ANTIBIOTIC CONCENTRATION IN SUBCELLULAR FRACTIONS OF THE KIDNEY

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Ampicillin, cephalothin, minocycline, and kanamycin were injected intraperitoneally at a dose of 100 mg/kg into male Wistar rats weighing 200~250 gm. The drugs were chosen as representatives of penicillins, cephalosporins, tetracyclines, and aminoglycosides respectively. Kidney homogenates were separated into a supernatant fraction consisting primarily of cytoplasm and microsomes, and a sediment fraction consisting primarily of lysosomes and mitochondria. Antibiotic concentrations were measured in both fractions. Minocycline and kanamycin showed a tendency to accumulate in the sediment fraction for a long time, whereas ampicillin and cephalothin did not. This pharmacokinetic study on subcellular fractions may provide an interesting and valuable approach to predicting functional and morphological kidney changes caused by antibiotics.

Introduction

Most of the many antibiotics in clinical use today are concentrated and excreted by the kidney. These heterogenous substances may disturb kidney function during the excretion process, and in some cases cause clinical renal failure. We designed a method to assay the activity of antibiotics in kidney fractions[6], which is an indication of subcellular pharmacokinetics.

Materials and Methods

Antibiotics examined in this study were ampicillin, cephalothin, kanamycin, and minocycline.

Sixteen male Wistar rats, weighing between 200 and 250 gram were used for each experiment. They were allowed free access to water and chow throughout the study. All received a single injection of antibiotic in a dose of 100 mg per kilogram of body weight. Animals were sacrificed in groups of four each at 30 minutes, 60 minutes, 120 minutes, and 180 minutes after drug administration. Cooled 1 mM EDTA-0.3 M sucrose solution (pH 7.0) was used for the preparation of homogenate[10]. The solution had been dysinfected and the following procedures were performed under aseptic conditions.

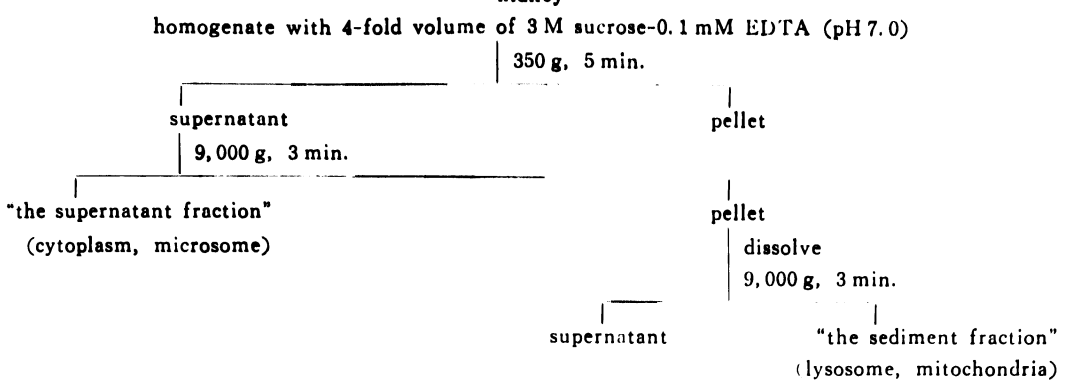
Each animal was placed on the table under ether anesthesia and the peritoneal cavity was opened. The aorta was punctured and blood was withdrawn into a heparinized syringe. After perfusion with the above-mentioned solution both kidneys were removed.

Fibrous capsules and hilar structures were removed and each kidney was weighed and sliced. Four ml of solution per gram of wet tissue was then added and the tissue was homogenated in a Potter homogenizer with a teflon pestle.

The homogenate was centrifuged at 350 g for 5 minutes to remove the undestroyed cells and nuclei[10]. The supernatant was then centrifuged at 9,000 g for 3 minutes. The supernatant of this centrifuge was collected as "the supernatant fraction". The pellet was dissolved to the original volume of the solution and centrifuged again at 9,000 g for 3 minutes. The supernatant was discarded. The pellet was dissolved into the same volume and used as "the sediment fraction" (Figure 1). The supernatant fraction is consisted primarily of cytoplasm and microsomes while the sediment fraction was lysosomes and mitochondria[10].

