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Biodistribution and plasma protein binding of zoledronic acid

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Running title: Biodistribution of zoledronic acid

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Nonstandard abbreviations:

AUC, area und the concentration time curve; LOD, limit of detection; LOQ, limit of quantitation; QAL, quantitative autoradioluminography

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Abstract

The bisphosphonate zoledronic acid is a potent inhibitor of osteoclast-mediated bone resorption. To investigate drug biodistribution and elimination, ^{14}C-zoledronic acid was administered intravenously to rats and dogs in single or multiple doses and assessed for its *in vitro* blood distribution and plasma protein binding in rat, dog, and human. Drug exposure in plasma, bones, and non-calcified tissues was investigated up to 240 days in rats and 96 hours in dogs using radiometry after dissection. Drug biodistribution in the rat and within selected bones from dog was assessed by autoradiography. Concentrations of radioactivity showed a rapid decline in plasma and non-calcified tissue but only a slow decline in bone, to ~50% of peak at 240 days post dose, whereas the terminal half-lives (50–200 days) were similar in bone and non-calcified tissues, suggesting redistribution of drug from the former rather than prolonged retention in the latter. Uptake was highest in cancellous bone and axial skeleton. At 96 h post dose, the fraction of dose excreted was 36% in rat and 60% in dog; 94–96% of the excreted radioactivity was found in urine. Blood/plasma concentration ratios were 0.52–0.59 and plasma protein binding of zoledronic acid was moderate to low in all species. The results suggest that a fraction of zoledronic acid is reversibly taken up by the skeleton, the elimination of drug is mainly by renal excretion, and the disposition in blood and non-calcified tissue is governed by extensive uptake into and slow release from bone.

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Bisphosphonates are therapeutic agents for the treatment of bone resorptive diseases. This class of drugs possesses a strong affinity to bone tissue, and markedly inhibits osteoclast activity (Cremers *et al*., 2005). The more potent nitrogen containing bisphosphonates interfere with cellular signal transduction pathways in osteoclasts by inhibiting protein prenylation (Luckman *et al*., 1998). Following intravenous dosing, bisphosphonates show initially a rapid disappearance from the systemic circulation with several short elimination phases which are followed by long elimination phases ($t_{½}$ months to years) (Lin, 1996). Elimination occurs almost exclusively by the kidney through glomerular filtration (Singer & Minoofar, 1995) and drug clearance is decreased in patients with renal impairment (Berenson *et al*, 1997; Saha *et al.,* 1994). Bisphosphonates show negligible biotransformation (Lin *et al.,* 1994). The rate and extent of skeletal uptake and release of drug is dependent on the osseous turnover rate as well as the intrinsic bone affinity of the bisphosphonate (Kasting & Francis, 1992). The distribution of bisphosphonates within the skeleton is not homogeneous. Bisphosphonates target mainly sites of bone turnover where bone mineral is exposed to the surrounding fluids. Bisphosphonate plasma concentrations during the early period post administration generally are dose proportional, but the very low concentrations long term post dose mainly reflect bone remodeling and do therefore not directly relate to the applied dose level or clearance processes (Gertz *et al.*, 1993). Accordingly, an accurate determination of bisphosphonate terminal half-lives is difficult, and $AUC_{0-\infty}$ and clearance parameters extrapolated using apparent terminal half-lives derived from a short observation period are open to question.

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The protein binding of bisphosphonates is typically low to moderate and may be calcium and pH dependent (Lin *et al.,* 1993; Lin, 1996). Bisphosphonates are known to produce renal effects ranging from transient proteinuria and alterations in creatinine clearance (Pecherstorfer *et al*, 1996) to acute renal failure (Bounameaux *et al*., 1983). Reducing dose, rate of infusion, and dosing frequency can mitigate these effects (Kanis *et al*, 1983). One of the newer and highly efficacious nitrogen containing bisphosphonate drugs is zoledronic acid (Figure 1). It ranks highest in its inhibitory potency in *in vitro* assays of bone resorption and calcium turnover (Green *et al*., 1994) as well as in assays of tumor cell invasion (Boissier *et al*., 2000). Following intravenous administration, zoledronic acid concentrations in patients' plasma show a rapid decline from the observed end-of-infusion peak to about 1% of peak by 24 hours post dose, followed by prolonged, very low drug plasma concentrations declining to below the limit of bioanalytical methodology over a period of days to weeks (Chen *et al*., 2002). The urinary excretion of zoledronic acid, consisting exclusively of unchanged drug and representing approximately 40% of the administered dose, is essentially complete over the first 24 hours post dose. This suggests that about 60% of dose is retained in the skeleton. Bone remodeling processes will slowly release retained drug back into the systemic circulation from where it is renally excreted. Zoledronic acid renal clearance is about 50% of the glomerular filtration rate in patients with normal renal function (Skerjanec *et al*., 2003). Transient rises in serum creatinine have been observed in a small fraction (<1%) of patients with postmenopausal osteoporosis receiving once-yearly 5 mg doses of intravenous zoledronic acid (Black *et al*., 2007). The reported risk of adverse renal events is higher in cancer patients due to their disease state and associated comorbidities,

