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Determination of Xanthonolol by High Performance Liquid Chromatography for Pharmacokinetic Studies in Rats

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ABSTRACT

A rapid and simple HPLC method was developed for the determination of xanthonolol concentration in plasma. The sample preparation utilized liquid-liquid extraction before injection into the HPLC system. Phenazine was used as the internal standard. Separation was obtained using a reversed-phase column under isocratic conditions. The mobile phase consisted of a 65% McIlvaine buffer containing 0.05% triethylamine adjusted to pH 6.4 with phosphoric acid, 18% acetonitrile, and 17% methanol. Xanthonolol was detected at the ultraviolet wavelength 235 nm. The lower limit of quantization was 50 ng/mL. The assay was applied to a pharmacokinetic study in rats. The plasma concentration of xanthonolol versus time data were best fitted to a two-compartment open model with first-order elimination processes. After the intravenous administration of xanthonolol at three different dosages of 5, 10 and 15 mg/kg, the respective pharmacokinetic parameters, such as apparent volume of distribution, half-life, and clearance, showed no significant difference at three different dosages. Also, the area under the plasma concentration time curves for three dosages increased proportionally with dose. Therefore, the pharmacokinetics of xanthonolol was found to be linear over the dose range studied.

Key words: xanthonolol, HPLC method, pharmacokinetics

INTRODUCTION

Xanthonoids are a group of natural substances, which are widely distributed in the families *Gentianaceae* and *Guttiferae*. Among their various biological properties, they have been shown to be potent as inhibitors of platelet aggregation and as vasorelaxing agents[1,2]. Particularly, researchers suggested the inhibitory effect on the contractile response caused by high potassium ion and norepinephrine in rat thoracic aorta of norathyriol, a 1,3,6,7-tetrahydroxy xanthone obtained from *Gentianaceae*, was mainly due to the inhibition of the calcium ion influx through both voltage-dependent and receptor-operated calcium channels[2]. A series of xanthonoxypropanolamine-related compounds, flavonoxy- propanolamines, have been shown to exist antihypertensive activity in spontaneously hypertensive rats[3]. Since propanolamine is chemically one of a key group of beta adrenoceptor blockers and since xanthones may possess calcium ion blocking-dependently hypotensive effect, xanthonolol, a derivative of 3-hydroxyxanthone (Figure 1), was synthesized and tested for its possible beta adrenergic and calcium channel blocking and vasodilating activities[4-7]. Very little study has been done on pharmacokinetic and its effects in animals. According to the other research about the similar structural compound (5,6-dimethyl-xanthenone-4-acetic acid, DMXAA), satisfactorily fitted to a two-compartment model with capacity-limited kinetics in the mouse and rat[8,9]. The capacity-limited elimination of DMXAA exhibited a disproportionate increase in AUC with increasing dose. Hence, we administered the different dosages of xanthonolol to evaluate the pharmacokinetic model in rat.

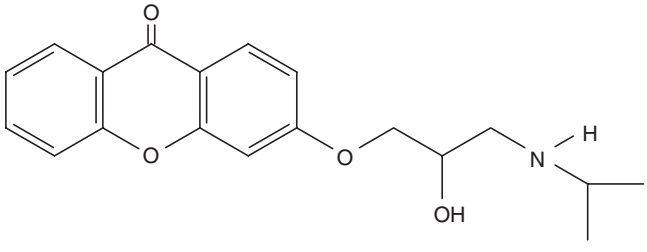

Figure 1. Structure of xanthonolol.

Quantitative analyses of the xanthone and its derivatives have been performed by HPLC[8,10]. From the previous study, the linearity over the concentration range used for the analysis of xanthone and 3-methoxyxanthone in nanocapsule formulations was not sensitive enough for the plasma specimen by UV detector. DMXAA was analyzed by HPLC using fluorescence detection. In the present study, we have developed a specific, accurate and reproducible HPLC method using UV detection to determine the concentration of xanthonolol in rat plasma and examined the pharmacokinetic profile of xanthonolol in rats to establish a drug administration regimen applicable for clinical trial.

MATERIALS AND METHODS

I. *Reagents and Materials*

Xanthonolol was synthesized in the laboratory of pharmaceutical chemistry (Kaohsiung Medical University,

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Taiwan). Identification of the compound was established by means of the spectral data (IR and NMR)[6]. The internal standards phenazine, triethylamine, disodium hydrogen phosphate dehydrate, and citric acid were purchased from Merck (Germany). All other reagents were analytical reagent grade. Methanol was obtained from Fisher scientific (U.S.A.), acetonitrile was obtained from Tedia Company (U.S.A.), and distilled water was purified *via* Mill-Q reagent water system from Millipore (U.S.A.). All other solvents used were of HPLC grade.

