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Disposition of carboxyfluorescein in the rat

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Summary

The pharmacokinetics of 6-carboxyfluorescein (CF) have been studied in the rat. CF was rapidly removed from blood in a biphasic manner and was found to have a small volume of distribution together with a clearance approaching renal blood flow. Purification of commercial CF resulted in a significantly larger volume of distribution, but insignificant changes in clearance and half-lives, as estimated using data from the commonly employed fluorimetric assay. Purified CF pharmacokinetics were linear following intravenous doses of 0.5–2 $mg \cdot kg^{-1}$; however, at 4 $mg \cdot kg^{-1}$ both clearance and volume of distribution were found to be dose-dependent. In rats anaesthetized with urethane, CF clearance was 37% lower than in rats anaesthetized with pentobarbitone. This difference in clearance was reflected by a large increase in elimination half-life during urethane anaesthesia.

Introduction

6-Carboxyfluorescein (CF) has been extensively used as an aqueous phase solute to examine the effects of liposome encapsulation on drug disposition primarily because of a relatively simple but sensitive assay which can differentiate between free and encapsulated CF (Weinstein et al., 1977) and the prolonged latency of CF in certain compositions of liposomes (Weinstein et al., 1977; Gregoriadis and Davis,

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1979; Van Renswoude et al., 1979). CF has been reported to rapidly disappear from the plasma after intravenous (i.v.) dosing in mice (Kirby et al., 1980); however, the disposition has not been fully characterized and consequently it has not been possible to study the absorption of CF from liposomes administered extravascularly.

CF is routinely assayed in plasma using a fluorimetric procedure which does not include a selective extraction step. Investigations of the distribution of CF in cell culture (Ralston et al., 1981) have indicated that commercially available CF contains fluorescent contaminants which transfer to cells at significantly different rates than the pure material. As a consequence many recent studies involving CF have employed a purification procedure for the dye. However, other workers have reported that the level of contamination is minimal (Senior and Gregoriadis, 1982a) and have considered purification unnecessary (Senior and Gregoriadis, 1982b; Senior et al., 1983; Tumer et al., 1983). We have studied the pharmacokinetics of commercially available and laboratory-purified CF to assess whether the purification procedure is a necessary prerequiste to in vivo studies.

Previous investigations (Clark et al., 1981) with the related compound fluorescein indicated that nonlinear pharmacokinetics were exhibited at relatively low doses in the dog. Clearly the exhibition of non-linearity in the i.v. disposition of CF could markedly complicate the characterization of CF absorption. Additionally, anaesthetics have been shown to promote nonlinearity in the disposition of co-administered thiamin (Pipkin and Stella, 1982).

In this paper we report the pharmacokinetic parameters describing i.v. CF disposition and their dependence upon CF dose in the rat. The effects of two anaesthetic agents, urethane and pentobarbitone, on the disposition of CF have also been studied. Additionally we report a comparison of the disposition of commercially available and purified CF.

Materials and methods

6-Carboxyfluorescein (CF) (Eastman-Kodak, Rochester, NY) was used either as received or purified using the charcoal/LH-20 procedure of Ralston et al. (1981). Urethane or pentobarbitone were prepared in 0.9% saline and administered intraperitoneally to male Wistar rats (200–250 g) at doses of 1.8 $g \cdot kg^{-1}$ and 67 $mg \cdot kg^{-1}$, respectively. After 1–1.5 h pentobarbitone anaesthesia was maintained by subsequent doses of 8 $mg \cdot kg^{-1}$ every 15–20 min or as needed. A longitudinal midline ventral incision was made along the neck of each rat to expose a carotid artery and a jugular vein which were then cannulated with polythene tubing (0.58 mm, i.d.; 0.96 mm, o.d.). 100 μl doses of CF dissolved in isotonic phosphate buffer pH 7.4 were administered over 5–10 s via the jugular vein. The body temperature of each rat was monitored by a rectal thermometer and maintained at 37°C by heat from an incandescent lamp suspended above the animal. 75 μl samples of blood were taken from the carotid artery at various times after dosing, diluted with 3 ml of pH 7.4 isotonic phosphate-buffered saline containing 0.01% potassium chloride, then centrifuged to remove red blood cells. The concentration of CF in the supernatant

was determined by fluorimetry using excitation and emission wavelenghts of 490 and 517 nm, respectively.