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exposure to other potentially nephrotoxic agents, and higher doses and more frequent dosing (Lewiecki and Miller, 2007). In a trial of monthly intravenous doses of zoledronic acid to treat skeletal metastases in patients with lung cancer and other solid tumors, the proportion of patients with decreased renal function was not significantly different between the 4-mg zoledronic acid (15-min infusion) and placebo groups (10.9% versus 6.7%). (Rosen *et al*., 2003).

Differences in kidney retention and plasma protein binding have been proposed as potentially contributing to perceived differences in the renal safety of bisphosphonates currently in clinical use (Bergner *et al*., 2007; Lewiecki & Miller, 2007). For a better understanding of the biodistribution and excretion of zoledronic acid, including exposure of the drug to the kidney, studies in rat and dog models were performed. The study in the rat (intravenous doses on 16 consecutive days) was designed to approximate the treatment regimen of cancer patients (multiple myeloma or bone metastases) who receive a single intravenous administration of zoledronic acid every 3 to 4 weeks (12 to 16 doses per year). The *in vitro* blood distribution and plasma protein binding of zoledronic acid were determined for rat, dog and human. Differences in plasma protein binding between zoledronic acid and ibandronate have been proposed as an additional potential contributing factor to the perceived differences in the renal safety of these two drugs (Body *et al*., 2005). Therefore, plasma protein binding of ibandronate and zoledronic acid was tested side by side under controlled assay conditions to obtain robust comparative data.

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Methods and Materials

Radiolabels and stock solutions. Zoledronic acid and ibandronate in ^{14}C-labelled form (Figure 1) were prepared by the Isotope Laboratory of Novartis Pharma AG (Basel, Switzerland). The specific radioactivity of zoledronic acid was 1.5 to 1.9 MBq/mg for the study in dog and the blood distribution investigations and 3.9 to 6.1 MBq/mg for the studies in rat. In the plasma protein binding study, the specific radioactivity was 6.9 and 4.3 MBq/mg for zoledronic acid and ibandronate, respectively. For intravenous dosing, ^{14}C-labelled zoledronic acid was dissolved in 0.9% sodium chloride to a concentration of 0.06 mg/g (rat) and 0.15 mg/g (dog). The pH was adjusted to 7.0 using a 1.5 mM solution of NaOH. For the *in vitro* investigations, aqueous stock solutions were prepared by serial dilution.

Animal studies. All animal studies were performed in accordance with Swiss animal welfare regulations. The individual intravenous doses of zoledronic acid were 0.15 mg/kg for all animals. This dose is similar to the maximum dose tested in the oncology phase 3 clinical trials of 8 mg i.v. per patient, (0.13 mg/kg for a 60 kg patient).

Studies in rats. Male albino rats (Tif: RAIf(SPF)) 6-8 weeks old (190-250 g) were given a diet of NAFAG pellets No. 890 (Nahr und Futtermittel AG, Gossau, Switzerland) and had free access to tap water. Zoledronic acid was administered via a single bolus injection into the tail vein. For the distribution studies a single 0.15 mg/kg dose, and for the multiple dose studies 0.15 mg/kg daily on 16 consecutive days were administered. The short term (4 days) excretion of zoledronic acid in urine and feces was studied after the single dose administration.

