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Use of fluorescence HPLC for determining extrarenal creatinine clearance in rats

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Summary

In order to clarify whether plasma creatinine concentration is overestimated due to the presence of non-creatinine chromogens when determined by the Jaffé method and also to elucidate the significance of tubular secretion in relation to creatinine clearance, the creatinine clearance in rats was measured by the use of a fluorescence HPLC method for the specific determination of creatinine. When creatinine was determined by the Jaffé method, plasma creatinine concentration was always overestimated. When creatinine was determined by the fluorescence HPLC method, the clearance of endogenous creatinine was found to be hardly influenced by tubular secretion, and it appears to be a reliable index of GFR in rats with normal renal function. The clearances for endogenous and exogenous creatinine were well correlated to inulin clearance, although the clearance of exogenous creatinine at plasma concentrations ranging from 22.6 to 289.9 μg/ml was slightly higher than the inulin clearance. The phenomenon of extrarenal clearance of exogenous creatinine was studied carefully in rats with normal renal function under steady-state conditions and after a single injection. The contribution of extrarenal clearance to overall clearance was 37.9% in the former case and 21.1% in the latter case. Although there is about a 2-fold difference between these values, extrarenal clearance appears to be significant in rats with normal renal function, and plasma clearance of exogenous creatinine cannot be necessarily regarded as a valid index of GFR.

Introduction

Although it is considered that inulin clearance is suitable for the determination of glomerular filtration rate (GFR) (Kampmann and Hansen, 1981), endogenous creatinine clearance is often used in routine clinical work and several methods have been reported for the determination of creatinine clearance (Chiou and Hsu, 1975a and b; Bjornsson, 1979; Hull et al., 1981; Hallynck et al., 1981; Thomis et al., 1982). However, discrepancies between endogenous creatinine clearance and inulin clearance have sometimes been reported (Skov, 1970; Hood et al., 1971; Hagstam et al., 1974; Bauer and Brooks, 1979; Carrie et al., 1980). This was mainly attributed to the tubular secretion of creatinine (Healy, 1968; Kampmann and Hansen, 1981; Prescott, 1982), but also partly to the chemical procedures used for the estimation of serum creatinine concentration (Healy, 1968; Bennet and Porter, 1971). In addition to these facts, extrarenal clearances of creatinine in rats (Jones and Burnett, 1972; Jones and Burnett, 1975; Watanabe et al., 1981b and c; Hirate et al., 1982),

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in dogs (Giovannetti et al., 1973) and in man (Giovannetti et al., 1973; Jones and Burnett, 1974; Gonella et al., 1975; Mitch and Walser, 1978; Mitch et al., 1980; Hankins et al., 1981; Huang et al., 1982) were also reported; however, this phenomenon was limited to the cases of disordered renal function.

Therefore, to clarify these relations further, creatinine clearances in rats were measured by a fluorescence high-performance liquid chromatography (HPLC) method for specific creatinine determination and compared with inulin clearance. The tubular secretion behavior of endogenous creatinine was also examined and compared with that of exogenous creatinine. Finally, the phenomenon of extrarenal clearance of exogenous creatinine was studied carefully in rats with normal renal function under the steady-state conditions and the single-bolus injections.

Experimental

Analytical method

The plasma and urine creatinine concentrations were determined by a modification of the fluorescence HPLC method (Hiraga and Kinoshita, 1981) and the modification is only for the mobile phase, i.e. Hiraga and Kinoshita (1981) used 6 eluent buffer solutions with the use of step gradient unit for the separation of ten guanidino compounds. In the present study, mono-mobile phase was used for the separation of creatinine only. Details were as follows: an ISC-05/S0504 packed column (strong cation exchange resin, 5 μm particle size, 50×4 mm i.d., Shimadzu Seisakusho) was used for the separations, and it was operated at 55°C in a CTO-2A column oven (Shimadzu Seisakusho). The eluent buffer, 0.35 N sodium citrate (pH 5.0), was delivered by a Shimadzu LC-3A pump (Shimadzu Seisakusho) at a constant flow rate of 0.7 ml/min at 100 kg/cm^2. The column effluent was first mixed with 0.75 N sodium hydroxide and then with 0.6% ninhydrin solution in a CRB-3A reaction chamber (Shimadzu Seisakusho) at 55°C. This alkaline solution and the ninhydrin solution were delivered at constant flow rates of 0.6 ml/min and 0.4 ml/min, respectively, by using PRR-1A single plunger pumps (Shimadzu Seisakusho). The fluorescence intensity of effluent fractions was measured with an RF 510 LC spectrofluoromonitor (Shimadzu Seisakusho) with excitation and emission at 395 and 500 nm, respectively.