II. *Instrumentation*

A high performance liquid chromatographic (HPLC) system consisted of Hitachi instruments equipped with a L-7100 HPLC pump, an L-4000H UV detector (Hitachi, Japan) wavelength at 235 nm, and a reversed-phase Lichrosper 100 RP-18 column (5 μm, 125 × 4 mm i.d., E. Merck, Germany). The mobile phase consisted of a 65% McIlvaine buffer containing 0.05% triethylamine adjusted to pH 6.4 with phosphoric acid, 18% acetonitrile, and 17% methanol. The solution was filtered and degassed by vacuum filtering through a 0.45-μm membrane filter. The chromatography was preformed at room temperature and the flow rate of the mobile phase was set at 1.0 mL/min. The injection volume was 20 μL.

III. *Drug Standards*

Standard solutions of xanthonolol were prepared in methanol at final concentrations ranging from 0.05 to 10 μg/mL. Plasma standards were prepared using drug-free rat plasma spiked standard solution to obtain the appropriate final concentrations. The internal standard solution (IS, phenazine 5 μg/mL, 25 μL) was prepared in methanol.

IV. *Drug Administration and Preparation of Plasma Samples*

The Wistar rats weighing about 200-250 g were used in the pharmacokinetic study (Laboratory Animal Center of National Science Council). These animals were divided into three groups and administered 5, 10 and 15 mg/kg of xanthonolol, respectively. Rats were anesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection. Xanthonolol solution prepared was administered *via* the tail vein. Blood samples were drawn from the jugular artery at 0, 5, 15, 30, 60, 120, 180, 240, 360, 540 and 720 min after xanthonolol administration. The blood samples were then centrifuged at 3,000 rpm for 10 min. Plasma was immediately separated and stored at -20˚C until analysis.

V. *Analytical Procedure and Assay Validation*

Aliquots of blank, spiked or plasma samples (0.25 mL) were added 25 μL of IS solution (5 μg phenazine/mL methanol) and 5 mL of ethyl ether/n-hexane (2/1, v/v). The mixture was horizontally shaken for 10 min and centrifuged at 3,000 rpm for 10 min. The organic layer was transferred to another tube and evaporated to dryness by vacuum pump. The dry residue was reconstituted in 250 μL of methanol and the clear supernatant was injected into the HPLC system.

Standards of rat plasma were prepared and analyzed during method validation. Linearity of standard curves, intra- and inter-assay precision and accuracy were determined from these data. The limit of quantization (LOQ) of xanthonolol was determined. LOQ is the smallest concentration where signals can be distinguished from the noise level[11]. The recovery of extraction was determined by the peak area ratio from processed plasma sample to those from xanthonolol standards in methanol solution for each standard concentration.

VI. *Analysis of Pharmacokinetic Data*

Concentrations were analyzed using the PCNONLIN computer program (V4.0, SCI software, U.S.A.) to determine the type of compartment model using the correlation coefficient of observed and predicted data. The following pharmacokinetic parameters were assessed: the area under the plasma concentration-time curves from time zero to infinity ($AUC_{0\rightarrow\infty}$), elimination rate constant (k), half-life for the elimination phase ($t_{1/2\beta}$), clearance (Cl), the volume of distribution (Vd_{ss}) and the mean residence time (MRT) were obtained. All data were expressed as mean ± standard derivation. The $AUC_{0\rightarrow\infty}$ were calculated by the trapezoidal rule ($AUC_{0\rightarrow t}$) and extrapolated to time infinity by the addition of $AUC_{0\rightarrow\infty}$. The k value was the terminal slope which was calculated by linear regression of the logarithmic value of the terminal phase. The Cl value was calculated as Dose/[$AUC_{0\rightarrow\infty}$], and the Vd_{ss} was obtained from the summation of central and tissue compartments. MRT was calculated from AUMC/AUC.

RESULTS AND DISCUSSION

The development of the HPLC method was initiated due to the effect of certain variables including the selection of organic phase, the concentration of the buffer, and the pH of the mobile phase. According to the retention time, peak height and peak asymmetry factor, the suitable conditions of the mobile phase was established as follow consisting of a 65% McIlvaine buffer containing 0.05% triethylamine adjusted to pH 6.4 with phosphoric acid, 18% acetonitrile, and 17% methanol. The retention times for xanthonolol and IS were 5.5 min and 15.0 min, respectively.

