AGE-DEPENDENT CHANGES IN WARFARIN TISSUE DISTRIBUTION

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Whole blood levels, serum protein binding and tissue concentration following intravenous administration of warfarin were investigated in 1-d-old, 1-, 3- and 8-week-old rats to determine the drug disposition in the growth process. It was shown that the clearance of warfarin in 1-d-old or 1-week-old rats was considerably lower than that in 3- or 8-week-old rats. The decrease in clearance in infant and young rats was considered to be caused by the immaturity of the physiological function of the liver to remove exogenous compounds. The distribution volume in 1-d-old or 1-week-old rats was larger than that in 3- or 8-week-old rats. The percentages of serum free warfarin in 1-d-old and 1-week-old rats were about twice those in 3- and 8-week-old rats. The increased distribution volume in infant rats was considered to be caused by a lower serum protein binding in these rats.

Keyword — warfarin; newborn rat; distribution; serum protein binding; age-dependent change; whole blood level

INTRODUCTION

The influence of aging on the disposition of drugs is not only very interesting but is an important problem. Many investigators have studied the changes in drug disposition in men or experimental animals from adulthood to the aged but only few such studies have been performed from the newborn to adulthood.

With clinical drug therapy, many pediatricians have reported differences in pharmacokinetic properties such as distribution volume,¹⁾ half-life time²⁾ and plasma protein binding of drugs³⁾ between newborn infants and adult men. They pointed out the necessity to vary the dosage of some drugs based on the age of patients.⁴⁾ Since these studies used newborn infants who have had already received some drugs transplacentally or become ill before the experiments were started, it was very difficult to determine the changes that occurred in the drug disposition in the growth process. Warfarin is an acidic model drug which is highly bound to serum protein and its pharmacokinetic properties in adult rats are well documented.⁵⁻⁸⁾

In this paper, we investigated the disposition of warfarin in 1-d-old, 1-, 3- and 8-week-old rats to evaluate the changes in the disposition of warfarin in the growth process.

MATERIALS AND METHODS

Chemicals — 3- α -Acetonyl [α -¹⁴C] benzyl-4-hydroxy-coumarin (racemic ¹⁴C-warfarin) was purchased from Amersham International Ltd., Amersham, England, and was diluted with non-radioactive racemic warfarin (Sigma, St. Louis, MO, U.S.A.) to 15.93 μ Ci/0.926 mg/ml in saline solution. All other chemicals were of analytical grade and were used without further purification.

Animals — Pregnant Wistar rats and 8-week-old male Wistar rats were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. One day old, 1- and 3-week-old male rats were obtained after the delivery of the pregnant rats in our laboratory. The body weights of 1-d-old, 1-, 3- and 8-week-old rats were 4.6–7.0, 9.5–17.1, 31.0–46.0 and 280–300 g, respectively.

Whole Blood Levels of Warfarin after Intravenous Administration — Rats were administered 17.2 μ Ci/mg/kg of warfarin intravenously. After removing part of the left jugular skin of 1-d-old and 1-week-old rats, intravenous injection of warfarin was carried out with a microsyringe. An adhesive agent (Aron Alpha®, Sankyo Co., Tokyo, Japan) was applied quickly in order to stanch the cut.⁹⁾ Three-week-old rats were

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chronically cannulated into the left external jugular vein with a polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ., U.S.A.) and 8-week-old rats were cannulated with silicon polymer tubing (i.d. 1.0 mm, o.d. 1.5 mm, Dow Corning, Tokyo, Japan) according to the method of Upton.¹⁰⁾ Intravenous administration of warfarin to rats was carried out through these cannulae. For 1-d-old or 1-week-old rats, blood samples (10 and 50 μ l) were taken from the right jugular vein. One blood sample was obtained from each rat. For 3- or 8-week-old rats, blood samples (about 100 μ l) were withdrawn through the cannulae 7 times from each rat.

Tissue Levels of Warfarin after Intravenous Administration — Rats were sacrificed 20 min and 36 h after the intravenous administration of 1 mg/kg of warfarin and then the brain, lung, liver, kidney and muscle were quickly removed, rinsed with ice-cold saline, blotted dry, weighed and homogenized with 9-fold volumes of ice-cold saline to obtain 10% homogenates.