To 100μl of plasma or urine, 100 μl each of 30% trichloroacetic acid solution and distilled water were added, and the mixture was centrifuged at 3000 rpm for 10 min. One hundred μl of this supernate was used for the analysis of creatinine. Fig. 1 shows a chromatogram of a rat plasma sample. The limit of detection was 2.5 μg/ml (peak height of twice the noise level) and calibration plots of peak height against drug concentration were linear over the range of 2.5–2000 μg/ml. The recovery from rat plasma was above 95% with satisfactory reproducibility.

For the comparison of this fluorescence HPLC method with the Jaffé method (Bonsnes and Taussky, 1945), creatinine was also measured with alkaline picrate in both samples; one of them was treated with Lloyd's reagent (Hare, 1950) and the other was not. These procedures were as follows: to 1 ml of plasma or urine, 0.5 ml of distilled water and 1 ml of 20% trichloroacetic acid solution were added, and the mixture was centrifuged at 3000 rpm for 10 min. To 1 ml of this supernate, 2 ml each of 0.5 N sodium hydroxide and 1% picric acid solution were added. After 10 min, the optical density was measured at a wavelength of 520 nm with a Hitachi 139 UV-VIS spectrophotometer. When Lloyd's reagent was used, 0.2 ml of saturated oxalic acid and 20 mg of Lloyd's reagent were added to 1 ml of supernate following the deproteinization. The tubes were shaken for 5 min and then centrifuged at 3000 rpm for 5 min. To this precipitate, 1 ml of distilled water, 2 ml of 0.5 N sodium hydroxide and 1% picric acid solution were added. The mixture was shaken for 5 min and centrifuged at 3000 rpm for 5 min, then the optical density of this supernate was measured. The plasma and urine inulin concentrations were determined by a slight modification of the method of Dische and Borenfreund (1951).

Clearance study under the steady-state condition

The standard renal clearance procedure was utilized. Male Wistar rats weighing 210–350 g

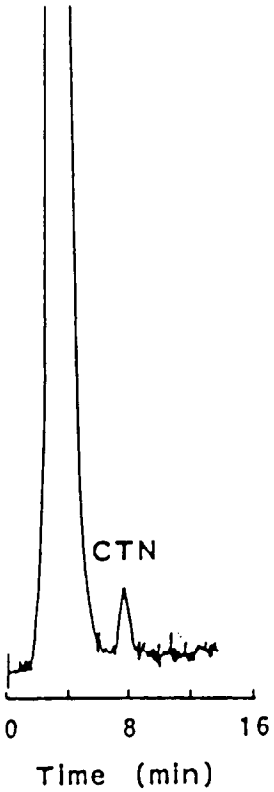


Fig. 1. Chromatogram of a rat plasma sample. CTN, endogenous creatinine.

were fasted for 24 h before the experiment and then cannulas were inserted into the femoral vein and artery, and the bladder under light ether anesthesia. Priming and maintenance doses were calculated by means of the following equations and dissolved in normal saline:

Priming dose (mg) = body weight (kg) × distribution volume (ml/kg) × expected plasma concentration (mg/ml)

Maintenance infusion rate (mg/min) = body weight (kg) × predicted clearance (ml/min/kg) × expected plasma concentration (mg/ml)