Antibiotics were assayed by an agar plate diffusion method. The organism employed for bioassay was *Staphylococcus aureus* FDA 209 P, JC-1 which had been grown in heart infusion broth for 18 hours. Two ml of the suspension was incubated into 100 ml of melted modified Mueller-Hinton

Fig. 1 The fractionating procedure



agar and 4 ml of this seeded agar was poured into a petri dish with a diameter of 6 cm which had been previously seated with 4 ml of agar. Two stainless-steel cylinder cups of 8 mm in diameter were placed on each plate. The kidney fractions were frozen and dissolved to disrupt the cell organelles. Known amounts of the antibiotics multiplied with 1 mM EDTA-0.3 M sucrose solution was used for reference standard solutions. They were dissolved into an equal volume of 1% Triton-X. Each cup was filled with either a reference standard antibiotic solution or sample and incubated for 18 hours at 37℃, following which time the diameter of the zonal growth inhibition was measured. Assays were made in a triplicated or more.

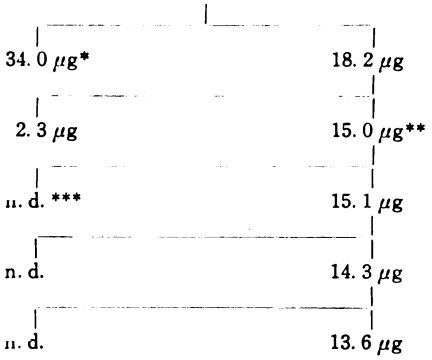
The mean of the measurements for each sample or standard was calculated and a standard curve was drawn with four standard points on semi-logarithmic paper. The values of fractions were calculated as antibiotic activity per gram of wet weight of the kidney.

Concentrations were plotted against time on semi-logarithmic coordinates. The levels declined after injection in a monoexponential fashion. Least-square regression analysis was made and half-life ($t_{1/2}$) was calculated.

Washout test of antibiotic given intraperitoneally: A kidney homogenate from a rat injected intraperitoneally with amikacin 200 mg/kg was centrifuged for 5 minutes by 350 g, and then for 3 minutes at 9,000 g. The pellet was dissolved in the original volume of the solution and again centrifuged for 3 minutes at 9,000 g. The dissolution and centrifugation were repeated for

Fig. 2 Washout of antibiotics from the sediment fraction

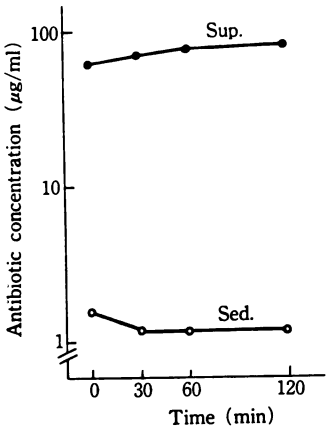
A kidney from a rat received amikacin was homogenated and centrifuged



* The supernatant fraction value
** The sediment fraction value
*** Not detected

Fig. 3 Stability of antibiotics

Homogenate containing 1,000 μg/ml of tetracycline was incubated at 37℃. Fractionation was done at various times thereafter.