Determination of Warfarin — The warfarin concentration in blood and tissue homogenate was determined by measuring radioactivity using a scintillation spectrometer following extraction and purification by a slightly modified thin layer chromatographic method reported by Yacobi *et al.*¹¹⁾ Fifty μ l of blood were added to an equal volume of distilled water and then 900 μ l of saline were added. The diluted blood sample and 1 ml of each tissue homogenate were acidified with 100 μ l of 3 N hydrochloric acid and extracted with 3 ml of ethylene dichloride. The phases were separated by centrifuging after shaking for 20 min. Two ml of the organic phase were transferred to a glass tube and evaporated under nitrogen gas without heating. Warfarin in the glass tube was then dissolved in about 50 μ l of acetone and all of the solution was applied to a thin layer chromatography (TLC) sheet (Kieselgel 60 F₂₅₄, Merck, Darmstadt, GFR). The glass tube was washed two times with 50 μ l of acetone. This acetone solution was also applied to the TLC sheet. Chromatography was carried out with ethylene dichloride-acetone (9:1). The ¹⁴C-warfarin spot was visualized under ultraviolet (UV) light and compared with a spot of unlabelled standard warfarin. The warfarin spot was scraped out and suspended into 10 ml scintillation fluid [2, 5-diphenyl-oxazole (PPO) 5 g, 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)

300 mg, toluene 700 ml, Triton X-100 300 ml] in a vial. The radioactivity was determined with an Aloka LSC-903 liquid scintillation system (Aloka Co., Ltd., Tokyo, Japan).

Determination of Total Radioactivity in Blood and Tissues — The 5 μ l of blood samples and 300 μ l of the 10% tissue homogenates were dissolved in 500 μ l and 1 ml of Soluene-350 (Packard Instrument Co., Dowers Grove, U.S.A.), respectively. After heating at 50 °C for 1 h, these samples were decolorized with 200 μ l hydrogen peroxide and then added to 10 ml of the scintillation fluid.

Serum Protein Binding of Warfarin — Serum protein binding was determined by an ultrafiltration method. Blood samples were obtained from rats to which no warfarin or anticoagulants were given and were centrifuged at 3000 rpm for 10 min to obtain serum samples. Three μ l of 0.5 mg/ml of ¹⁴C-warfarin solution were added to 500 μ l of the pooled serum samples. The mixture was incubated for 20 min at 25 °C and then ultrafiltered using a filter EMIT Free Level (Filter I, Syva Co., Palo Alto, Calif., U.S.A.) at 5000 rpm for 2 min at 25 °C. The serum before ultrafiltration and the ultrafiltrate were assayed for radioactivity.

RESULTS AND DISCUSSION

Whole Blood Levels of Warfarin Following Intravenous Administration

Since several metabolites of warfarin were found in plasma after the administration of warfarin to rats,¹²⁾ we purified the warfarin in blood samples from other radioactive metabolites by a thin layer chromatographic method following organic solvent extraction. Whole blood levels of warfarin following intravenous administration to 1-d-old, 1-, 3- and 8-week-old rats are shown in Fig. 1 and the pharmacokinetic parameters were estimated on the basis of a two-compartment open model (Table I) using a non-linear least squares program MULTI.¹³⁾ The purpose of this study was to evaluate the changes of the warfarin disposition in the growth process. Since we could not experiment for long periods, we selected 36 h for the experimental period. It is well known that the physiological function of the liver and kidney to remove exogenous compounds is much lower at the neonatal period than in older men or experimental animals.^{14,15)} Whole blood levels of warfarin in the β -phase

FIG. 1. Whole Blood Levels of Warfarin Following Intravenous Administration to 1-d-Old ($\circ$), 1- ($\square$), 3- ($\bullet$) and 8-Week- ($\blacksquare$)-Old Rats at a Dose of 1 mg/kg

Lines are calculated by a nonlinear least squares method (Gauss-Newton method, weight $(i) = 1/C_i^2$ where C_i is the whole blood level). Each point represents the mean $\pm$ S.D. of 4 or 5 rats. The points without vertical bars have smaller S.D. than the symbols.