Individual blood concentration–time profiles showed biexponential declines and were analyzed according to a two-compartment open model using the non-linear least squares regression program, NONLIN-74 (Metzler et al., 1974). Weighting factors of $1/C^2$ were used in the analyses. Examination of residuals plots and error variances indicated this weighting gave better fits to the data than using weighting factors of either 1 or $1/C$. From these analyses a number of model-dependent

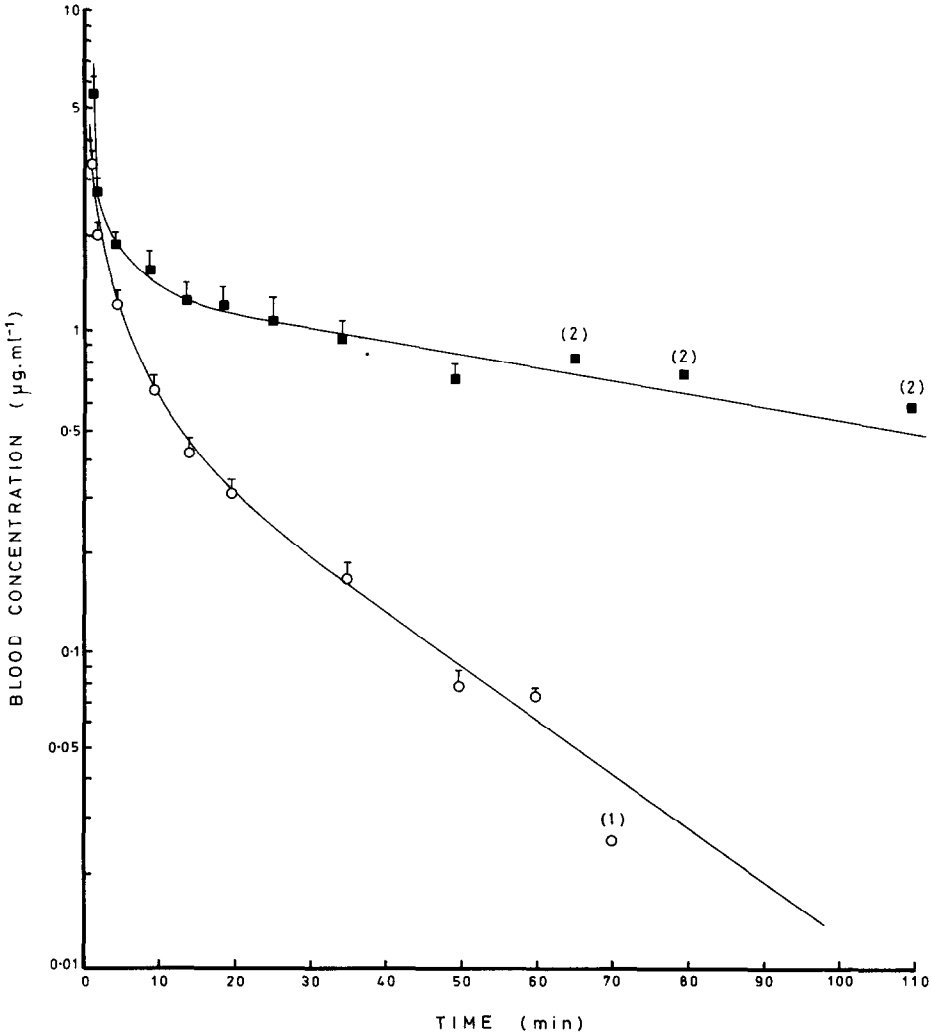

Fig. 1. Mean blood concentrations (± S.E.M.) of CF after i.v. administration (1 mg·kg^{-1} of impure CF) to rats under either urethane (■, n = 5) or pentobarbitone (○, n = 8) anaesthesia. Numbers in parentheses are the group sizes when less than indicated previously.

pharmacokinetic parameters were calculated including initial ($t_{1/2,\alpha}$) and terminal half-lives ($t_{1/2,\beta}$) and the apparent volume of distribution of the central compartment (V_C). The model-independent parameters volume of distribution at steady-state ($V_{d_{SS}}$) and blood clearance (Cl_B) were determined from areas under the curve (AUC_∞) and first moments curve ($AUMC_\infty$) from time zero to infinity. These areas were calculated using the linear trapezoidal rule.

Since pharmacokinetic parameters tend to be geometrically distributed (Sheiner and Beal, 1981, 1982; Jusko 1981) a log-normal transformation was applied to the pharmacokinetic parameters and hence the variation around each parameter estimate is represented as upper and lower 95% confidence limits. All statistical tests were performed on the transformed data and applied using a 5% significance level.