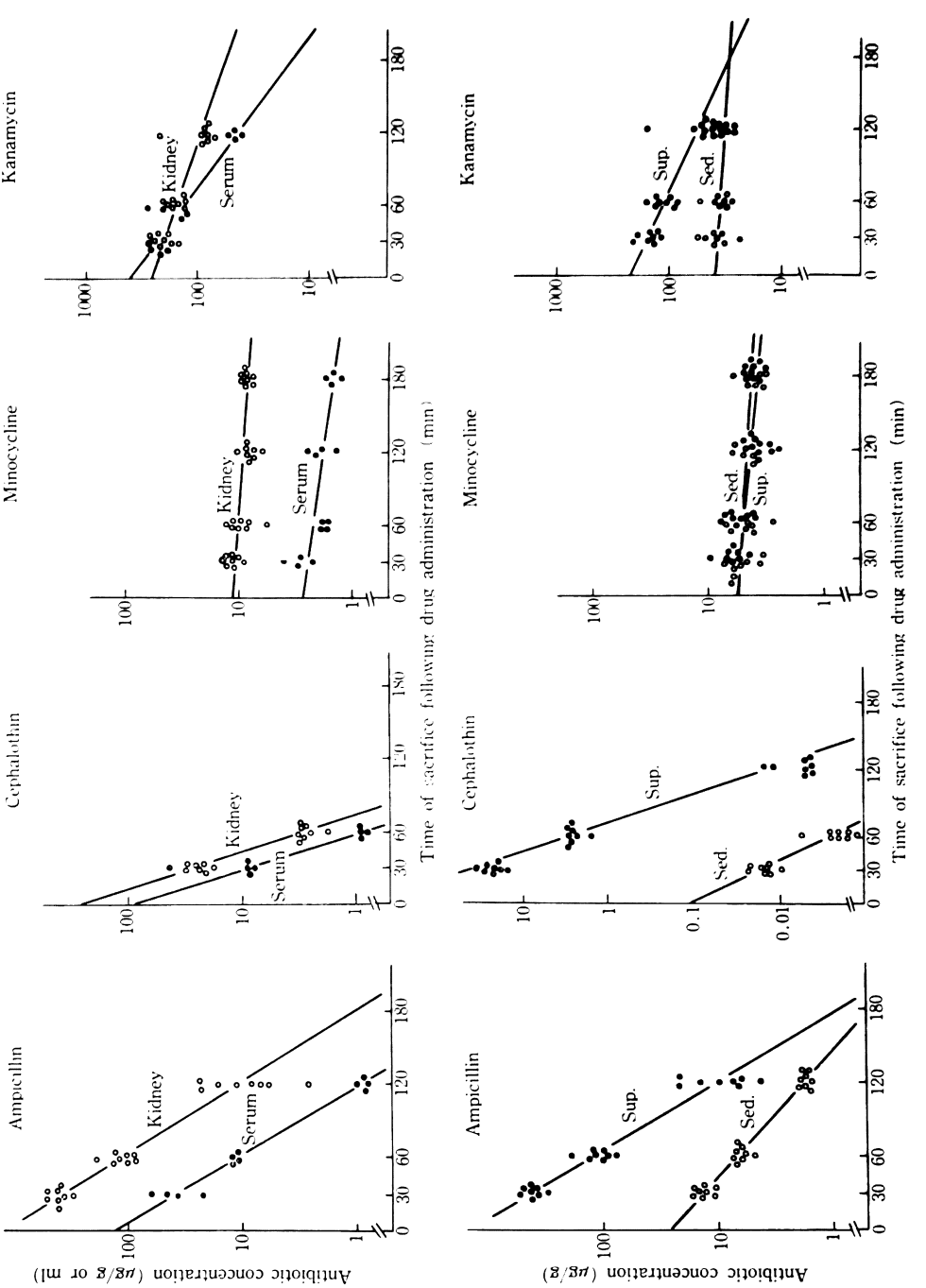


Fig. 4 Time course of antibiotic activity after intraperitoneal injection
100 mg/kg of antibiotics was injected to male Wistar rats. The vertical axis means the antibiotic activity equivalent to μg per ml of plasm or gm of wet kidney tissue.

several times.

Incubation of antibiotic with homogenate *in vitro*;

Two mg of tetracycline was added to 20 ml of the supernatant fraction obtained from kidney homogenate by centrifuging for 5 minutes at 350 g. The fraction contained all cell components except nuclei. The mixture was incubated at 37℃. Four ml of the mixture were withdrawn serially. Then the samples were divided into fractions and assayed.