TABLE I. Pharmacokinetic Parameters for Warfarin after Intravenous Administration to 1-d-Old, 1-, 3- and 8-Week-Old Rats (Value for Parameter $\pm$ Standard Deviation)

Parameter	1-d-old ($n = 7$) ^a	1-Week-old ($n = 7$)	3-Week-old ($n = 7$)	8-Week-old ($n = 7$)
A ($\mu\text{g/ml}$)	3.36×10^0 $\pm 1.35 \times 10^{0d}$	4.50×10^0 $\pm 7.61 \times 10^{-1d}$	4.41×10^0 $\pm 3.46 \times 10^{-1d}$	7.23×10^0 $\pm 1.43 \times 10^0$
B ($\mu\text{g/ml}$)	1.99×10^0 $\pm 1.22 \times 10^{-1d}$	3.08×10^0 $\pm 7.86 \times 10^{-2d}$	4.11×10^0 $\pm 5.97 \times 10^{-2d}$	3.69×10^0 $\pm 1.92 \times 10^{-1}$
α (min^{-1})	8.77×10^{-2} $\pm 4.49 \times 10^{-2}$	7.38×10^{-2} $\pm 1.73 \times 10^{-2}$	6.72×10^{-2} $\pm 8.05 \times 10^{-3}$	6.62×10^{-2} $\pm 1.85 \times 10^{-2}$
β (min^{-1})	0^b	0^b	2.35×10^{-4} $\pm 6.80 \times 10^{-6d}$	3.63×10^{-4} $\pm 2.48 \times 10^{-5}$
K_{12} (min^{-1})	5.51×10^{-2} $\pm 4.86 \times 10^{-2}$	4.38×10^{-2} $\pm 1.89 \times 10^{-2}$	3.44×10^{-2} 9.04×10^{-3}	4.29×10^{-2} $\pm 1.97 \times 10^{-2}$
K_{21} (min^{-1})	3.26×10^{-2} $\pm 1.86 \times 10^{-2}$	3.00×10^{-2} $\pm 7.66 \times 10^{-3}$	3.25×10^{-2} $\pm 4.10 \times 10^{-3d}$	2.26×10^{-2} $\pm 6.94 \times 10^{-3}$
K_{10} (min^{-1})	0^b	0^b	4.86×10^{-4} $\pm 8.57 \times 10^{-5d}$	1.06×10^{-3} $\pm 4.46 \times 10^{-4}$
$V_d(\text{ml/kg})^c$	5.02×10^2 $\pm 3.08 \times 10^{1d}$	3.24×10^2 $\pm 8.27 \times 10^{0d}$	2.43×10^2 $\pm 3.54 \times 10^{0d}$	2.71×10^2 $\pm 1.41 \times 10^1$
AUC ($\mu\text{g} \cdot \text{min/ml}$)	— ^b	— ^b	1.75×10^{-4} $\pm 5.64 \times 10^{2d}$	1.03×10^4 $\pm 8.73 \times 10^2$
CL (ml/min/kg)	— ^b	— ^b	5.71×10^{-2} $\pm 1.84 \times 10^{-3d}$	9.73×10^{-2} $\pm 8.26 \times 10^{-3}$

a) Number of input data, each of which is the mean for 4 or 5 rats. b) See Results and Discussion in detail. c) $V_d(\text{ml/kg}) = (\text{Dose})/B$ d) Significantly different from the value for 8-week-old rats at $p < 0.01$.

FIG. 2. The Relationship between the Age of Rats and the Distribution Volume (V_d) (A) and the Total Body Clearance (CL) (B)

The points without vertical bars have smaller S.D. than the symbols.