TABLE 1

EFFECT OF ANAESTHETIC ON THE I.V. PHARMACOKINETICS OF CF[a] IN THE RAT

Parameter	Anaesthetic Agent		Statistical difference [b]
	Urethane (n = 5)	Pentobarbitone (n = 8)	
$t_{1/2,\alpha}$(min)	3.63 (11.28/1.17)	1.68 (2.2/1.29)	NS
$t_{1/2,\beta}$(min)	116.72 (244.37/55.76)	14.34 (18.36/11.20)	$P < 0.001$
V_c ($l \cdot kg^{-1}$)	0.22 (0.42/0.12)	0.26 (0.34/0.2)	NS
Vd_{SS}($l \cdot kg^{-1}$)	0.75 (1.16/0.48)	0.56 (0.69/0.45)	NS
Cl_B($ml \cdot min^{-1} \cdot kg^{-1}$)	23.9 (42.35/13.5)	38.05 (42.8/33.89)	$P < 0.05$

Upper and lower 95% confidence limits are shown in parentheses.
[a] Dose: 1 mg kg^{-1} of impure CF.
[b] Student's *t*-test.

TABLE 2

COMPARISON OF THE I.V. PHARMACOKINETICS OF PURIFIED AND IMPURE CF IN THE RAT

Parameter	Purity of CF		Statistical difference [b]
	Pure (n = 6)	Impure (n = 8)	
$t_{1/2,\alpha}$(min)	1.53 (2.18/1.07)	1.68 (2.2/1.29)	NS
$t_{1/2,\beta}$ (min)	16.95 (20.90/13.74)	14.34 (18.36/11.20)	NS
Vc ($l \cdot kg^{-1}$)	0.36 (0.46/0.28)	0.26 (0.34/0.2)	$P < 0.05$
$V_{d_{SS}}$ ($l \cdot kg^{-1}$)	0.78 (0.87/0.7)	0.56 (0.69/0.45)	$P < 0.01$
Cl_B ($ml \cdot min^{-1} \cdot kg^{-1}$)	44.10 (53.45/36.40)	38.05 (42.8/33.89)	NS

Upper and lower 95% confidence limits are shown in parentheses.
[a] Dose: 1 $mg \cdot kg^{-1}$.
[b] Student's *t*-test.

Results

Determinations of CF concentrations in spiked buffer solutions and in buffer solutions containing spiked blood samples established that the binding of CF to red blood cells under the conditions of the assay was negligible. Blood levels of CF

