Usability of Repeated Liver Biopsies for Estimation of Drug Residue in Calf Liver

Yuji TAKAHASHI, Masaaki IIDA, Kenji ODA, and Yasumasa KIDO

Research Institute for Animal Science in Biochemistry and Toxicology, 2277 Shimokuzawa, Sagami-hara, Kanagawa 229, Japan

(Received 6 March 1986/Accepted 26 May 1986)

ABSTRACT. Usability of repeated liver biopsies was examined for the estimation of drug residual level in the calf liver. The liver biopsy was carried out under ultrasonic observation. Twenty four calves were given 20,000 units of benzylpenicillin (PC-G) per kg body weight. Twelve calves were biopsied and sacrificed within 2 hrs after the biopsy at 3 and 7 days after the administration, and 7 calves were sacrificed at 3, 7, 14 and 21 days after the administration to take the liver samples (the serial sacrifice group). Five calves were biopsied repeatedly 15 times during 21 days following the administration to obtain the liver parenchyma (the repeated biopsy group). There was no significant difference ($p=0.05$) in PC-G concentrations between the biopsy samples and the samples obtained by sacrifice immediately after the biopsy, nor between the samples of "the repeated biopsy group" at 3 and 7 days after the administration and those of "the serial sacrifice group" at the corresponding points. Pharmacokinetic parameters and the decay lines calculated from the mean concentrations in both groups were almost identical. These results suggest that the estimation of drug residual level in the calf liver taken by repeated biopsies in place of the serial sacrifice is useful to determine the withdrawal time of drugs remaining predominantly in the liver.—**KEY WORDS:** benzylpenicillin, calf liver, drug residue, liver biopsy.

Jpn. J. Vet. Sci. 48(5): 873–878, 1986

Estimation of the withdrawal time of a drug needs a large number of experimental animals when it is performed by the method of the serial sacrifice of the animals administered a drug with a clinical dosage and several times higher than that [1, 4, 5]. It also requires a great deal of labor and cost to estimate exactly the withdrawal time.

Recently, the pharmacokinetic method was applied to the determination of the withdrawal time of a drug of which concentration in tissue decreases parallel to that in blood [7, 8]. However, this method can not be applied to a drug of which concentration in tissue does not correlate with that in blood. To establish the proper withdrawal time for such a drug, the direct measurements of the residual level in target organ are necessary at the suitable intervals following the drug withdrawal. It was already

reported that the repeated renal biopsies were useful to estimate the withdrawal time of drugs remaining in the pig and calf kidneys [14, 15].

In the present study, it was found that the repeated liver biopsies were useful for the estimation of the withdrawal time through the studies on the usability and reliability of the biopsy samples for the measurement of benzylpenicillin (PC-G) residual level in the calf liver.

MATERIALS AND METHODS

Animals and experimental protocol: Duopen (Glaxo Co., Ltd., England), a mixed preparation of PC-G procaine and PC-G benethamine in the ratio of 1:1, was given subcutaneously (6 calves) or intramuscularly (18 calves) with a dose of 20,000

units/kg body weight to 24 calves (body weight: 186 ± 41 kg, mean $\pm$ S.D.). The 13 calves were sacrificed at 3 (4 calves), 7 (5 calves), 14 (2 calves) and 21 (2 calves) days after the intramuscular administration to obtain the liver samples (the serial sacrifice group). The 5 calves were repeatedly biopsied at one day before the intramuscular administration, and 0.5, 1, 2, 4, 8, 12 hrs, 1, 2, 3, 4, 7, 9, 11, 14 and 21 days after the administration (the repeated biopsy group). The 6 calves administered PC-G subcutaneously were biopsied and sacrificed within 2 hrs after the biopsy at 3 and 7 days after the administration to obtain the biopsied and sacrificed liver samples. And also, the 6 calves of "the serial sacrifice group" that were sacrificed at 3 and 7 days after the administration were biopsied within 2 hrs before the sacrifice.

Biopsy method: The liver biopsy was performed under ultrasonic observation as reported earlier [16]. The scan with the ultrasonic apparatus (SSD-248, Aloka Co., Ltd.) was carried out on the right thoracic wall of the calf which was loosely restrained with a rope in the narrowed stanchion to search for the suitable biopsy site on the wall where the liver located, and where the bile and large vessels were not observed just under the site. Ten ml of 1 percent procaine was subcutaneously and intramuscularly injected into the biopsy site. A 1 to 3 cm skin incision was made and the biopsy needle with a stylet (250 mm long; 4.8 mm diameter, Fujihira Kogyo Co., Ltd.) was firmly thrust into the thorax. The stylet was moved upward about 10 cm and the needle was inserted into the liver to obtain the parenchyma. After the removal of the needle, an antiseptic (povidone-iodine solution) was applied to the incision site. When the biopsy was repeated in the same body, the biopsy site was changed to the portion more than 40 mm apart from the site biopsied before.