declined very slowly in 1-d-old and 1-week-old rats in this experiment. Therefore, we analyzed the data by fixing β to 0, so that the area under the blood concentration curve (AUC) and total body clearance in both 1-d-old and 1-week-old rats became infinite and 0, respectively. The relationship between the age of rats and the total body clearance and the distribution volume are shown in Fig. 2. It is apparent that the elimination of warfarin from blood in 1-d-old and 1-week-old rats was considerably slower than that in 8-week-old rats ($p < 0.01$). The elimination in 3-week-old rats was also significantly slower than that in 8-week-old rats ($p < 0.01$). The values for total body clearance in 1-d-old, 1-, 3- and 8-week-old rats were 0, 0, $5.71 \times 10^{-2} \pm 1.84 \times 10^{-3}$ and $9.73 \times 10^{-2} \pm 8.26 \times 10^{-3}$ ml/min/kg, respectively. The distribution volume in 1-d-old rats ($5.02 \times 10^2 \pm 3.08 \times 10^1$ ml/kg) was about twice that in 8-week-old rats ($2.71 \times 10^2 \pm 1.41 \times 10^1$ ml/kg) ($p < 0.01$) and those in 1-

and 3-week-old rats were $3.24 \times 10^2 \pm 8.27 \times 10^0$ and $2.43 \times 10^2 \pm 3.54 \times 10^0$ ml/kg. In 1-d-old and 1-week-old rats, the metabolites of warfarin were barely detectable in blood. However, in 3- and 8-week-old rats, sufficient amount of the metabolites existed in the blood. It was reported that in newborn infants, the distribution volume was higher and the half-life time was longer than the distribution volume in older men with furosemide¹⁾ and amobarbital.²⁾ Similar results were obtained also in this experiment with warfarin disposition in rats. To clarify the reason for the differences of the warfarin disposition between newborn (or young) and adult rats, serum protein binding and tissue distribution of warfarin were investigated.

Serum Protein Binding of Warfarin

Table II summarizes the serum protein binding of warfarin. The percentages of free warfarin in 1-d-old, 1-, 3- and 8-week-old rats were 1.078 ± 0.112 , 0.871 ± 0.083 , 0.463 ± 0.291 and

TABLE II. Warfarin Binding to Serum Protein in 1-d-Old, 1-, 3- and 8-Week-Old Rats

1-d-old ^{a)}	1-Week-old ^{a)}	3-Week-old ^{b)}	8-Week-old ^{a)}
98.92 ^{c)} ±0.112	99.22 ^{c)} ±0.083	99.54±0.288	99.57±0.165

a) Mean % ± S.D. of 4 cases. b) Mean % ± S. D. of 7 cases. c) Significantly different from the value of 8-week-old rats at $p < 0.01$.

TABLE III. Tissue-to-Blood Concentration Ratios of Warfarin in 1-d-Old, 1-, 3- and 8-Week-Old Rats at 20 min and 36 h Following Intravenous Administration