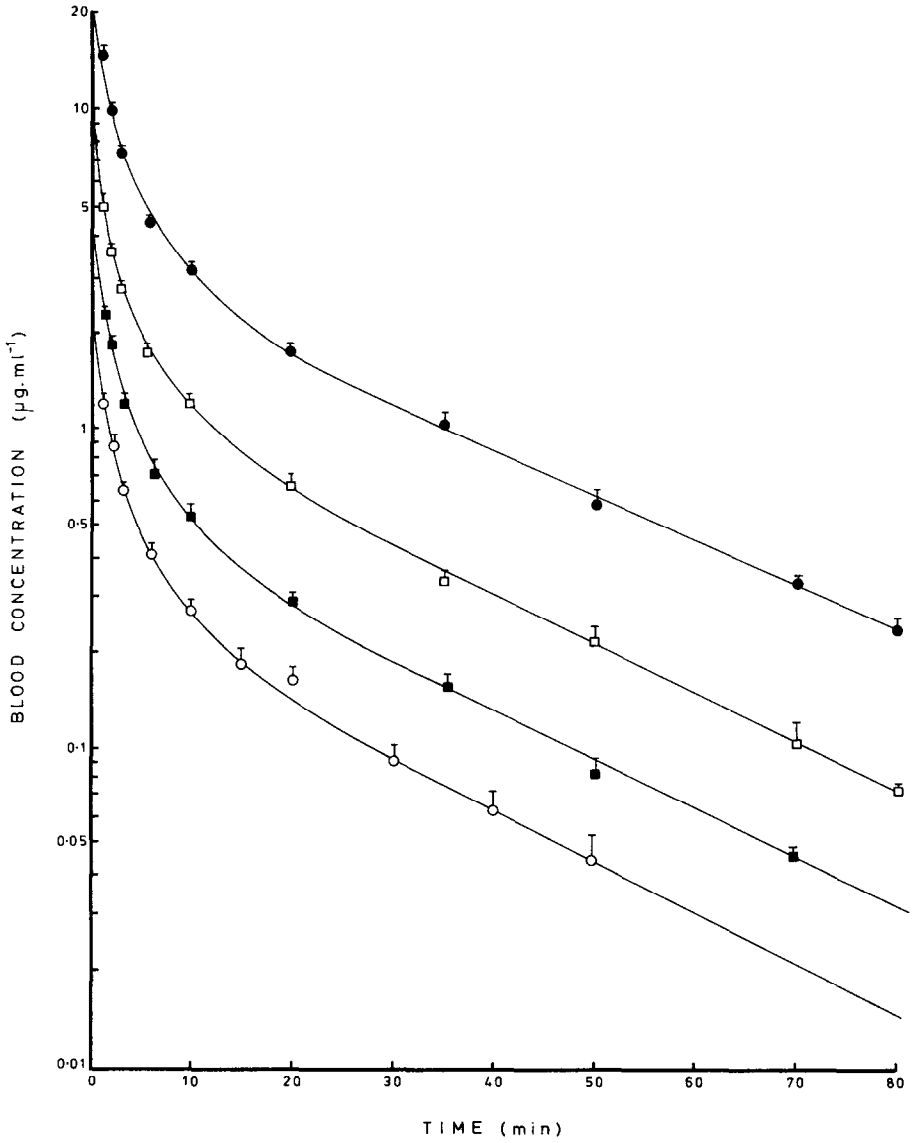

Fig. 2. Mean blood concentrations (±S.E.M.) of CF after i.v. administration of: 0.5 (○), 1 (■), 2 (□) and 4 $mg \cdot kg^{-1}$ (●) of purified CF.

TABLE 3

PHARMACOKINETICS OF CF FOLLOWING VARIOUS I.V. DOSES IN THE RAT

Parameter (n = 6)	Dose of CF ($mg \cdot kg^{-1}$)			
	0.5	1	2	4
$t_{1/2,\alpha}$ (min)	2.01 [a] (2.44/1.67)	1.53 [a] (2.12/1.11)	2.17 [a] (3.04/1.54)	1.84 [a] (2.64/1.59)
$t_{1/2,\beta}$ (min)	17.32 [b] (20.38/14.44)	16.90 [b] (20.38/14.14)	18.73 [b] (23.10/15.75)	19.80 [b] (21.00/18.73)
V_c ($l \cdot kg^{-1}$)	0.35 [c] (0.42/0.29)	0.36 [c] (0.45/0.28)	0.34 [c] (0.41/0.29)	0.26 * (0.32/0.20)
$V_{d_{ss}}$ ($l \cdot kg^{-1}$)	0.77 [d] (0.90/0.65)	0.78 [d] (0.86/0.70)	0.75 [d] (0.86/0.66)	0.60 * (0.68/0.53)
Cl_B ($ml \cdot min^{-1} \cdot kg^{-1}$)	43.30 [e] (58.20/49.17)	44.10 [e] (52.55/37.00)	39.23 [e] (44.46/34.55)	28.85 * (33.40/24.90)

Upper and lower 95% confidence limits are shown in parentheses. Multiple comparisons were made using Student-Newman Keuls procedure.

[a-e] Parameters statistically equivalent for all doses.

* Parameters at 4 $mg \cdot kg^{-1}$ were different from those of the 3 lower doses.

declined in a biexponential manner following various i.v. bolus doses as shown in Figs. 1 and 2. Fig. 1 shows the mean blood concentrations of impure CF after i.v. administration at 1 $mg \cdot kg^{-1}$ to rats anaesthetized with either urethane or pentobarbitone. Urethane, compared with pentobarbitone, caused a 37% decrease ($P < 0.05$) in the clearance of CF together with a large increase ($P < 0.001$) in the terminal half-life. However, the volume of distribution parameters V_C and $V_{d_{ss}}$ were not significantly different between the two groups (Table 1).

The pharmacokinetic parameters for pure and impure CF are compared in Table 2. The volumes of distribution V_C and $V_{d_{ss}}$ were significantly larger (38% and 39% respectively) for the purified material. Conversely, Cl_B and the initial ($t_{1/2,\alpha}$) and terminal ($t_{1/2,\beta}$) half-lives were not significantly different.

Fig. 2 shows the mean blood concentrations of pure CF following i.v. doses of 0.5, 1, 2 and 4 $mg \cdot kg^{-1}$ in rats anaesthetized with pentobarbitone. The mean pharmacokinetic parameters of CF at these doses are given in Table 3. Whilst there was no evidence for dose-dependency of half-lives; Cl_B, V_C and $V_{d_{ss}}$ were all significantly smaller at 4 $mg \cdot kg^{-1}$ than at the three lower doses. Clearance was 32% smaller at 4 $mg \cdot kg^{-1}$ compared with the three lower doses, whilst V_C was 26% smaller, and $V_{d_{ss}}$ 22% smaller, at 4 $mg \cdot kg^{-1}$.

