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A comparative study of diazepam levels in bone marrow versus serum, saliva and brain tissue

T. Takatori*, S. Tomii, K. Terazawa, M. Nagao, M. Kanamori, and Y. Tomaru**

Department of Legal Medicine, Hokkaido University School of Medicine, Sapporo 060, Japan

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Summary. The distribution of diazepam in biological fluids and tissues of rats was examined 1, 2, 4 and 8 h after intraperitoneal administration by using a radioimmunoassay with specific anti-diazepam antibody. The diazepam levels in serum, saliva, brain and bone marrow decreased over a period of 2 h and levelled off 4 h after administration. The diazepam concentration in bone marrow was much higher than in serum, saliva and brain, suggesting an accumulation of diazepam in this tissue. This indicates that bone marrow could be a very useful material for the detection of diazepam in skeletonized remains. The diazepam concentrations in bone marrow, serum, saliva and brain showed a linear relationship (r = 0.860–0.997), indicating that a valid estimate of diazepam concentration in blood can be made from bone marrow samples.

Key words: Diazepam – Radioimmunoassay – Rat – Bone marrow – Serum – Saliva

Zusammenfassung. 1, 2, 4 und 8 Stunden nach intraperitonealer Injektion wurde die Verteilung von Diazepam in Körperflüssigkeiten und Geweben von Ratten untersucht. Die Untersuchung erfolgte mit Hilfe eines Radioimmunoassays und mit einem spezifischen Antidiazepam-Antikörper. Die Diazepamspiegel im Serum, im Speichel, im Gehirn und im Knochenmark fielen über eine Zeitdauer von 2 Stunden ab und zeigten nach 4 Stunden eine Nivellierung. Die Diazepamkonzentration im Knochenmark war viel höher als im Serum, im Speichel und im Gehirn. Dieser Befund muß an eine Akkumulation von Diazepam in diesem Gewebe denken lassen. Dieser Befund weist auch darauf hin, daß Knochenmark ein nützliches Material für den Nachweis von Diazepam bei skeletierten Überbleibseln sein kann. Die Diazepamkonzentrationen im Knochenmark, im Serum, im Speichel und im Hirngewebe zeigten eine lineare Korrelation (r = 0.860–0.997). Dieser Befund weist darauf hin, daß eine zuverlässige Abschätzung der Diazepamkonzentration im Blut aufgrund der Befunde im Knochenmark erfolgen kann.

Schlüsselwörter: Diazepam – Radioimmunoassay – Ratte – Knochenmark – Serum – Speichel

Introduction

In postmortem situations where uncontaminated blood samples are not available for toxicological analysis, other tissues must be used. However, if the body remains are almost skeletonized and the soft tissues are highly putrefied, bone marrow can be used as a sample. Winek et al. [1, 2] and Iwasaki et al. [3] reported that levels of some drugs in blood and bone marrow showed good correlation, indicating that bone marrow can be used to estimate the levels of some drugs and poisons in blood.

In this paper diazepam levels in blood, saliva, brain and bone marrow of rats have been investigated by using radioimmunoassay as described previously [4].

Materials and methods

Materials. [^{3}H]Diazepam (Sp. Act. 2.85 TBq/mmol, 76.8 Ci/mmol) was purchased from New England Nuclear Co. U.S.A. Scintisol 500 and all other reagents were obtained from Wako Pure Chemical Ind., Osaka Japan. Rats were supplied by Hokudo Animal Center, Sapporo, Japan. Anti-diazepam antiserum was obtained by repeated immunization of rabbits with temazepam (oxydiazepam)-3-hemisuccinate conjugated to bovine serum albumin as described previously [4].

Radioimmunoassay procedure. Radioimmunoassay (RIA) is based on competitive binding of unlabeled and ^{3}H-labeled diazepam to specific anti-diazepam antibodies. The method used was as previously described [4] except that Scintisol 500 was used as the scintillator.