The priming doses of creatinine ranged from 3 to 250 mg and corresponding maintenance infusion rates ranged from 0.075 to 6 mg/min. The priming doses of inulin ranged from 12 to 200 mg and corresponding maintenance infusion rates ranged from 0.012 to 2 mg/min. To examine the ratio of urinary excretion rate to maintenance infusion rate, the maintenance infusion rate of inulin increased with increasing the maintenance infusion rate of creatinine. After awakening of the rats, 1 ml of the priming dose was given intravenously and the maintenance dose was delivered at 0.1 ml/min through a constant infusion pump, in which creatinine and inulin were infused simultaneously in the case of the measurement of exogenous creatinine clearance and only inulin was infused in the case of the measurement of endogenous creatinine clearance. Drug renal clearance (CL_R^{ss}) was calculated as $CL_R^{ss} = UV/C$, where U, C, and V represent urine and plasma concentrations of the drug, and urine flow rate, respectively. Clearances were determined every 20 min. Arterial blood samples were drawn at the midpoint of each urine collection period. The number of determinations for each rat was 5. When exogenous creatinine experiments were done, exogenous creatinine concentrations in plasma and excretion rates in urine were corrected by subtracting their endogenous creatinine concentrations and excretion rates obtained in the samples prior to dosing from total creatinine concentrations and excretion rates, respectively, in all experiments.

Single-bolus injection study

Procedures for blood and urine collections were the same as described above. Following bolus injection of creatinine into the femoral vein, plasma and urine samples were collected periodically from the femoral artery and bladder, respectively. Animals where urine flow rate was markedly decreased compared to the stationary rate were excluded from the data analysis. The pharmacokinetic parameters were calculated by use of the non-linear least-squares program MULTI (Yamaoka et al., 1981).

Statistical analysis

All means of the data are presented with their standard error (S.E.). Student's *t*-test was utilized to determine a significant difference.

Results and Discussion

Comparison of endogenous creatinine levels determined by the present HPLC and Jaffé methods

The creatinine concentrations in pooled plasma and urine of rats as determined by the two methods are presented in Table 1. The plasma endogenous creatinine concentration obtained by the present method is significantly lower than those found by the both Jaffé methods ($P < 0.05$). However, there is no significant difference between the present and Jaffé methods as regards the urinary endogenous creatinine concentration. It seems clear that the plasma concentration is overestimated in the Jaffé method due to the presence of non-creatinine chromogens. In addition, there is a significant difference between Lloyd's reagent-treated and non-treated samples as regards the plasma concentration.

Table 2 shows the mean values of the endogenous creatinine concentrations in plasma and urine of 10 rats and its clearance as determined by different analytical methods. When the plasma and urinary concentrations were determined by the Jaffé method without Lloyd's reagent, the values deviated positively from those determined by the present HPLC method ($P < 0.05$).

Endogenous creatinine clearances determined by the Jaffé method without Lloyd's reagent deviated negatively from those determined by the HPLC method ($P < 0.05$). On the other hand, the differences of creatinine clearance determined by the HPLC method and Jaffé method with Lloyd's reagent were slight. As previously reported by Doolan et al. (1962) and Healy (1968), present results also suggests that the different chemical procedures for the estimation of creatinine give different results.

Endogenous and exogenous creatinine clearances and their relation to inulin clearance

In the present study, endogenous plasma creatinine concentration was 4.39 ± 0.14 μg/ml (mean ± S.E., $n = 55$), endogenous creatinine clearance was 5.37 ± 0.17 ml/min/kg and the clearance ratio to inulin was 0.99 ± 0.02. As shown in Fig. 2, the endogenous creatinine clearance and clearance ratio were lower than those of exogenous creatinine infusion at plasma creatinine concentrations below 300 μg/ml. This was mainly due to the tubular secretion of creatinine in the exogenous creatinine infusion (Mandel et al., 1953; Kampmann and Hansen, 1981). In the exogenous creatinine infusion, clearance tended to decrease with increasing plasma creatinine concentration and became almost constant at plasma concentrations above 300 μg/ml. It is likely that this was due to the saturation of tubular secretion of creatinine with increasing plasma concentration (Fingl, 1952). The clearance of exogenous creatinine and its clearance ratio to inulin at plasma concentrations ranging from 22.6 to 289.9 μg/ml