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Distribution by dissection. At each sampling time three rats were anesthetized with ether and bled out after cardiac puncture. The tissues were harvested by dissection at 5 min, 4 h, and 24 h after the first dose, 24 h after the 8th dose, then 1, 16, 31, 64, 128, and 240 days after the 16th daily dose (n=3 rats/time point). For radiometry samples were prepared as described by Botta et al. (1985), bones were dissolved in 25% HCl. The limit of quantification (LOQ) for the determination of total radioactivity was defined as 1.8 fold the total background count. The area under the tissue or plasma concentration-time curve (AUC) of radioactivity over the period 0 to 256 days was calculated using the linear trapezoidal method. The following tissues were analyzed: blood, plasma, salivary, thyroid, thymus, lung, heart, aorta, liver, pancreas, spleen, adrenal, kidney, white fat, testis, muscle, sciatic nerve, bone marrow, stomach, small intestine, skin, brown fat, eye, brain, cranium, vertebrae thoracales, and tibia. For the tibia (including fibula) the amount of drug related radioactivity in the whole bone (concentration times organ weight) was determined at different sampling points, in order to correct for organ growth during the observation period.

Distribution by autoradiography. Tissue distribution was qualitatively assessed by whole-body autoradiography up to 12 months after a single 0.15 mg/kg intravenous dose. Immediately after sacrifice, the rats were deep-frozen in a mixture of dry ice and hexane at approximately -75°C and then embedded in a 2% pre-cooled semi-liquid gel of sodium carboxymethylcellulose. Multiple sections of 40 µm thickness were obtained at varying depths at approximately -20°C in a Cryomacrocut (Reichert-Jung, Nussloch, Germany) according to the method of Ullberg (1977). After freeze-drying, the sections were fixed

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onto transparent adhesive tape, autoradiographs were obtained after two days of exposure of selected sections to imaging plates as described for the dog (below).

Excretion studies. For the excretion experiments rats were housed singly on steel grids in metabolism cages. Urine (ice-cooled vials) and feces were collected quantitatively and separately in daily intervals up to 96 h after a single dose. Day 1 urine was collected in two fractions (0 to 8 h and 8 to 24 h).

Studies in dogs. The distribution and excretion of zoledronic acid was studied in three 91-month-old male beagle dogs. Old dogs were used since they – in contrast to rats - achieve skeletal maturity, the growth plates close and bone growth ceases, resulting in a bone metabolism corresponding to that in adult humans. The average weight of the dogs was 13.1 kg. Prior to and after administration of zoledronic acid the dogs had free access to food (pellets no. 3353, Provimi Kliba SA, Kaiseraugst, Switzerland) and tap water. Each dog received 0.15 mg/kg of ^{14}C-zoledronic acid as a 15 min intravenous infusion.

Excretion studies. Urine (ice-cooled vials) and feces were collected quantitatively and separately in daily intervals up to 96 h after dosing, urine on day 1 was collected in two fractions (0 to 8 h and 8 to 24 h).

Distribution by dissection and autoradiography. At 96 h after the infusion of ^{14}C zoledronic acid the dogs were anaesthetized by an injection of 10 mL of a 10% solution of ®Pentothal (Abbott AG, 6300 Zug, Switzerland) in distilled water into the cephalic vein and then bled by severing the carotid artery. Tissues were harvested and assayed by radiometry and by quantitative autoradioluminography (QAL) (Schweitzer *et al.*, 1987, Johnston *et al.*, 1990). Radiometry samples were prepared as described by Botta et al. (1985). The limit of quantification (LOQ) for the determination of total radioactivity was

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defined as 1.8 fold the total background count (28 dpm), corresponding to 0.02 nmol/mg in urine and blood/plasma, and to 0.01 nmol/mg in tissues, based on an average tissue sample weight of 200 mg. For QAL analysis, selected bones were dissected immediately after sacrifice, snap-frozen and embedded, and multiple sections of 40 µm thickness were obtained at varying depths in a CM 3600 PLC cryomicrotome (Leica Microsystems GmbH, Nussloch. Germany) according to the method of Ullberg (1977). Following 48 h of dehydration at -23°C the sections were exposed to Fuji BAS 5000 Imaging plates (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 4 days at room temperature in a lead shielded box in order to minimize the background signal. The duration of exposure was chosen to allow detection of approximately 1 dpm/mg. At the end of the exposure, the imaging plates were first left at room temperature in the dark for 3 min, then transferred into a Fuji BAS 5000 confocal phosphorimager and scanned in steps of 25 µm. The resulting photostimulated light data files were corrected by background subtraction and processed electronically with a MCID/Elite (7.0) image analyzer (Imaging Research, St. Catherines, Ontario, Canada). The limit of detection (LOD) was taken as the sum of the mean of the background (n=10 measurements) and 3 standard deviations on this mean; the limit of quantitation (LOQ) was taken as 3 times the LOD. Under the conditions of this study, the LOD and LOQ were 0.038 and 0.11 nmol/g, respectively, in the central cavity of the bones, and 0.24 and 0.74 nmol/g, respectively, in the compact bone, spongy bone, and periosteum. The image files were processed using the Adobe Photoshop Elements 2.0 software (Adobe Systems Inc., San Jose, CA, USA).