The chromatographic system was also applied to a pharmacokinetic study. Hence, the extraction procedure was an important step for the isolation of the analytes from the rat plasma sample. After several trials, it was found that the one-step liquid-liquid extraction of xanthonolol in plasma was satisfactory. The extraction efficiencies of xan-

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Table 1. Precision and accuracy of intra- and inter-day validation in rats plasma

Expected Concentration (μg/mL)	Intra-day Observed (mean ± SD)	Intra-day Error (%)	Intra-day Precision (CV, %)	Inter-day Observed (mean ± SD)	Inter-day Error (%)	Inter-day Precision (CV, %)
0.05	0.061 ± 0.001	21.4	1.65	0.069 ± 0.004	37.6	6.40
0.1	0.112 ± 0.007	12.3	6.26	0.110 ± 0.005	9.7	4.72
0.2	0.206 ± 0.004	3.2	1.68	0.212 ± 0.003	6.0	1.61
0.5	0.484 ± 0.007	-3.2	1.39	0.480 ± 0.019	-4.0	4.04
1.0	0.969 ± 0.017	-3.1	1.72	0.953 ± 0.039	-4.7	4.04
2.0	2.018 ± 0.038	0.9	1.90	2.027 ± 0.086	1.4	4.22

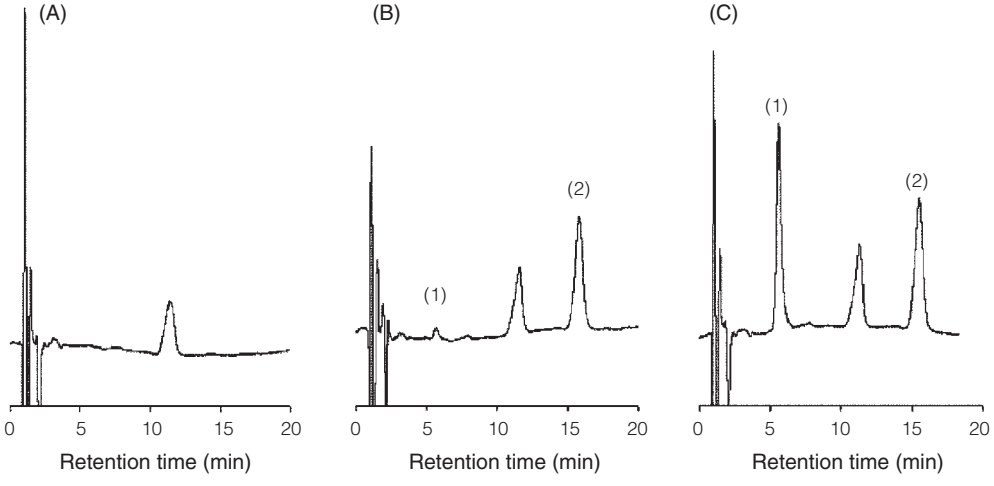

Figure 2. HPLC chromatograms of xanthonolol in rat plasma. A. blank plasma; B. (1) xanthonolol 0.05 μg/mL and (2) phenazine (IS, 0.5 μg/mL); C. (1) xanthonolol 1 μg/mL and (2) phenazine (IS, 0.5μg/mL).

thonolol from plasma were determined for each standard concentration from validation standards. The mean recovery was 86.0% for rat plasma (n = 6). Therefore, the extraction method was reproducible and suitable for the analysis of plasma samples. Under the chromatographic conditions described above; symmetrical and well resolved peaks were obtained for xanthonolol and IS. Typical chromatograms of xanthonolol in rat plasma are shown in Figure 2. No interfering peaks from endogenous plasma substances were observed for drug-free plasma.

In this assay, xanthonolol in rat plasma could be measured reliably over a wide range of concentration from 0.05 to 2.0 μg/mL using 0.25 mL of plasma samples. The linearity between 0.05 and 2 μg/mL was determined using the least-square linear regression with concentration and peak area ratio. The regression equation was $y = 0.8189x - 0.0366$ ($R^2 = 0.9988$, n = 6), where y is the concentration of xanthonolol in plasma, x is the peak area ratio, and R^2 is the determination coefficient. The LOQ for xanthonolol under these conditions was 0.05 μg/mL.

The precision expressed as the coefficient of variation (CV) was calculated for both inter- and intra-day for each standard concentration. All data show the excellent reproducibility of the sample analysis. The CV value was less than 6.5% at 50 ng/mL and complied with the requirements of assay validation. Accuracy was calculated as a relative error (%) for each standard concentration. Results of precision and accuracy for rat plasma are shown in Table 1.