Methods. Male Wistar rats (200–300 g) were used for all experiments. Twenty rats were divided into 4 groups. Serum, saliva, brain and bone marrow from each animal were collected 1, 2, 4

Offprint requests to: K. Terazawa

* *Present address:* Department of Forensic Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

** *Present address:* Scientific Crime Laboratory, Hokkaido Police Headquarters, Sapporo 060, Japan

and 8 h after intraperitoneal administration of diazepam dissolved in saline containing 0.3% Tween 80 [5] (83 μg diazepam/kg body weight equivalent to human doses of 5 mg/60 kg). Blood was withdrawn from retro-ocular plexes 30 min after administration, saliva was collected with a capillary glass tube and brain and bone were collected after sacrificing the animals by decapitation. Samples were stored at −20°C until use.

These experiments were approved by the Animal Care and Use Committee, Hokkaido University School of Medicine, Sapporo 060, Japan.

Serum and saliva (60–80 μl) samples were diluted 3 times with 0.05 *M* phosphate buffered saline (pH 7.4, PBS) and centrifuged at 3000 rpm for 15 min at 4°C using a Hitachi 20PR-50 refrigerated centrifuge. The supernatant was used as a sample. Brain tissue (1 g wet weight) was minced with scissors and homogenized with 5 vol 0.1 *N* HCl using a universal homogenizer [6]. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. Bone marrow (200–300 mg) collected from femur and tibia was finely ground with 5 vol 0.1 *N* HCl in a mortar and centrifuged at 10,000 rpm for 30 min at 4°C. Supernatants from brain and bone marrow were diluted twice with 0.05 *M* PBS before use.

Results

Figure 1 shows the concentration of diazepam in serum, saliva, brain and bone marrow of rats with the elapse of time after intraperitoneal administration. The levels decreased gradually for 2 h after administration and then leveled off.

The rate of decrease in the diazepam concentration in serum was faster than that in saliva, brain and bone marrow. The concentration of diazepam 4 h after administration was found to be 0.28 ± 0.05 ng/ml, about one-thirteenth of the level (3.75 ± 0.62 ng/ml) 1 h after administration (Fig. 1).

The concentration of diazepam in bone marrow was 1.3–7.8 times greater than in serum. The concentration of diazepam in saliva and brain 1 h after administration was 1.16 ± 0.03 ng/ml and 2.25 ± 0.11 ng/g, respectively, but the diazepam levels in saliva and brain 4 h after administration were 0.36 ± 0.02 ng/ml and 0.37 ± 0.02 ng/g which were higher than the serum levels.

Comparisons of diazepam levels in serum, saliva and brain versus bone marrow, in serum and saliva versus brain and in serum versus saliva are shown in Figs. 2–7. The correlation coefficients of the 3 groups in each comparison are listed in Table 1, although concentrations do not seem to indicate a linear correlation.

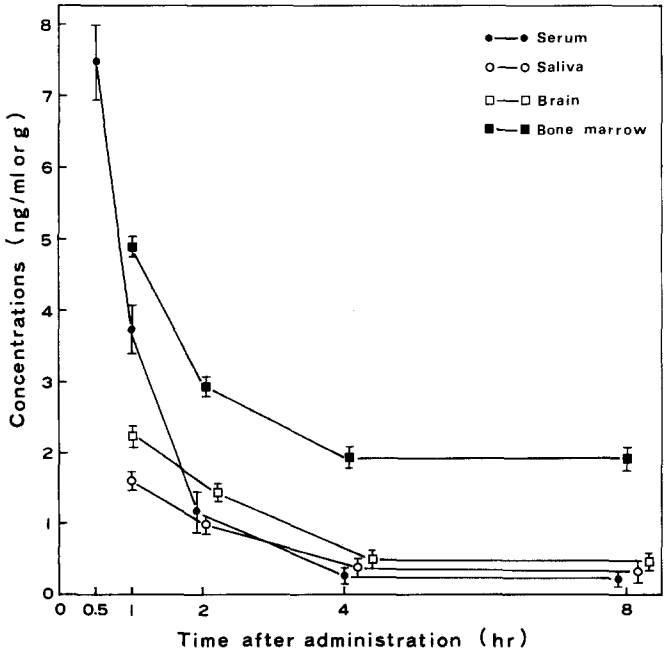

Fig. 1. Change in concentrations of diazepam in serum, saliva, brain and bone marrow after administration