Radiometry. Radioactivity in the rat and the dog blood, plasma, tissues, urine and feces samples was determined by liquid scintillation counting using a TriCarb liquid

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scintillation system (Packard Instruments, Downers Grove, USA) according to Botta et al. (1985). The LOQ was defined as 1.8 fold the background radioactivity.

***In vitro* biodistribution.** Blood and plasma were obtained from male albino rats of the Hanover Wistar strain and from male beagle dogs. Human blood and plasma was from healthy male volunteer donors. Lithium heparin was used as anticoagulant. Animal blood and plasma was used as pools (n = 3). For human blood, three individual blood samples were used.

Blood distribution. Whole blood was spiked with zoledronic acid to concentrations of 30 – 5000 ng/mL, incubated for 30 min at 37°C, and centrifuged for 15 min at 1000 g for plasma separation. Drug related radioactivity in blood (C_b) and plasma (C_p) was determined in triplicate by the combustion method. The hematocrit (H) was determined in triplicate. The fraction in plasma was determined as $(C_p/C_b) * (1\text{-}H)$.

Plasma protein binding. Separation of plasma protein bound and unbound drug was performed by ultrafiltration. Initially the recovery and free permeation of zoledronic acid and ibandronate in PBS were analyzed: PBS was spiked and three times 1 mL aliquots were introduced into Centrifree® devices (molecular cut-off 30 kDa, Amicon Inc., Beverly, MA, USA) and spun at 2000 *g* for 1 min (filtrate ≤ 500 µL). Samples were taken from the spiked solution, the filtrate and retentate and analyzed by liquid scintillation counting and filtrate and retentate weights were determined. The radioactivity recovery in the device was above 90% and the free permeation through the membrane (concentration in filtrate/concentration in retentate) was ≥ 0.95, suggesting no relevant bias due to the separation technique. The plasma pH was adjusted to 7.4 ± 0.1 (using 5% lactic acid or 1 N NaOH) before spiking to concentrations of 2 – 2000 ng/mL zoledronic acid or

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ibandronate. After incubation for 30 min at 37°C under constant gentle agitation, the spiked plasma samples (n = 3) were centrifuged at 2000 *g* for 10 min in the pre-warmed Centrifree devices. Total radioactivity was determined by liquid scintillation counting in the ultrafiltrate (C_u, concentration of unbound drug) and in the sample introduced into the reservoir before ultrafiltration (C_p, concentration in plasma). The unbound fraction in plasma was calculated as C_u / C_p.

Values of 30 dpm above background (^{14}C) were defined as the LOQ for radioactivity analysis; all reported values were above this limit.

Results

Tissue distribution in rats. Five minutes after a single intravenous dose of ^{14}C-zoledronic acid (0.15 mg/kg) to adolescent rats, the highest concentration of drug related radioactivity was detected in plasma (5383 pmol/g), which declined very rapidly (29 pmol/g at 4 h and 3.9 pmol at 24 h) due to extensive distribution and renal elimination. The highest tissue concentration was initially observed in the kidney (3218 pmol/g at 5 min after dosing). This also dropped rapidly within 4 h to 403 pmol/g and 183 pmol/g at 24 h post dose. At 4 h the highest concentrations were detected in the calcified tissues, which in contrast to non-calcified tissue continued to show uptake of zoledronic acid related radioactivity up to 24 h post dose (Inset, Figure 2A). This preferential and extensive uptake of zoledronic acid into bones was more pronounced after multiple intravenous doses of 0.15 mg/kg of ^{14}C-zoledronic acid (daily on 16 consecutive days) to adolescent rats. There was no indication of saturation of available binding capacity in bones: zoledronic acid concentrations in the calcified tissues were 6