Preservation of tissue samples: The biopsy

sample was weighed immediately after the biopsy and stored in 5 ml of methanol at -25°C until the assay.

About 100 g of the right marginal portion of the liver was taken from the animals of "the serial sacrifice group" and frozen at -25°C until the assay.

Drug assay: Five or 20 ml of methanol was added to 100 mg or 2.0 g tissue sample, respectively, and homogenized. After centrifugation at 3,000 rpm for 20 min, the supernatant was evaporated to dryness under reduced pressure at 40°C . The residue was dissolved in 0.5 ml of phosphate buffer (51 mM KH_2PO_4 , and 42 mM Na_2HPO_4 , pH 6.0) in the case of 100 mg sample and in 10 ml in the case of 2.0 g sample. The PC-G concentration was determined by microbioassay with *Bacillus stearothermophilus* var. *calidolactis* C-953 [10].

Stability test of PC-G in a small amount of sample: Homogenized liver samples retaining PC-G were weighed (approximately 100 mg) and stored in 5 ml methanol at -25°C . The triplicate determination was performed at 0, 7, 14, 21, 28 and 42 days during the storage.

Distribution test of PC-G in calf liver: About 100 g of the left and right marginal, and central portions, and surroundings of the portal vein and bile duct were obtained from the 6 livers retaining PC-G. The samples were sliced finely with a knife and mixed well. PC-G concentrations in 2.0 g of liver samples were determined.

Statistics: Statistical evaluation of the analytical results between the 100 mg and 2.0 g samples, and between the biopsied and sacrificed samples were computed by Two-Way Layout ANOVAR [11]. The results of the stability and distribution tests were also evaluated by the same method. Statistical analysis of PC-G concentrations between the samples of "the repeated biopsy group" and those of "the serial sacrifice

group" obtained at 3 and 7 days after the administration was performed using unpaired Student's *t* test [12]. Pharmacokinetic parameters were calculated by Damping Gauss-Newton method [13]. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

All of the 91 biopsies was successfully performed without any clinical manifestation even if the biopsy was repeated 16 times for 22 days in the same animal. The average weight of the biopsy samples obtained with

Table 1. Reproducibility of the analytical method of PC-G concentration

Animal No.	n ^{a)}	PC-G concentration (U/g)		
		mean	S.D.	C.V.(%)
19 ^{b)}	10 ^{c)}	2.57	0.27	10.5
14 ^{c)}	10	0.246	0.009	3.7
28 ^{d)}	10	0.076	0.006	7.9

a) Number of samples.

b, c, d) The animals were sacrificed at 3, 7 and 14 days after the administration, respectively.

e) Each 100 mg sample was used for analysis.

Fig. 2. Stability of PC-G in the 100 mg liver sample stored in 5 ml of methanol at -25°C . Each point represents the mean $\pm$ S.D. of triplicate determinations.

the biopsy needle for the horse liver was 101 ± 44 mg ($n=91$, mean $\pm$ S.D.).

The analytical method applied to this study was able to detect the residual level of 0.03 units/g when 100 mg of tissue was used for the analysis, and recover $90 \pm 7\%$ ($n=5$) of PC-G added to 100 mg of a control

Fig. 1. Comparison of PC-G concentrations between 100 mg and 2.0 g samples obtained from the animals treated with PC-G. The regression equation calculated by the least squares method was $y=1.04x-0.01$. The correlation coefficient was 0.99.

Fig. 3. Residual level (U/g) of PC-G in various portions of a calf liver. A: Surroundings of portal vein and bile duct. B, C: Left and right marginal portions. D: Central portion.

sample. Under the same analytical condition as the recovery test, the coefficient of variation was $\leq 10.5\%$ (Table 1). The residual level of PC-G indicated no significant difference between the 100 mg and 2.0 g of liver samples retaining PC-G (Fig. 1). From the result of the stability test, it was exhibited that PC-G in the 100 mg liver sample was stable in 5 ml of methanol for more

Fig. 4. Comparison of PC-G concentrations between the samples taken by biopsy and by sacrificing the animals within 2 hrs after the biopsy. ○, △: samples taken at 3 and 7 days after the subcutaneous administration, respectively, ●, ▲: samples taken at 3 and 7 days after the intramuscular administration, respectively. The regression equation calculated by the least squares method was $y = 1.04x - 0.07$. The correlation coefficient was 0.97.

than 1 month at -25°C (Fig. 2). The distribution test indicated that PC-G injected to a calf distributed evenly in the various portions of the liver, namely, the marginal and central portions and surroundings of the portal vein and bile duct (Fig. 3). PC-G concentrations in the biopsy samples and the samples obtained by sacrifice immediately after the biopsy were almost same, suggesting the uniform distribution of PC-G in the liver tissue (Fig. 4).