Results

As is shown in Figure 2 the concentration of intraperitoneally injected amikacin in the sediment fraction is relatively stable on repeated washout if the pellet was once washed with the buffer. For this reason we used the value obtained after only a single washing as the value of the sediment fraction.

Figure 3 shows that there was no detectable change in activity of antibiotics in either fraction at 37℃. This indicates that the antibiotics are stable in the homogenate and that variation in the time required to complete the procedure does not affect the result.

Antibiotic activities after a single injection are shown in Figure 4.

Ampicillin; The blood concentration of ampicillin falls rapidly with the kidney concentration falling in parallel with the blood concentration. The concentrations in the supernatant and in the sediment do not, however, move in parallel. This indicates a tendency of ampicillin to accumulate in the sediment fraction in comparison with cephalothin.

Cephalothin; Concentrations of cephalothin in both blood and kidney decrease more rapidly than ampicillin. The disappearance rate of the antibiotic in the supernatant and that in the sediment are similar, and the concentration ratio of supernatant to sediment is about 1,000 vs. 1. These findings suggest that cephalothin has no affinity to the sediment fraction.

Minocycline; Excretion of minocycline is prolonged. The concentrations in the blood and the kidney did not decrease as rapidly as ampicillin or cephalothin. The levels in both supernatant and sediment remain ralatively stable over the course of the experiment.

Kanamycin; Blood level decreases gradually. Kidney level decreases more slowly. The kanamycin concentration in the supernatant fraction decreases gradually but that in the sediment fraction remains stable.

Half-lives of antibiotics in blood and fractions are described in Table 1.

Discussion

Nephrotoxicity is an important complication of antibiotic therapy. Since approximately 24 per cent of the cardiac output at rest passes through the kidney each minutes, they are exposed to high concentrations of antibiotics through both glomerular filtration and tubular secretion[2].

Among the various classes of antibiotics, aminoglycosides are most toxic. They produce strikingly high concentrations especially in the cortex which results in an intrarenal gradient pattern from the cortex to medulla[9] The accumulation and subsequent toxic changes are most prominent in the proximal tubules within the renal cortex[3]. Electron microscopic examinations on the kidney after gentamicin treatment consistently show an accumulation of drug in lysosomes. The proximal tubular cells have numerous large lysosomes containing concentric membranous whorles or laminations called myeloid bodies[1,3,5,7,8,9,13].

Table 1 Half-life of antibiotics

	Ampicillin	Cephalothin	Minocycline	Kanamycin
Serum	0.274* (0.249～0.305)**	0.149 (0.143～0.155)	3.381 (2.071～ 9.202)	0.637 (0.564～0.733)
Kidney	0.290 (0.264～0.320)	0.155 (0.138～0.175)	6.469 (3.986～17.150)	1.405 (1.062～2.075)
Sup.	0.277 (0.250～0.309)	0.123 (0.116～0.129)	5.089 (2.970～17.760)	1.047 (0.804～1.450)
Sed.	0.514 (0.480～0.554)	0.177 (0.145～0.226)	10.849 (4.443～24.556)	7.348 (1.062～2.075)

* hours

** 95% confidence limit

Tetracycline is an another nephrotoxic antibiotic especially in patients with pre-existing renal diseases. Accumulated tetracycline fluorescein has been observed in mitochondria[4]. To understand the influence of these antibiotics on renal function, we initially attempted to determine the antibiotic affinity to subcellular organelles.

The accumulated drugs per se do not necessarily harm the renal cells. Nephrotoxic effects may be the result of complicated intracellular interactions. Nevertheless, one may consider that those antibiotics which interact with and accumulate in the large organelles are potentially nephrotoxic.

VERA-ROMAN[13], in a study of gentamicin nephrotoxicity, measured the levels of the antibiotic and found significant quantities of gentamicin within various intracellular organelles. Few other data are available on antibiotic concentrations in subcellular fractions of the kidney. Although differences in the elimination rates from the serum and the verious organs has been repeatedly reported, available data do not provide information on the separate kinetics of antibiotics in the renal subcellular fractions.