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to 7-fold higher at 24 h after the 8th dose and 10 to 12 fold higher at 24 h after the 16th dose compared to the single dose. ^{14}C-zoledronic acid related radioactivity in the calcified tissues declined very slowly over the observation period of 240 days (Figure 2A). The AUC_{0-256d} values of the bone tissues were 100 to 1500-fold higher than in the non-mineralized tissues and at least 4000-fold higher than in plasma (Table 1, Figure 2). 16 days after the last dose, the drug concentrations in rat plasma and all non-mineralized tissues (listed in methods section) dropped below 0.4 nmol/g, whereas concentrations in bone remained high at >10 nmol/g up to 240 days after dosing. Considering the entire 240-day observation period , the concentrations in the analyzed bones (cranium, vertebrae thoracales, and tibia) decreased by a factor of about 2. Based on this observation period approximate half-lives of 150 to 200 days were estimated. When the observed tibia concentrations were corrected for bone growth, no elimination was discernible within the 240 days post-treatment period. Qualitatively similar results were obtained by whole body autoradiography after a single intravenous dose of 0.15 mg/kg of ^{14}C-zoledronic acid (Figure 3). Five minutes after dosing, high concentrations of drug related radioactivity were found in the blood, the highly perfused organs such as liver and kidney, and bone, whereas at 1 month after dosing (Figure 3B) and up to the final autoradiogram taken at 12 months post dose, visible exposure was confined to bones.

Tissue distribution in dogs. In skeletally mature dogs, 96 h after administration of a single intravenous infusion of zoledronic acid, the plasma and blood radioactivity concentrations as well as concentrations in brain and muscle were below the LOQ (Figure 4). Measurable concentrations were found in all of the other tissue specimens assayed, suggesting a high volume of distribution. As observed for the rat, there was a striking

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difference between the concentrations measured in non-calcified tissues versus bone tissues. Exposure was higher in bones of the axial skeleton compared to the appendicular bones or the head (including teeth). Analysis of the distribution of radioactivity within selected bones of the dog by quantitative autoradiography revealed high labeling in the cancellous bones. Distribution was mainly into the more dynamic portions of bone and areas with a high surface area, such as spongy bone. Uptake of drug in compact bone was lower, and only little labeling was detected in the central cavity of compact bones (Figure 5). Laniary and molar teeth, mandibula, and maxilla showed similar radioactivity concentrations, 323, 468, 320, and 534 pmol/g respectively, and were in the lower range of drug uptake, which extended from 241 pmol/g in radius and 1619 pmol/g in the sternum to 2358 pmol/g in the lumbar vertebrae. In non-calcified tissues the lowest concentrations were found in blood, muscle and brain (all below LOQ), whereas the highest concentrations were present in the thyroid (294 pmol/g), kidney medulla (250 pmol/g), knee cartilage (156 pmol/g), and kidney cortex (146 pmol/g). (Figure 4).

Excretion of zoledronic acid in rats and dogs. After administration of a single intravenous dose of 0.15 mg/kg zoledronic acid to adolescent rats, 36.0% of the dose was recovered in urine and feces within 96 h (Figure 6A). Of this, 96% was present in the urine (34.6% of the dose) and 4% in the feces (1.4% of the dose). In skeletally mature dogs, the total cumulative mean recovery was 60.5% within 96 h of dosing, with 94% of this being excreted in the urine (Figure 6B).

In vitro blood distribution and plasma protein binding. In blood of human, dog, and rat, zoledronic acid was mainly located in the plasma fraction. The mean fraction of drug

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in plasma was 90 ± 6%, 95 ± 5%, and 91 ± 9% for rat, dog, and human, respectively, corresponding to mean blood to plasma concentration ratios of 0.54, 0.52, and 0.59. Plasma protein binding of zoledronic acid was moderate in rat plasma and low in dog and human plasma (Table 2). The unbound fraction was in the range of 12 – 20% in rat plasma and no concentration dependency was evident. In dog and human plasma the extent of binding was similarly low, the unbound fraction ranging from 51 to 64% and 60 to 77%, respectively. In dog and human plasma, the unbound fraction appeared to increase with increasing concentration (Table 2).. The protein binding of ibandronate was slightly but consistently lower compared to that of zoledronic acid in rat, dog, and human plasma across the entire concentration range tested, 2 – 2000 ng/mL, which encompasses the drug concentrations observed after clinical doses of the two drugs (Table 2, C_{max} values for zoledronic acid (4 mg, i.v., 15 min infusion) and ibandronate (6 mg, i.v., 30 min infusion), in human are around 320 ng/mL (Skerjanec et al., 2003, Bergner *et al.*, 2007)).