TABLE 1

ENDOGENOUS CREATININE CONCENTRATIONS IN PLASMA AND URINE OF RATS AS DETERMINED BY DIFFERENT ANALYTICAL METHODS [a]

	Concentration (μg/ml; mean ± S.E.) determined by:		
	Fluorescence HPLC	Jaffé method	
		non-Lloyd's reagent	Lloyd's reagent
Plasma	3.26 ± 0.09	11.1 ± 0.07	4.56 ± 0.12
Urine	374 ± 4.7	376 ± 6.2	353 ± 2.4

[a] These values are means of 5 determinations on pooled plasma and urine of 6 rats.

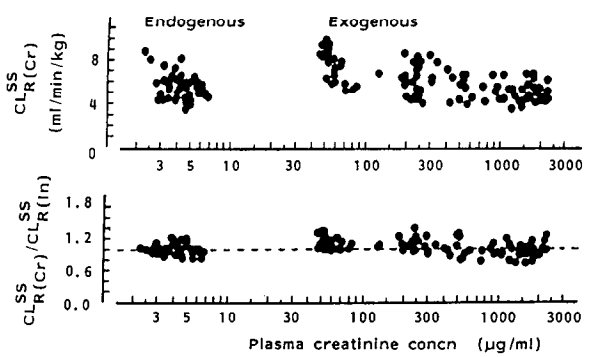


Fig. 2. Relations between the creatinine clearance and creatinine plasma concentration (upper) and between the clearance ratio of creatinine to inulin and plasma concentration of creatinine (lower). The dotted line indicates a clearance ratio of unity.

TABLE 2

ENDOGENOUS CREATININE CONCENTRATIONS IN PLASMA AND URINE, AND ITS CLEARANCES AS DETERMINED BY DIFFERENT ANALYTICAL METHODS ($n = 10$, mean ± S.E.)

	Concentration (μg/ml) determined by:			Endogenous clearance		
	Fluorescence HPLC	Jaffé method		Fluorescence HPLC	Jaffé method	
		non-Lloyd's reagent	Lloyd's reagent		non-Lloyd's reagent	Lloyd's reagent
Plasma	4.45 ± 0.22	16.3 ± 1.4 (3.75 ± 0.38)	4.70 ± 0.57 (1.08 ± 0.15)	4.56 ± 0.73	2.14 ± 0.38 (0.49 ± 0.06)	4.44 ± 0.53 (1.12 ± 0.17)
Urine	831 ± 112	1324 ± 169 (1.64 ± 0.12)	842 ± 103 (1.04 ± 0.09)			

[a] Values in parentheses are the ratio of Jaffé methods to HPLC method.

were 6.68 ± 0.24 ml/min/kg and 1.10 ± 0.02 ($n = 54$), whereas the values were 5.32 ± 0.17 ml/min/kg and 0.96 ± 0.02 ($n = 50$) at plasma concentrations above 300 μg/ml. There were significant differences between the values of both of these parameters at plasma exogenous creatinine concentrations of up to 300 μg/ml and of above 300 μg/ml ($P < 0.05$). Further, there were also significant differences in both parameters between plasma exogenous creatinine concentrations of up to 300 μg/ml and endogenous creatinine, but there was no statistically significant difference between the case of plasma exogenous creatinine concentrations above 300 μg/ml and endogenous creatinine. Consequently, it appears that the low concentration of endogenous creatinine in contrast to the high concentration attained by exogenous creatinine infusion results in less tubular secretion, so that the apparent clearance is more nearly equivalent to the true GFR. The coefficients of variation for the steady-state concentrations of exogenous creatinine ranged from 11.8 to 24.7% ($n = 8 \sim 29$).