There was no significant difference between the PC-G concentrations in the sam-

Fig. 5. The residual concentrations of PC-G in calf livers after the intramuscular administration. The open circles (○) and the filled circles (●) represent the means of "the repeated biopsy group" and "the serial sacrifice group", respectively. S.D. is shown by vertical bar. The number of the samples of "the repeated biopsy group" at each point except at 21 days were 5. a) $n=4$, b) $n=5$, c) $n=2$.

Table 2. Residual levels of PC-G in calf livers of "the repeated biopsy group" and "the serial sacrifice group"

	Days after administration			
	3	7	14	21
"Repeated biopsy group"	$1.30 \pm 0.72^{\text{a}}(5)^{\text{b}}$	$0.45 \pm 0.23(5)$	$0.07 \pm 0.05(5)$	$< 0.03(4)$
"Serial sacrifice group" ^{c)}	$1.13 \pm 0.56(4)$	$0.32 \pm 0.19(5)$	$0.04(2)$	$< 0.03(2)$

a) Mean $\pm$ S.D. (U/g).

b) Number of samples.

c) Each 100 mg sample was used for analysis.

Table 3. Pharmacokinetic parameters of PC-G in calf liver

	"Repeated biopsy group"	"Serial sacrifice group"
$C_0(\text{U/g})^{\text{a)}}$	2.90 ^{d)}	2.90
$\alpha(\text{day}^{-1})^{\text{a)}}$	0.27	0.31
$T_{1/2}(\text{day})^{\text{b)}}$	2.6	2.2
$\text{AUC}^{\text{c)}}$	6.70	5.86

a) PC-G concentration in liver = $C_0 e^{-\alpha t}$.

b) Half-life of PC-G in liver.

c) Area under the curve (U·day/g).

d) Calculated from the mean concentration at each time.

ples of "the repeated biopsy group" at 3 and 7 days after the administration and those of "the serial sacrifice group" at the corresponding points (Table 2). The pharmacokinetic parameters and decay lines calculated from the mean concentrations in the livers of both groups were almost identical (Table 3, Fig. 5).

DISCUSSION

For the study on drug residue, it is necessary to establish an adequately sensitive, accurate and reproducible analytical procedure. The criteria on the analytical method are instituted only for the feed additives as the detection limit to be less than 0.1 units/g, the recovery rate to be more than 70%, and the coefficient of variation to be less than 10% [1]. Since the criteria for the detection of drug residue in domestic animals have not been established, the criteria for the feed additives are usually applied to this purpose [6]. In the analytical method of PC-G in this study, it was found that the detection limit was 0.03 units/g, the recovery rate was over 90%, and the coefficient of variation was 3.7–10.5%.

It was considered that the analytical error would occur during the analytical process of the biopsy sample since the amount was very small. Therefore, the concentrations of

PC-G in the 100 mg sample obtained by the biopsy and 2.0 g sample obtained from the sacrifice animal were compared. The result (Fig. 1) indicated that the sample amount would not influence the analytical values.

It was reported that the potency of PC-G in bovine plasma stored at -30°C for 2 months decreased about 25% while it was kept well in milk [9]. In this study, the loss of potency of PC-G in the liver was not observed for more than 1 month when the tissue sample was stored in methanol at -25°C (Fig. 2), suggesting that PC-G in the biopsy sample stored under the condition mentioned above was stable enough for the reliable determination of the residual level in the liver.

It was also clarified that the biopsy sample taken from any portion of the liver was applicable to determine PC-G concentration because of its uniform distribution in the liver (Fig. 3).

It has been reported that the concentrations of lipid in calf liver and copper in sheep liver obtained by biopsy agreed well with those in liver taken by sacrifice [2, 3]. However, there is no report on the drug residue in liver samples obtained by repeated biopsies from the same body. In the present results, it was shown that the samples obtained by biopsy and by sacrifice immediately after the biopsy contained similar residual levels of PC-G (Fig. 4). And also, no difference ($p=0.05$) was observed between the intramuscular and subcutaneous administrations (Fig. 4). Further, PC-G concentrations did not differ ($p=0.05$) between the samples of "the repeated biopsy group" at 3 and 7 days after the administration and those of "the serial sacrifice group" at the corresponding times (Table 2). The residual level of PC-G in the calf liver determined by the repeated biopsies was almost identical with that obtained by the conventional method (Table 3, Fig. 5).

In conclusion, the present results suggest that the repeated liver biopsies could be applicable to the estimation of the withdrawal time of drugs remaining in the calf liver, in place of the conventional method and also available to the pharmacokinetic analysis in the calf liver.