Time	Age	Brain	Lung	Liver	Kidney	Muscle
20 min	1-d-old	$8.44 \times 10^{-2a)}$	2.42×10^{-1}	$4.86 \times 10^{-1b)}$	2.68×10^{-1}	$2.50 \times 10^{-1a)}$
		$\pm 1.17 \times 10^{-2}$	$\pm 1.05 \times 10^{-1}$	$\pm 4.80 \times 10^{-2}$	$\pm 5.63 \times 10^{-2}$	$\pm 4.94 \times 10^{-2}$
	1-Week-old	$3.76 \times 10^{-2a)}$	2.14×10^{-1}	3.29×10^{-1}	2.25×10^{-1}	$1.68 \times 10^{-1b)}$
		$\pm 4.30 \times 10^{-3}$	$\pm 4.06 \times 10^{-2}$	$\pm 8.54 \times 10^{-2}$	$\pm 4.75 \times 10^{-2}$	$\pm 3.46 \times 10^{-2}$
	3-Week-old	1.92×10^{-2}	2.22×10^{-1}	$2.16 \times 10^{-1a)}$	1.87×10^{-1}	8.84×10^{-2}
		$\pm 4.10 \times 10^{-3}$	$\pm 2.42 \times 10^{-2}$	$\pm 2.73 \times 10^{-2}$	$\pm 3.07 \times 10^{-2}$	$\pm 1.41 \times 10^{-2}$
	8-Week-old	1.86×10^{-2}	2.83×10^{-1}	3.42×10^{-1}	2.28×10^{-1}	7.99×10^{-2}
		$\pm 2.90 \times 10^{-3}$	$\pm 9.44 \times 10^{-2}$	$\pm 5.56 \times 10^{-2}$	$\pm 3.46 \times 10^{-2}$	$\pm 1.06 \times 10^{-2}$
36 h	1-d-old	6.98×10^{-2}	$2.02 \times 10^{-1a)}$	$3.72 \times 10^{-1b)}$	2.30×10^{-1}	$3.27 \times 10^{-1b)}$
		$\pm 2.15 \times 10^{-2}$	$\pm 7.07 \times 10^{-2}$	$\pm 1.15 \times 10^{-1}$	$\pm 6.88 \times 10^{-2}$	$\pm 8.30 \times 10^{-2}$
	1-Week-old	5.30×10^{-2}	$2.74 \times 10^{-1b)}$	$2.43 \times 10^{-1b)}$	3.61×10^{-1}	$2.56 \times 10^{-1b)}$
		$\pm 7.90 \times 10^{-3}$	$\pm 9.79 \times 10^{-2}$	$\pm 5.41 \times 10^{-2}$	$\pm 7.88 \times 10^{-2}$	$\pm 6.38 \times 10^{-2}$
	3-Week-old	2.93×10^{-2}	$1.28 \times 10^{-1a)}$	$3.90 \times 10^{-1b)}$	$1.52 \times 10^{-1b)}$	1.01×10^{-1}
		$\pm 5.40 \times 10^{-3}$	$\pm 3.17 \times 10^{-2}$	$\pm 6.26 \times 10^{-2}$	$\pm 6.37 \times 10^{-2}$	$\pm 1.80 \times 10^{-2}$
	8-Week-old	3.62×10^{-2}	5.69×10^{-1}	7.11×10^{-1}	3.77×10^{-1}	1.27×10^{-1}
		$\pm 9.20 \times 10^{-3}$	$\pm 1.35 \times 10^{-1}$	$\pm 1.79 \times 10^{-1}$	$\pm 1.17 \times 10^{-1}$	$\pm 3.13 \times 10^{-2}$

Data are mean $\pm$ S.D. of 3 or 4 rats. a) Significantly different from the value of 8-week-old rats at $p < 0.01$. b) Significantly different from the value of 8-week-old rats at $p < 0.05$.

$0.432 \pm 0.165\%$ respectively. The values in 1-d-old and 1-week-old rats were about twice those in 3- and 8-week-old rats ($p < 0.01$). These results suggest that the decrease in serum protein bindings in infant rats increased the transfer of warfarin to tissues and led to the increase of distribution volume.

Tissue Distribution of Warfarin

Table III summarizes the apparent tissue-to-blood concentration ratios (T/B values) of warfarin in 1-d-old, 1-, 3- and 8-week-old rats 20 min and 36 h after intravenous administration. The T/B values in both 1-d-old and 1-week-old rats at 20 min and 36 h were similar except for the liver at 20 min in 1-d-old rats and brain. Warfarin seemed to be distributed homogeneously in 1-d-old and 1-week-old rats.

At 20 min when warfarin in tissues did not equilibrate with that in blood, the T/B values of the brain in 1-d ($8.44 \times 10^{-2} \pm 1.17 \times 10^{-2}$) and 1-week ($3.76 \times 10^{-2} \pm 4.30 \times 10^{-3}$) old rats were significantly higher than those in 3-week ($1.92 \times 10^{-2} \pm 4.10 \times 10^{-3}$) and 8-week ($1.86 \times 10^{-2} \pm 2.90 \times 10^{-3}$) old rats ($p < 0.01$). The T/B values of the muscle in 1-d ($2.50 \times 10^{-1} \pm 4.94 \times 10^{-2}$) and 1-week ($1.68 \times 10^{-1} \pm 3.46 \times 10^{-2}$) old rats were significantly higher

than those in 3-week ($8.84 \times 10^{-2} \pm 1.41 \times 10^{-2}$) and 8-week ($7.99 \times 10^{-2} \pm 1.06 \times 10^{-2}$) old rats ($p < 0.05$). It was reported that the maturity of the blood-brain barrier depended upon growth.¹⁶⁾ In this experiment, the high T/B values of the brain in 1-d-old and 1-week-old rats might be caused by the difference in blood-brain-barrier permeability to warfarin. The high T/B values of the muscle in 1-d-old and 1-week-old rats might be caused by the following two possibilities. One is the difference of the blood flow rate in muscle and the other is the difference of the muscular cell membrane permeability to warfarin. Hirate *et al.* reported similar results with creatinine.⁹⁾ They suggested that the high T/B value of the muscle in newborn mice was based upon higher cell membrane permeability rather than blood flow rate.