Brod and Sirota (1948) reported that the discrepancy between the endogenous and exogenous creatinine clearances can be attributed to the contribution of non-creatinine chromogens in the determination of creatinine, in addition to the tubular secretion of creatinine. The fluorescence HPLC method used in the present study is specific for the determination of creatinine, because the guanidine compound is well separated on the column and ninhydrin combines with guanidine compounds without disturbance by amino acids in strongly alkaline media (Hiraga and Kinoshita, 1981). Hence, the possibility that non-creatinine chromogens contributed to this discrepancy can be ruled out. Hood et al. (1971) speculated that the elevated plasma creatinine produced by exogenous infusion might stimulate the tubular secretion mechanism. The present results are consistent with this supposition. Endogenous and exogenous creatinine clearances in the case of disordered renal function may thus be strongly influenced by the tubular secretion (Hood et al., 1971), since the plasma creatinine concentrations in the case of disordered renal function are expected to be elevated.

Extrarenal clearance of exogenous creatinine

If clearance is calculated solely on the basis of serum creatinine level data (Bjornsson, 1979; Hallynck et al., 1981; Hull et al., 1981; Kampmann and Hansen, 1981; Thomis et al., 1982), the existence of extrarenal clearance of creatinine may crucially affect the reliability of creatinine clearance determination. Although the existence of extrarenal elimination of creatinine has been reported, previous reports suggested that extrarenal clearance was limited to cases of disordered renal function in rats, in dogs, and in man, and that it was negligible in the case of normal renal function, as mentioned in the Introduction.

The ratio of excretion rate to the maintenance infusion rate in our normal rats is shown in Fig. 3. In the case of creatinine this ratio fell below unity with increasing plasma concentration, in contrast to the case of inulin. This finding suggests that extrarenal clearance or tubular re-absorption of exogenous creatinine may also occur in rats with normal renal function.

Chiou (1982) reported the tubular re-absorption of creatinine in man. Vree et al. (1981) reported a good correlation between endogenous creatinine clearance and urine flow in man with both normal and disordered renal functions. However, as shown in Fig. 4, exogenous creatinine clearance did not correlate with urine flow, in the present case, so the tubular re-absorption may be negligible in normal rats. Consequently, extrarenal clearance of exogenous creatinine may be occurring. This is of particular interest because no extrarenal elimination of creatinine following intravenous administration has previously been suggested in the case of normal renal function.

The discrepancy regarding extrarenal elimination of creatinine between the present and previous studies may be attributed to the dose of exogenous creatinine used, the specificity of the analytical method for creatinine determination or the experimental conditions (single injection or steady-state). In the previous reports, the dose of ^{14}C-labeled creatinine used was too small to affect the endogenous level and physiological conditions. Plasma creatinine concentration attained in the present study was much higher. On the other

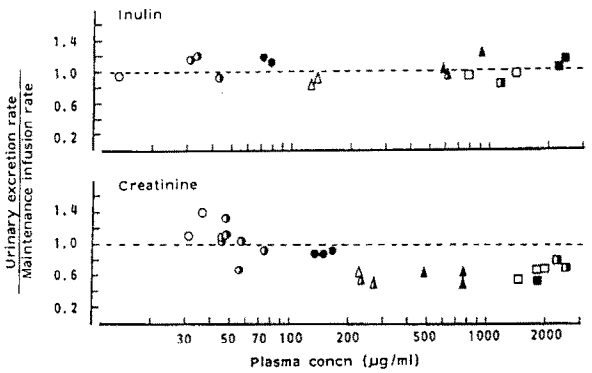


Fig. 3. Relation between the ratio of excretion rate to maintenance infusion rate and plasma concentration for creatinine and inulin. The dotted line indicates the ratio of unity. Each symbol shows the mean value of 5 determinations of a rat after the steady-state concentration was attained. Maintenance infusion rate (mg/min) for inulin: ○, 0.012; ◑, 0.05; ●, 0.1; △, 0.25: ▲, 0.5; ▲, 1; □, 1.14; ◨, 1.5; ■, 2. Maintenance infusion rate (mg/min) for creatinine: ○, 0.075; ◑, 0.015; ●, 0.375; △, 0.75; ▲, 0.965; ▲, 1.5; □, 3; ◨, 4.5; ■, 6.

hand, the analytical method used in the present study is specific for creatinine determination. The discrepancy between our results and Watanabe et al.'s (1981a) might be due to the difference of experimental conditions or analytical method.