REFERENCES

1. Director General, Bureau of Animal Industry, Ministry of Agriculture and Forestry, and Director General, Fisheries Agency. 1977. Studies on Residue. pp. 61-65. In: Establishment of Standards for Evaluation of Feed Additives, Japan Scientific Feed Association, Tokyo (in Japanese with English summary).
2. Donald, G. E., Paull, D. T., Langlands, J. P. 1984. Liver biopsy as a technique for assessing copper status of sheep. *Aust. Vet. J.* 61: 121-122.
3. Gaal, T., and Husveth, F. 1983. Comparison of the liver biopsy sample and the "whole liver" in respect of lipid content and fatty acid composition of lipids. *Acta Vet. Hungarica, Abstract.* 31: 51-56.
4. Hayama, T. 1985. Drug residue in food-producing animals. pp. 54-59. In: Veterinary Pharmacology, Yookendo, Tokyo (in Japanese).
5. Jones, L. M., Booth, N. H., and McDonald, L. E. 1977. Drug and chemical residues in the edible tissues of animal. In: Veterinary Pharmacology and Therapeutics 4th ed. (Booth, N. H. ed.), AMES.
6. Kido, Y. 1985. Residual Analyses. pp. 121-122. In: Residual Analyses of Feed Additives and Drugs for Food-producing Animals (Yonemura, T., Uchiyama, A., Hayama, T., and Kido, Y. eds.), Kindaishuppan, Tokyo (in Japanese).
7. Kokue, E. 1985. Pharmacokinetics. pp. 51-73. In: Residual Analyses of Feed Additives and Drugs for Food-producing animals (Yonemura, T., Uchiyama, A., Hayama, T., and Kido, Y. eds.), Kindaishuppan, Tokyo (in Japanese).
8. Mercer, H. D., Baggof, J. D., and Sams, R. A. 1977. Application of pharmacokinetic methods to the drug residue profile. *J. Toxicol. and Environ. Health.* 2: 787-801.
9. Molle, G., and Hoffmann, B. 1984. Beobachtungen zur Stabilität der Wirkstoffkonzentrationen verschiedener Antibiotika in Blutplasma und Milch vom Rind. *Dtsch. Tierärztl. Wschr.* 91: 225-227.
10. Ouderkirk, L. A. 1977. Detection of residual penicillins in milk by using a *Bacillus stearothermophilus* disk assay. *AOAC.* 60: 1116-1118.
11. Sakuma, A. 1975. Two-way layout. pp. 44-58. In: Bioassay-Design and Analysis, Univ. Tokyo Press (in Japanese).
12. Sakuma, A. 1975. Comparison of two group. pp. 19-22. In: Bioassay-Design and Analysis, Univ. Tokyo Press (in Japanese).
13. Sakuma, A. 1981. Gauss-Newton Method. pp. 248-255. In: Statistical Methods in Pharmacometrics, Univ. Tokyo Press (in Japanese).
14. Takahashi, Y., Kido, Y., Naoi, M., and Kokue, E. 1985. The serial biopsy technique for estimation of drug residue in calf kidney using gentamicin as a model drug. *Jpn. J. Vet. Sci.* 47: 179-183.
15. Takahashi, Y., Kido, Y., Naoi, M., and Kokue, E. 1985. The estimation of drug residue in pig kidney by repeated biopsy. *Jpn. J. Vet. Sci.* 47: 705-709.
16. Takahashi, Y., Iida, M., Hashizume, M., and Kido, Y. 1986. Safety of repeated biopsies under ultrasonic observation on calves. *Jpn. J. Vet. Sci.* (in contribution).
17. Weber, N. E. 1983. Metabolism and kinetics in the regulation of animal drugs. *J. Anim. Sci.* 56: 244-251.

要 約

反復肝臓生検によるウシの肝臓中薬物残留評価法の有用性：高橋雄二・飯田正明・小田憲司・城戸靖雅（（財）畜産生物科学安全研究所）——ベンジルペニシリン20,000 units/kgを24頭のウシに投与し、5頭については投薬前および投薬後0.5, 1, 2, 4, 8, 12時間, 1, 2, 3, 4, 7, 9, 11, 14および21日に肝臓組織を採取した（反復生検群）。他のウシを投薬後3, 7, 14および21日に屠殺解剖し、それぞれ肝臓組織を得た（逐次屠殺群）。投薬後3および7日に屠殺解剖したウシについて屠殺前2時間以内に生検を行い、肝臓組織を採取した。生検試料と生検直後に屠殺解剖して得た屠体試料中のPC-G濃度の間に有意差（ $p=0.05$ ）は認められず、投薬後3および7日の反復生検群のPC-G濃度と同時点の逐次屠殺群との間にも有意差は認められなかった。また、両群の薬物動態学的パラメーターおよび減衰直線はよく一致した。以上から、ウシ肝臓中の薬物の休薬期間を設定する場合において反復肝臓生検による薬物残留評価法は従来の逐次屠殺法に代わる有用な方法であると考えられた。