We have tried to establish a method of determining the degree of antibiotic accumulation in the subcellular level. A preliminary report of this work has appeared previously[6]. It is an application of the method developed by MAUNSBACH[10] who noted that it is possible to separate brush border, lysosome and mitochondria. From a theoretical view point, it is desirable to determine separatedly the levels of antibiotics in nuclear, mitochondrial, lysosomal, microsomal, and cytoplasmic fractions. While this may be theoretically possible, on a practical level a certain amount of cross contamination is inevitable, regardless of the amount of care taken.

Another point that must be considered is the possibility that the antibiotics are, at least in part, permeable across the membrane of the organelles, which means that drug may pass from one fraction to another during the procedure. Too much complicated, time-consuming procedure must be avoided.

We sought an easily reproducible method to evaluate and compare the accumulation of antibiotics in the kidney. Stable values must be obtained in the repeated examinations. Figure 3 shows the stability of antibiotics during the experiment and also the reproducibility of this fractionating procedure.

The supernatant fraction consists primarily of cytoplasm and microsomes. By perfusing the kidney before removal from the animal we were able to minimize the contamination with blood and urine. The sediment fraction contains the large organelles such as lysosomes, mitochondria, brush borders, and myeloid bodies. Accumulation of antibiotics in the sediment fraction is not a simple process.

Aminoglycosides may accumulate in lysosome whereas tetracyclines may concentrate in the mitochondria[4]. The mechanism by which the accumulation occurs may be the result of various complicated processes such as attachment, passive diffusion, and active penetration into the organelles, or pino- and endo-cytosis of lysosomes.

We are studying a variety of antibiotics using the method described above. Ampicillin, cephalothin, minocycline, and kanamycin were presented in this paper. These antibiotics were selected as representatives of the penicillin, cephalosporin, tetracycline, and aminoglycoside classes of antibiotics and are widely used in the treatment of genitourinary infections. A characteristic kinetic pattern was obtained for each group of antibiotic.

A bioassay was used in this study which can not detect any of the inactivated or metabolised antibiotics. Previous reports, however, suggest that the concentration of inactive substances correlates as well with the concentrations of active forms[11].

Investigators have suggested that for ensuring effective treatment it is important to maintain the antibiotic concentration in blood and tissue above the minimum-inhibitory concentration for bacterial growth[12].

High antibiotic levels in blood and the supernatant fraction with low level in the sediment fraction may be a desirable characteristic for therapeutic antibiotics. In this regard our method provides a valuable index. The finding we observed are consistent with previous pharmacological, biological, toxicological, and morphological findings[1~5,7~9,11~13].

In conclusion this paper shows a technique for studying pharmacokinetics on a subcellular level. Although the antibiotic activities were bioassayed in this study, it may be possible to use other

assay methods such as radioactive tracer method, radioimmunoassay, enzyme immunoassay and high speed liquid chromatography. We are using these alternative methods in part. More detailed analyses of cellular fractions may be attainable in future.

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腎細胞分画の抗生物質濃度

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ウィスター系ラットに体重1 kg 当り 100 mg の抗生物質を腹腔内注射し，時間を追って腎をとりだし，リソゾームやミトコンドリアを主体とした沈渣分画と，サイトプラスマやミクロゾームを主体とした上清分画に分けて，各分画の抗生物質濃度を測定した。

抗生物質はペニシリンとしてアンピシリン，セファロスポリンとしてセファロシン，テトラサイクリンとしてミノサイクリン，アミノ配糖体としてカナマイシンを選んだ。アンピシリン，セファロシンでは血中濃度の下降に伴って各分画濃度も下降した。ミノサイクリンは血中濃度も腎の両分画濃度も高値を示した。カナマイシンは血中濃度が下降していくにもかかわらず沈渣分画中の濃度はほとんど下降しなかった。本研究は細胞分画レベルにおけるファルマコカイネティクスの追求が可能であることを示したもので，抗生物質が腎に及ぼす影響を追求する手段として興味がもたれる。