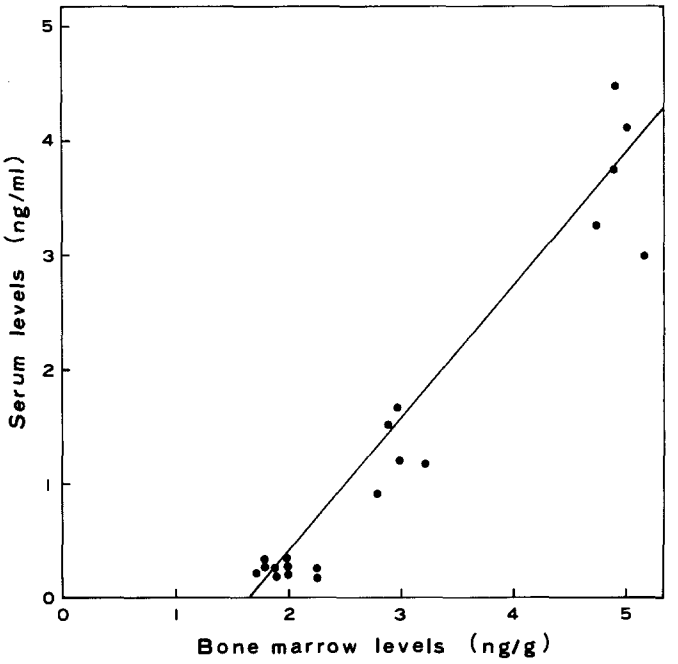

Fig. 2. Correlation of bone marrow and serum diazepam levels

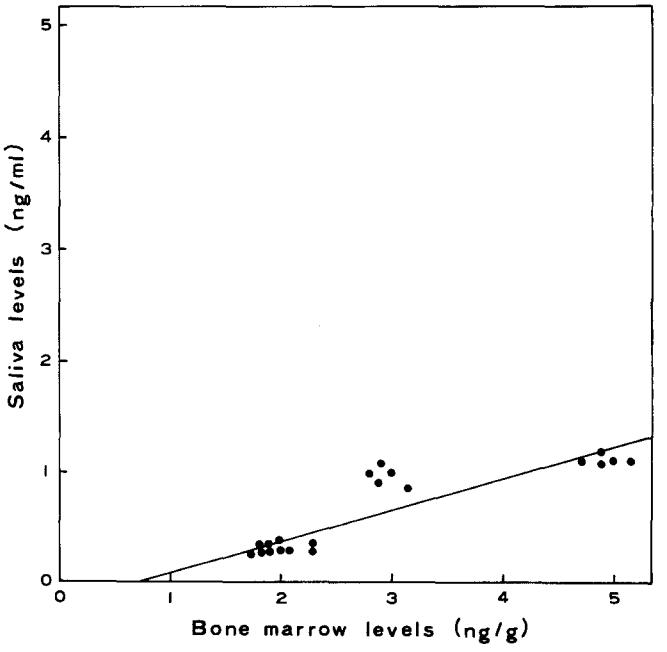

Fig. 3. Correlation of bone marrow and saliva diazepam levels

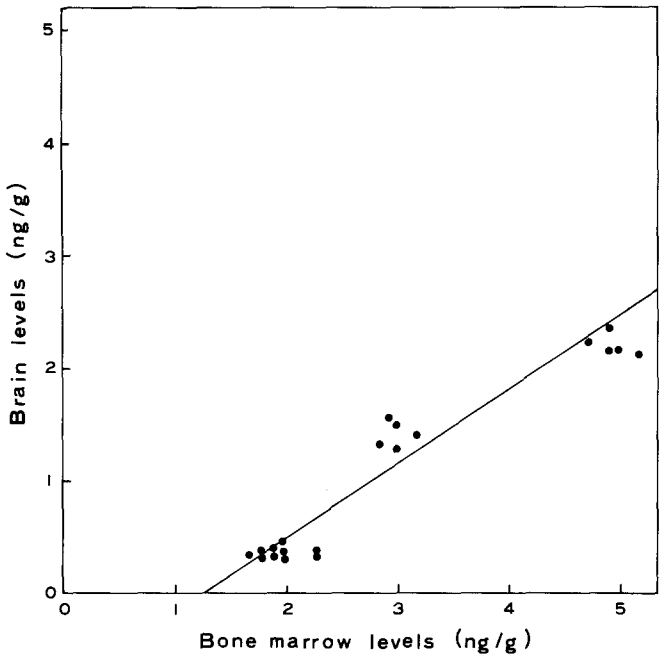

Fig. 4. Correlation of bone marrow and brain diazepam levels

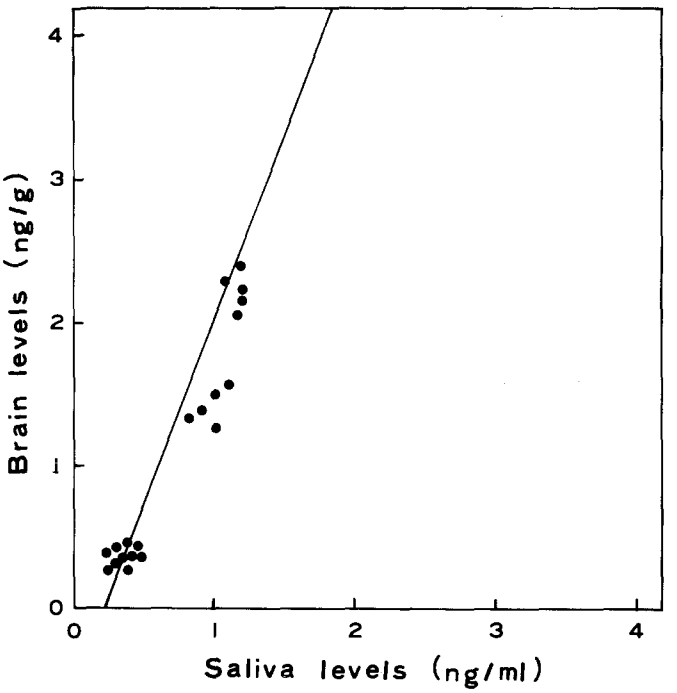

Fig. 6. Correlation of saliva and brain diazepam levels

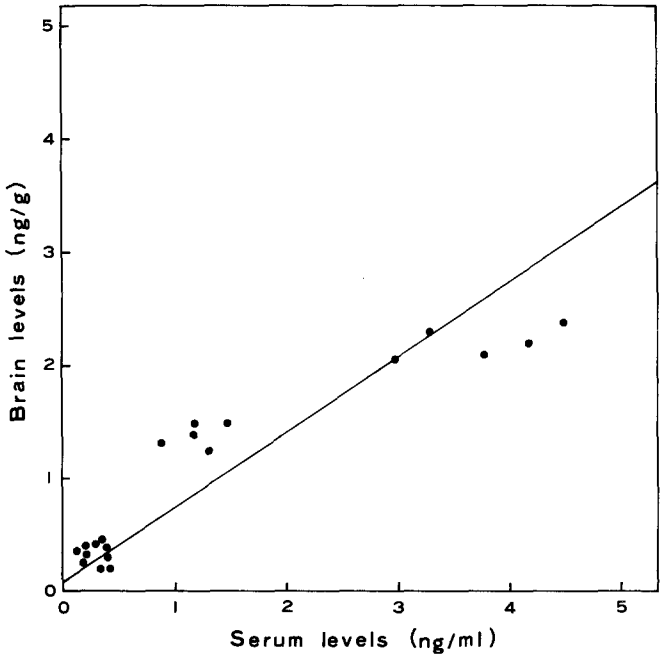

Fig. 5. Correlation of serum and brain diazepam levels

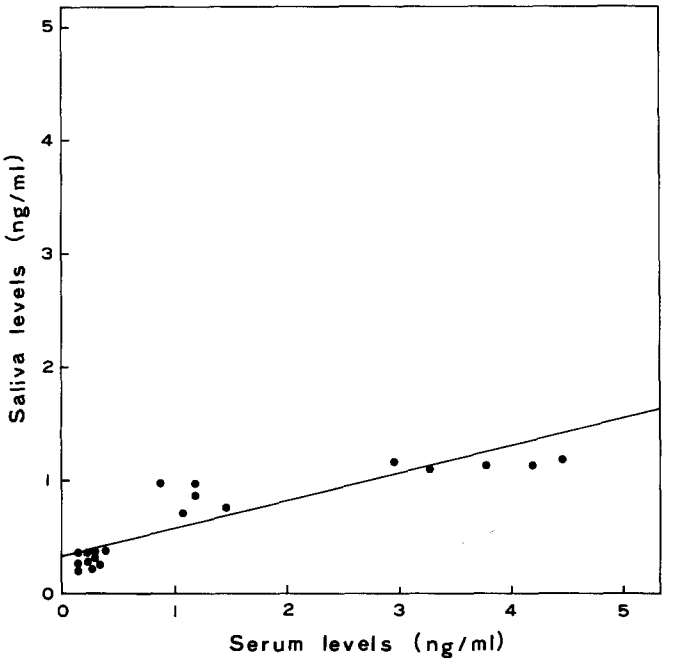

Fig. 7. Correlation of serum and saliva diazepam levels

Discussion

Diazepam (83 μg/kg body weight) was administered intraperitoneally to rats and the concentration of diazepam in serum, saliva, brain and bone marrow was determined by RIA with the elapse of time after administration. The body fluids and tissues of the rats in the control group contained no (endogenous) substances cross-reacting with the assay used (data not shown).