As can be seen in Fig. 3, the ratio of urinary excretion rate to maintenance infusion rate was 1.029 ± 0.055 (mean ± S.E.) at plasma concentrations below 200 μg/ml, but when the plasma concentration was increased further, this ratio declined to 0.621 ± 0.027. Jones and Burnett (1972) suggested that in rats that as the plasma level increased to 15 μg/ml, as it might be in cases of uremia, the quantity of creatinine available for gastrointestinal tract secretion or diffusion might become significant, but at the normal serum level of 10 μg/ml this phenomenon was negligible. Hankins et al. (1981) reported a similar threshold effect in man, i.e. the extrarenal clearance appeared when plasma creatinine concentration was higher than 6 μg/ml. In the present study, the ratio of excretion rate to maintenance infusion rate was lower than unity at plasma concentrations above 200 μg/ml, and this is considered to be due to extrarenal clearance, since the results in Fig. 4 suggested that tubular re-absorption was absent. Although the reason for the different ratios at lower and higher plasma concentrations is not clear, the results may reflect tubular secretion at the lower concentrations, and the occurrence of significant extrarenal clearance at the higher concentration (threshold effect), because exogenous

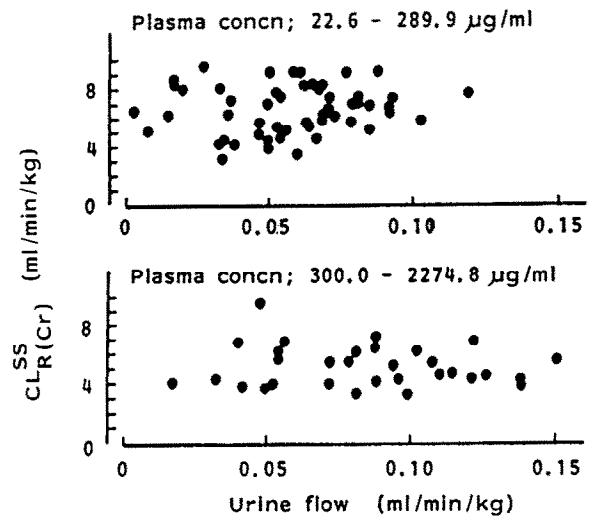


Fig. 4. Relation between the exogenous creatinine clearance and urine flow.

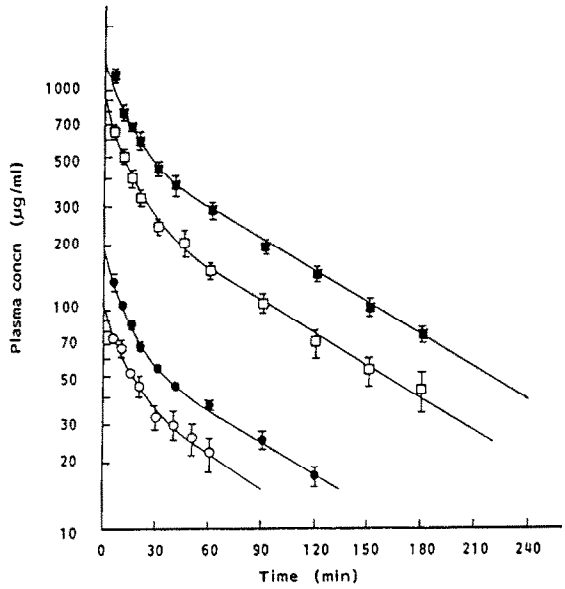


Fig. 5. Plasma concentration–time course of exogenous creatinine following bolus intravenous injection. Each point is the mean ± S.E. The curves were computer-fitted. Doses (mg/kg): ○, 25; ●, 50; □, 200; ■, 400.