There was no evidence of dose-dependency in any of the parameters following doses of 0.5–2 $mg \cdot kg^{-1}$. A plot of AUC_∞ versus dose is shown in Fig. 3 and highlights the dose-dependent pharmacokinetics at 4 $mg \cdot kg^{-1}$ but not at 0.5, 1 or 2 $mg \cdot kg^{-1}$.

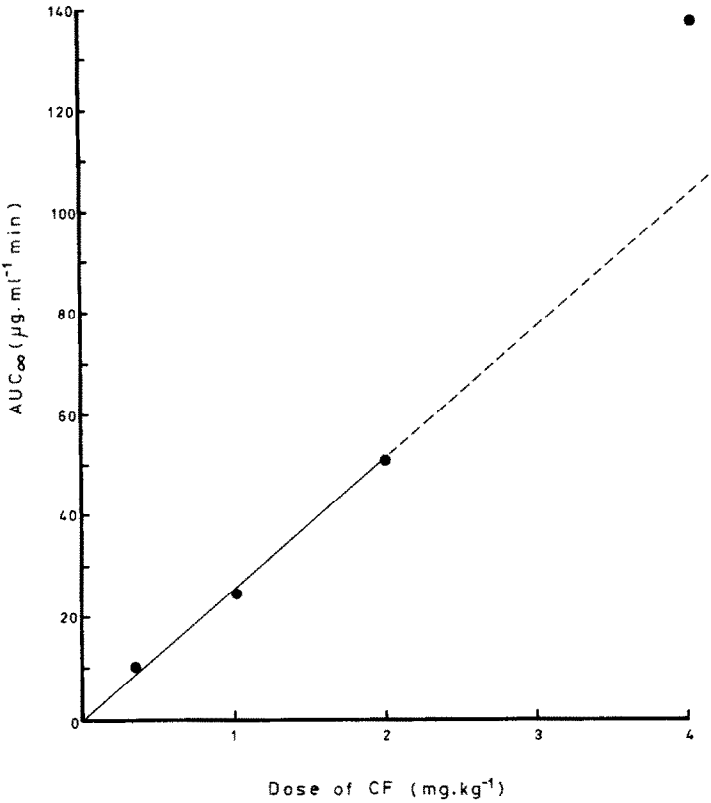

Fig. 3. Relationship between i.v. CF dose and AUC.

Discussion

This work has established dose-independent pharamacokinetics for pure CF when administered to rats at doses not exceeding 2 mg · kg^{-1}. The change from dose-independent to dose-dependent pharmacokinetics of pure CF at 4 mg · kg^{-1} may be due to a saturation of the clearance processes since the clearance is close to that of renal blood flow in the rat. The upper limit of dose-independent pharmacokinetics is, however, sufficient to permit pharmacokinetic studies of various CF-containing formulations administered by extravascular routes.

The exact mechanism by which urethane altered the clearance of CF is not known. Urethane has been reported to reduce blood pressure and increase the haematocrit (Van Der Meer et al., 1975), impair renal function (Severs et al., 1981), and produce hepatotoxicity (Rosoff, 1974). Pipkin and Stella (1982) reported that urethane anaesthesia decreased the clearance and increased the half-life, but did not alter the volume of distribution of thiamin; a compound which is highly cleared by renal mechanisms. Conversely, the half-life of phenytoin, which is predominantly cleared by the liver, was not altered by urethane (Umeda and Inaba, 1978). As urethane-induced changes would most likely have resulted in dose-dependent pharmacokinetics at much lower doses of CF further investigations were carried out using pentobarbitone anaesthesia.

Ralston et al. (1981) reported that commercially available CF contained both lipophilic and polar impurities which were removed during the purification process. These impurities were probably responsible for the significantly smaller V_C and $V_{d_{ss}}$ which we observed for the impure dye compared to the pure material. However, the polar contaminants were most likely of greater importance than the lipophilic products, as contamination with the latter would have been expected to increase the volumes of distribution. Contamination by non-fluorescent materials would also tend to increase the observed volumes of distribution of the impure material. As the disposition of commercially available CF differs from that of the purified material, this could complicate the interpretation of CF absorption or its release from liposomes and hence we believe that purification is a necessary prerequisite to extensive in vivo studies using this compound.

In conclusion, this work has established a range for dose-independent pharmacokinetics of CF which allow future biopharmaceutical studies to yield an understanding of CF absorption from extravascularly administered formulations. Furthermore, it has emphasised the need to be judicious in the choice of anaesthetic and to establish the influence of the anaesthetic on the pharmacokinetics of the drug in question.

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