Diazepam in man is metabolized to N-desmethyldiazepam, oxazepam and oxydiazepam (temazepam) [7–9]. Temazepam is present in low levels in blood, but is evacuated in urine as a conjugate with glucuronic acid [7–8]. Kvetina et al. [10] reported that temazepam can, to some extent, be recognized in perfused fluid of rat liver, but Marducci et al. [11] showed that temazepam is not found as a metabolite of diazepam in blood, indicating that the amount of temazepam is negligible in blood and brain of rats. These reports show that the benzodiazepine derivative determined in this study was not temazepam but almost certainly diazepam, although the cross-reactivity of

Table 1. Correlation coefficient of diazepam concentrations among serum, saliva, brain and bone marrow

	Serum	Saliva	Brain
Saliva	0.852		
Brain	0.939	0.986	
Bone marrow	0.997	0.860	0.949

the anti-diazepam antibody with temazepam was equal to the reaction with diazepam [4]. The antibody does not recognize either N-desmethyldiazepam or oxazepam and these two derivatives are therefore not detectable [4].

Diazepam in rat serum could be detected within 30 min after administration and its half-life was approximately 1 h (Fig. 1), while in human serum it is approximately 2–3 h [7]. Therefore, the concentration of diazepam in rat serum decreased faster within 1 h after administration than in human serum, but thereafter gradually decreased and leveled off.

Diazepam in rat saliva could also be detected 1 h after administration and thereafter decreased more gradually than in serum (Fig. 1). The levels of some drugs in saliva generally depends on saliva pH, but diazepam does not seem to be affected [12]. The determination of drugs in saliva is clinically important for therapeutic drug monitoring [13] and various drugs have been determined from saliva stains adsorbed onto filter paper [14–16]. In these cases, more sensitive methods for the determination of diazepam are required and RIA can sufficiently meet the requirements because 1 pg of diazepam can be detected per assay tube [4].

Changes in diazepam levels in brain was almost the same as for saliva (Fig. 1). Brain tissue is a target organ of benzodiazepine and specific receptors exist [17]. Benzodiazepine is easily and diffusely incorporated into brain from blood and is reported to have a higher level than plasma because of the existence of the benzodiazepine receptors in the brain [18]. The results of this study show similar results.