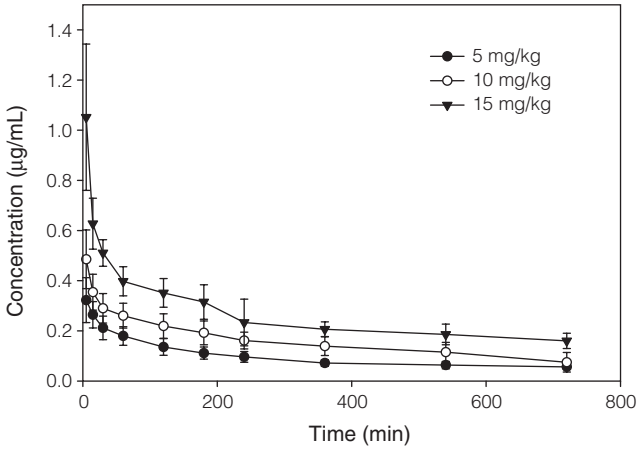

Figure 3. Plasma concentration-time curves of xanthonolol after intravenous administration at doses of 5, 10 and 15 mg/kg in rats (mean ± SD).

The analyticl procedure described here was applied to determine xanthonolol in plasma samples of rats from a pharmacokinetic study. After intravenous administrations of 5, 10, and 15 mg/kg xanthonolol, the plasma level of the drug declined with time in a biphasic pattern as shown in Figure 3. The plasma concentration of xanthonolol versus time data were best fitted by a two-compartment open model with first-order elimination processes. The initial

Table 2. Pharmacokinetic parameters of xanthonolol after intravenous administration at doses of 5, 10 and 15 mg/kg in rats (mean ± SD)

Parameters	5 mg/kg (n = 6)	10 mg/kg (n = 5)	15 mg/kg (n = 3)
$AUC_{0\rightarrow\infty}$ (μg · min/mL)	107.44 ± 24.99	189.67 ± 80.37	346.65 ± 49.31
$t_{1/2\beta}$ (min)	437.16 ± 120.60	421.19 ± 121.17	447.98 ± 130.57
V_{ss} (L)	5.75 ± 1.45	7.29 ± 1.33	6.84 ± 1.40
Cl (mL/min)	9.78 ± 2.48	13.11 ± 4.97	10.97 ± 1.60
MRT (min)	599.49 ± 148.21	597.30 ± 173.05	638.01 ± 180.66

distribution was very rapid compared to the terminal phase. The various pharmacokinetic parameters for xanthonolol were estimated as shown in Table 2. Following the three different doses (5, 10, and 15 mg/kg), the average elimination half-lives were 7.3, 7.0 and 7.5 hr, respectively. Comparing these parameters of xanthonolol including half-life, volume of distribution and clearance, no significant difference was shown ($p > 0.05$) at these dosages. From the plasma levels, the values of area under the plasma level-time curve ($AUC_{0\rightarrow\infty}$) at the three dosages were 107.44, 189.67, and 346.65 μg · min · mL^{-1}, respectively. A plot of the $AUC_{0\rightarrow\infty}$ at various doses showed linear relationship in Figure 4. However, the pharmacokinetic parameters did not show any significant difference between dose levels. The mean values of $AUC_{0\rightarrow\infty}$ were significantly correlated with the dose. It was different from the similar structure compound (DMXAA) which was satisfactorily fitted to a two-compartment model with capacity-limited kinetics and exhibited a disproportionate increase in AUC with increasing dose. As a whole, the pharmacokinetics of xanthonolol was found to be linear over the dose range studied. These parameters provide a reasonable and objective rationale for clinical research.

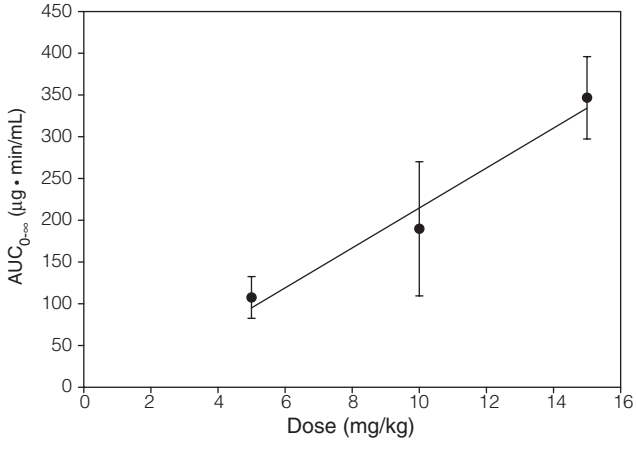

Figure 4. The relationship of $AUC_{0\rightarrow\infty}$ (mean ± SD) between the dosage after intravenous administration of xanthonolol at various doses (5, 10 and 15 mg/kg).

CONCLUSION

This was a rapid, sensitive, selective and reliable HPLC method for the quantitative determination of a derivative of 3-hydroxyxanthone, xanthonolol, and has been successfully used in plasma samples for a pharmacokinetic study. The plasma concentration of xanthonolol versus time data were best fitted to a two-compartment open model with first-order elimination processes and following linear pharmacokinetics.

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