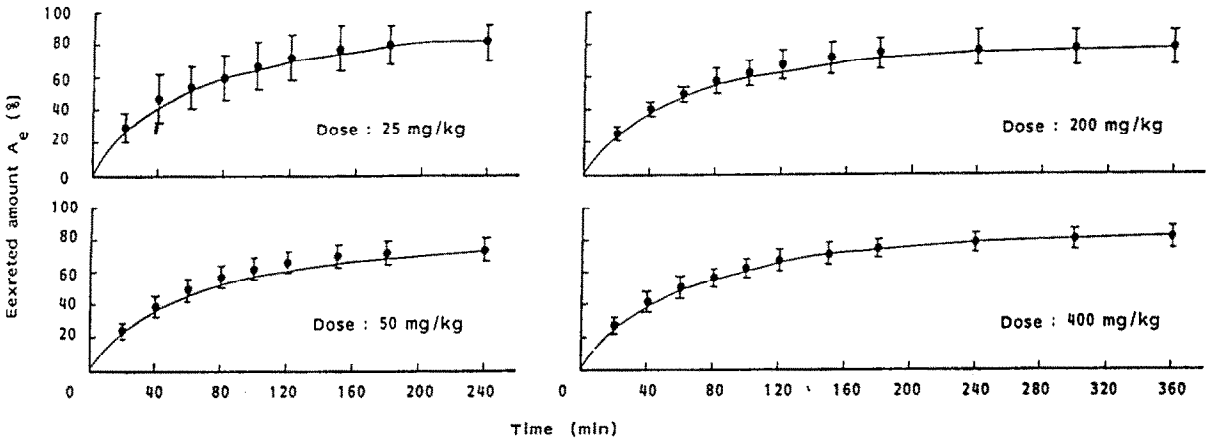


Fig. 6. Cumulative urinary excretion of exogenous creatinine following bolus injection. The curves were computer-fitted.

creatinine was secreted at plasma concentrations below 300 μg/ml and the process was saturated above this concentration.

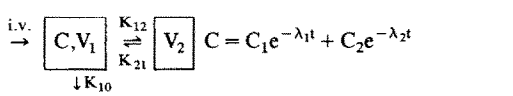
In view of the possible occurrence of extrarenal clearance as well, careful consideration of the disposition of exogenous creatinine is required. Figs.

TABLE 3

PHARMACOKINETIC PARAMETERS OF EXOGENOUS CREATININE FOLLOWING BOLUS INTRAVENOUS INJECTION [a] (mean ± S.E., $n = 5$)

Parameters	Dose (mg/kg)			
	25 [g]	50 [h]	200	400
C_1 (μg/ml)	54.3 ± 10.7	123 ± 15.7	580 ± 54.4	760 ± 30.6
C_2 (μg/ml)	46.6 ± 4.2	68.5 ± 3.7	310 ± 31.1	554 ± 69.3
λ_1 (min^{-1})	0.0941 ± 0.0114	0.1056 ± 0.0081	0.0958 ± 0.0040	0.0880 ± 0.0008
λ_2 (min^{-1})	0.0124 ± 0.0018	0.0112 ± 0.0014	0.0115 ± 0.0011	0.0111 ± 0.0008
k_{10} (min^{-1})	0.0238 ± 0.0053	0.0261 ± 0.0032	0.0270 ± 0.0014	0.0227 ± 0.0014
k_{12} (min^{-1})	0.0324 ± 0.0078	0.0450 ± 0.0034	0.0392 ± 0.0008	0.0330 ± 0.0028
k_{21} (min^{-1})	0.0502 ± 0.0045	0.0456 ± 0.0048	0.0411 ± 0.0044	0.0435 ± 0.0049
V_1 (ml/kg)	250 ± 18.4	267 ± 22.7	230 ± 17.0	310 ± 19.4
V_{SS} (ml/kg) [b]	409 ± 27.0	534 ± 35.9	459 ± 33.7	552 ± 44.1
AUC × 10^3 (μg/ml) [c]	4.63 ± 0.88	7.46 ± 0.52	33.5 ± 3.68	58.6 ± 4.20
CL (ml/min/kg) [d]	5.77 ± 0.96	6.81 ± 0.50	6.28 ± 0.72	6.97 ± 0.52
CL_R (ml/min/kg) [e]	4.92 ± 1.45	5.10 ± 0.35	4.48 ± 0.24	5.66 ± 0.72
CL_{NR} (ml/min/kg) [f]	1.03 ± 0.61	1.70 ± 0.62	1.83 ± 0.83	1.31 ± 0.44