The concentration of diazepam in bone marrow 4 and 8 h after administration was about two-fifths of that 1 h after administration, while under the same conditions the serum diazepam concentration was about one-fourteenth. Furthermore, the diazepam level in bone marrow after 8 h was 5–8 times greater than that in serum, saliva and brain (Fig. 1). These results indicate that diazepam accumulates in bone marrow over a long period (at least more than 8 h) and it is possible that diazepam can be detected in bone marrow whilst not detectable in serum, saliva or brain.

Diazepam concentrations in bone marrow showed a linear relationship to the levels in serum, saliva and brain (Figs. 2–4) among the 3 groups of plot in each comparison with correlation coefficients of 0.860–0.997 (Table 1). Therefore, in cases where uncontaminated blood samples are not available and where remains are skeletonized, a valid estimate of diazepam concentrations in blood and brain can be made from bone marrow samples.

References

1. Winek CL, Plustoka M, Wahba WW (1982) Plasma versus bone marrow flurazepam concentration in rabbits. Forensic Sci Int 19:155–163
2. Winek CL, Costanino AG, Wahba WW, Collon WD (1985) Blood versus bone marrow pentobarbital concentrations. Forensic Sci Int 27:15–24
3. Iwasaki M, Nakasono I, Ogata M, Kubo S, Suyama H (1987) Relationship between methamphetamine level in the marrow and serum. Eisei Kagaku 33:136–139 (in Japanese)
4. Tomii S, Takatori T (1984) Production and characterization of antibodies reactive with diazepam. Jpn J Clin Chem 13:281–285
5. Meier JR (1952) C^{14}-Cholesterol IV. Oxidation of carbon 4 and 26 to carbon dioxide by surviving tissues. J Biol Chem 198: 105–109
6. Faraj BA, Israili ZH, Kight NE (1976) Specificity of an antibody directed against d-methamphetamine. Studies with rigid and nonrigid analogs. J Med Chem 19:20–25
7. Desilva JAF, Koechlin BA, Bader G (1966) Blood level distribution patterns of diazepam and its major metabolite in man. J Pharm Sci 55:692–702
8. Schwartz MA, Koechlin BA, Postma E, Palmer S, Krol G (1965) Metabolism of diazepam in rat, dog and man. J Pharmacol Exp Ther 149:423–435
9. Klotz U, Antonin KH, Bieck PR (1976) Pharmacokinetics and plasma binding of diazepam in man, dog, rabbit, guinea pig, and rat. J Pharmacol Exp Ther 199:67–73
10. Kvetina J, Marcucci F, Fanelli R (1968) Metabolism of diazepam in isolated perfusion liver of rat and mouse. J Pharm Pharmacol 20:807–808
11. Marcucci F, Fanelli R, Mussini E, Garattini S (1970) Further studies of species difference in diazepam metabolism. Eur J Pharmacol 9:253–256
12. Feller K, LePetit G (1977) On the distribution of drugs in saliva and blood plasma. Int J Clin Pharmacol Res 15:468–469
13. Horning MG, Brown L, Nowlin J, Lertratanangkoon K, Kellaway P, Zion TE (1977) Use of saliva in therapeutic drug monitoring. Clin Chem 23:157–164
14. Dixon R, Crews T (1978) Diazepam: Determination in micro samples of blood, plasma and saliva by radioimmunoassay. J Anal Toxicol 2:210–213
15. Noe ER, Lathrop GD, Ainsworth III CA, Merritt JH (1976) Radioimmunoassay technology in mass drug screening: an evaluation of an absorbent paper disk transport system. J Forensic Sci 21:390–397
16. Shaler FP, Shaler RC, Mortimer CE (1978) Detection of drugs in a blood stain. I: Diphenylhydantoin. J Forensic Sci 23:701–706
17. Möhler H, Okada T (1977) Benzodiazepine receptor: Demonstration in the central nervous system. Science 198:849–851
18. Brestrup C, Squires RF (1977) Brain specific benzodiazepine receptors in rats characterized by high affinity ^{3}H-diazepam binding. Proc Natl Acad Sci USA 74:3805–3809