At 36 h when warfarin in tissues equilibrated with that in blood, there was a similar tendency in the T/B values of the brain and muscle to those at 20 min. These results indicate that the lower serum protein bindings in infant rats increased the transfer of warfarin from blood to the muscle and brain principally and that the high T/B values of the brain and muscle at 20 min in 1-d-old and 1-week-old rats were caused

TABLE IV. Contribution of Tissues to Warfarin Distribution at 20 min and 36 h Following Intravenous Administration

Time	Age	Brain	Lung	Liver	Kidney	Muscle ^{a)}	Blood ^{b)}
20 min	1-d-old	0.566	1.171	4.404	0.670	16.90	19.79
	1-Week-old	0.625	1.772	4.079	1.136	18.69	31.63
	3-Week-old	0.272	1.202	3.837	1.070	23.33	40.60
	8-Week-old	0.043	1.035	7.620	0.976	21.59	43.65
36 h	1-d-old	0.619	1.205	2.901	0.618	21.11	19.87
	1-Week-old	0.623	1.638	2.089	0.966	17.95	22.19
	3-Week-old	0.296	0.770	6.200	0.741	21.22	32.20
	8-Week-old	0.067	1.188	11.94	1.034	25.42	30.84

a) The weight of the muscle in 1-d-old, 1-, 3- and 8-week-old rats used was 25%, ⁴⁾ 25%, ⁴⁾ 50% and 50% of the body weight, respectively. b) The volume of the blood used was 1/13 of the body weight. Data are mean % of 3 rats. See Results and Discussion in detail.

by not only the difference in the cellular membrane permeability but also the difference in the serum protein binding. In 8-week-old rats, the T/B values of warfarin in the lung and liver were about twice those in other rats. This suggests that some substances to which warfarin binds are synthesized in the lung after 3 weeks of age. It was reported that in warfarin pharmacokinetics in rats, saturable hepatic warfarin uptake totally accounted for the apparent dose dependency when warfarin (over 0.01 mg/kg dose) was administered to rats.^{7,8)} On the other hand, Walter *et al.*¹⁸⁾ and Charest-Boule *et al.*¹⁹⁾ reported that there were some soluble fractions for binding of warfarin in rat liver and Levi *et al.*²⁰⁾ showed that the fraction in guinea pig liver increased with growth. In this experiment, the T/B value of the liver in 8-week-old rats was about twice that in 1-d-old, 1 or 3-week-old rats ($p < 0.05$). However, it remains to be clarified whether the factor might cause the age-dependent change of warfarin transfer to the liver.

Table IV summarizes the percentages of warfarin in tissues to evaluate the contribution of tissues to the distribution volume. Total warfarin in rats at 20 min and 36 h was calculated by the following equation using the parameters in Table I.

Total drug

$$= \text{Dose} - \text{CL} \cdot W \cdot (AUC - A/\alpha \cdot e^{-\alpha \cdot t} - B/\beta \cdot e^{-\beta \cdot t})$$

where W is body weight of rat (kg) and t is time

(min). In 1-d-old and 1-week-old rats, recovery from tissues was about 50% of the total warfarin in the body. This result is supported by the data that warfarin was distributed homogeneously in the body of 1-d-old and 1-week-old rats and that the sum of the sampling tissues was about 50% of the body weight. The contribution of the brain to warfarin distribution was very small and decreased with growth. The contributions of the lung, kidney and muscle were similar among the 4 ages and the muscle contributed largely to the distribution of warfarin in the body. These results are very important because some drugs are often administered to newborn infants.

We are now investigating other drugs which are administered to newborn infants on clinical drug therapy to determine whether results similar to those with warfarin are found.

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