[a] These parameters were analyzed based on the following two-compartment open model:



[b] $V_{ss} = (1 + k_{12}/k_{21})V_1$.

[c] Area under the plasma concentration (AUC) was calculated by the trapezoidal rule to the last data point and residual area from the last data point to infinite time was calculated by dividing the last concentration by λ_2.

[d] Total plasma clearance (CL) was calculated as the dose divided by AUC.

[e] Renal clearance (CL_R) was calculated as CL multiplied by cumulative fraction of those excreted into the urine (A_e).

[f] Non-renal clearance (CL_{NR}) was calculated by subtracting CL_R from CL.

[g] Number of rats used to data analysis were 3 due to incomplete urine collection.

[h] Number of rats used to data analysis were 4 due to incomplete urine collection.

5 and 6 show plots of plasma concentration–time course and cumulative urinary excretion (percent) following bolus intravenous administration of 25, 50, 200 and 400 mg/kg of creatinine. Table 3 lists the pharmacokinetic parameters; C_1 and C_2 are the coefficients of the first and second exponential terms and λ_1 and λ_2 are the exponents in the two exponential equations describing the plasma concentration following bolus intravenous administration. Although λ_2 was compatible with the value reported by Watanabe et al. (1981a), the other values were different. This is mainly due to the difference of sampling schedule. The plasma clearance (CL) and urinary excretion ratio (A_e) were almost constant at all doses (no statistical differences for CL and Ae among 4 doses), suggesting that the disposition of exogenous creatinine is linear, but the urinary excretion ratios were slightly lower than Watanabe et al.'s values.

However, we described above that exogenous creatinine was secreted at plasma concentrations below 300 μg/ml and the secretion was saturated above this concentration. In both Figs. 5 and 6, this saturated phase was not seen, in accordance with the results in Table 3. This was mainly considered to be due to a smaller contribution of tubular secretion to the total elimination of creatinine (ca. $< 10\%$). In the standard renal clearance measurement, constant urine flow could be obtained by constant infusion of the drug solution with no disturbance of distribution. On the other hand, in the present bolus injection study the tubular secretion might be masked by the distribution.

In the present study the contribution of extrarenal clearance to overall clearance of exogenous creatinine was 37.9% (at plasma concentrations above 200 μg/ml) in the case of standard renal clearance measurement and 21.1% in the case of the bolus intravenous injection. These values were calculated by subtracting the ratio of urinary excretion rate to maintenance infusion rate from unity in the former case and by subtracting accumulation fraction of dose excreted eventually into the urine from unity in the latter case. In addition, there were statistical differences ($P < 0.05$) between the values of CL_R and CL in Table 3 for 50 and 200 mg/kg.

This difference may be explained partly by the partial contribution of tubular excretion for bolus injection study, since for the standard renal clearance study, the tubular secretion of creatinine at plasma concentration above 200 μg/ml is almost negligible, as shown in Fig. 2. The existence of extrarenal clearance of exogenous creatinine was suggested in rats with normal renal function in both cases. Consequently, plasma clearance of exogeneous creatinine cannot be necessarily regarded as a valid index of GFR.

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