup>g</sup>	0.069	0.005	0.077	0.007	0.079	0.008	NS
MRT <sup>h</sup> (hours)	6.45	0.21	20.02	2.07	7.01	0.92	P < 0.05

(N = 6).

<sup>a</sup> Time for the peak serum concentration.

<sup>b</sup> The peak serum concentration.

<sup>c</sup> The terminal elimination rate constant.

<sup>d</sup> The terminal half-life.

<sup>e</sup> Area under serum concentration-time curve to the last observation.

<sup>f</sup> The volume of distribution.

<sup>g</sup> Total serum clearance.

<sup>h</sup> The mean residence time.

Alternatively, it was suspected that the surface active suppository vehicle, pluronic F-68, might solubilize AQCA in rectal fluid, thereby enhancing its dissolution rate. The results indicated that the rate and extent of rectal absorption of AQCA were better than those of the oral administration.

By applying the above dose, the absolute bioavailabilities of AQCA were 0.876 and 0.872 for oral and rectal routes, respectively. It is likely that these moderate absolute bioavailabilities were partially due to the incomplete absorption of AQCA.

In conclusion, a validated HPLC method for the determination of AQCA in serum was established. This method is simple, sensitive and accurate. For the application, preliminary pharmacokinetic parameters for AQCA after various administrations were determined thereby. These observations indicate that the rate and extent of oral and rectal absorption of AQCA were slower and lower than that of i.v. administration. Although the results of this study were not entirely satisfactory, the different dosage forms of AQCA for clinical use via various administrations could be still suggested. Actually, drugs enter an organism efficiently only when they are soluble both in water and in lipids<sup>(7)</sup>. So, how to improve the dissolution rate of AQCA in organisms in order to enhance the absolute bioavailabilities of various administrations for clinical use will be the subject of future research.

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# 高效液相層析法檢測血清中9,10—蔥醌酮—2—羧酸 濃度及其在藥物動力學研究之應用

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## 摘要

本研究發展出一簡單、高感度和精確之高效液相層析法，用以檢測家兔血清中9,10—蔥醌酮—2—羧酸(AQCA)濃度。本分析法以Lichrospher<sup>R</sup> 100RP-18為層析管柱，0.4%磷酸：乙氰(7:3) / 甲醇 = 45:55為移動相，流速為1.2 mL / min，紫外光檢測器之檢測波長為256 nm，甲基苯甲酸為內標準品。檢品經簡單處理後，注入管柱分析。最低檢測值為0.6 µg/mL，於0.6～18 µg/mL濃度間具有良好的線性檢量關係，確效試驗顯示，精密度佳(變異係數少於2%)，回收率為96.98%。

繼以此分析法應用於藥品動力分析研究之探討。十八隻家兔分成三組，取不同劑型之AQCA，以相同的單一劑量(10 mg/kg)，分別以血管注射、口服和直腸投藥，檢測各組各時段血清中的AQCA濃度，經Winnolin軟體處理資料，並以ANOVA試驗( $\alpha = 0.05$ )評定各組重要的藥品動力參數之異同。注射投藥時，半衰期( $t_{1/2}$ )、血清濃度—時間曲線下面積(AUC)分別為 $4.43 \pm 0.13$  hrs和 $141.79 \pm 7.84$  µg\*hr/mL。口服投藥的參數數據為血清中尖峰濃度( $T_{max}$ ) =  $7.17 \pm 0.41$  hrs;  $t_{1/2}$  =  $15.32 \pm 1.86$  hrs; 平均滯留時間(MRT) =  $20.02 \pm 2.07$  hrs; AUC =  $107.53 \pm 4.50$  µg\*hr/mL，顯示口服途徑的藥品吸收速率慢、滯留時間長、吸收總量低。直腸投藥的參數數據為 $T_{max}$  =  $1.58 \pm 0.20$  hrs;  $t_{1/2}$  =  $5.67 \pm 0.82$  hrs; AUC =  $121.18 \pm 6.19$  µg\*hr/mL，依數據比較，藥物吸收速率及吸收總量均比前者好。另者，在本劑量下，口服和直腸投藥的絕對生體可用率分別為0.876和0.872。這些參數上的差異可能是AQCA於不同的劑型與投藥部位、有不同的溶離性質所致。

**關鍵詞：**9,10—蔥醌酮—2—羧酸，藥物動力學，生體可用率，高效液相層析法。