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Discussion

The biodistribution studies in rat and dog demonstrate a several orders of magnitude higher affinity of zoledronic acid for calcified compared to non-calcified tissue. As shown in rats, bone exhibits a large storage capacity for drug which was not saturated after 16 daily doses, representing the cumulative annual dose of zoledronic acid in patients with metastasis to bone. In the skeleton of mature dogs the uptake of drug was higher in the axial skeleton compared to appendicular bones or the head. It was most pronounced in the dynamic parts of bone and in regions with a high surface area (cancellous bone), compared to compact bone or the non-calcified central cavity of bone. Teeth and jaws showed no exceptional differences in drug uptake compared to other bones.

Bisphosphonates are known to complex with hydroxyapatite, thereby leading to a high sequestration of the drug into bone (Cremers et al., 2005). The prolonged but extremely low exposure level of zoledronic acid related radioactivity in plasma and non-calcified tissues seen in the rat 240 days after the last dose likely reflects the slow release from bone subsequent to its initial rapid uptake, rather than a very low systemic clearance (Lin, 1996). This parallels the observations with other bisphosphonates (Lin et al., 1991). The accuracy of reported terminal half-lives of bisphosphonates may be strongly influenced by detection limits, observation times, and sampling schedules as well as differences in bone turnover due to age and disease state. Thus, the reported 2.2 h half-life of zoledronic acid in the dog (Martin-Jiminez *et al*. 2007), which was derived from a 9-hour observation period, does not represent the terminal elimination phase. The half-life of ibandronate in the rat kidney of 24 days, determined by Bauss & Russell (2004), has been

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cited as a potential reason for the purportedly better renal safety of this bisphosphonate (Body *et al*., 2005). However, when calculating the terminal kidney half-life of ibandronate by more appropriately using the last 2 (or 3) values rather than all 4 of the concentration values reported by Bauss & Russell (2004), it turns out to be 138 days (or 90 days), which is similar to the half-lives reported for other bisphosphonates, including zoledronic acid.

Bisphosphonates are generally not subject to hepatic metabolism (Lin, 1996; Cremers et al., 2005), which is in line with the observed relatively low concentrations of zoledronic acid in the liver compared to other non-osseous tissue in rats and dogs (Figures 2 and 4) and negligible fecal excretion of drug following intravenous dosing (Figure 6). Pamidronate and alendronate represent examples of other bisphosphonates where drug concentrations in rat kidney are higher than in the liver (Pongchaidecha & Daley-Yates, 1993; Lin, 1996).

Variability in the pharmacokinetic profiles of bisphosphonates between and within species is mainly attributable to differences in the rates of renal excretion and uptake into calcified tissues. Within 1 to 5 days after administration to humans, 30 to 60% of the bisphosphonate dose is renally excreted (Lin, 1996; Kino *et al*., 1999). This was also observed for zoledronic acid (Figure 6). Since uptake into bone and renal elimination are competing processes, a faster bone uptake should result in a lower amount of drug excreted by the kidney. Therefore, predominance of bone formation in the adolescent rats could explain their lower degree of renal excretion of zoledronic acid as compared to the skeletally mature dogs (Figure 6). In addition Lin *et al*. (1994) reported a higher apparent

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uptake clearance of alendronate by bone for rat compared to dog. A similar difference for zoledronic acid may contribute to the observed difference in excretion in rat and dog.

Zoledronic acid did not partition strongly into blood cells. Binding to plasma proteins was moderate in rat plasma and low in dog and human plasma. In the absence of active transport processes, the extent of tissue distribution of a drug is driven by the ratio between tissue binding and plasma protein binding. For zoledronic acid, and likely bisphosphonates in general, the balance is greatly in favor of the tissues due to the high affinity of the drug for bone. Plasma protein binding is low and clearly not capable of significantly restricting the uptake of these drugs by bone. Lin *et al*. (1991) reported that the apparent uptake clearance of alendronate by bone was higher in rat compared to dog, despite the more than 10-fold higher plasma protein binding in rat.

Both rat and dog show the lowest exposure to zoledronic acid related radioactivity in blood and plasma compared to all other tissues analyzed (Table 1, Figures 2 and 4). Earlier reports have suggested that high bisphosphonate doses accompanied by high concentrations in plasma overload renal elimination mechanisms, and the retained compound can damage renal cells (Body *et al*., 2005). These reports also have speculated that a higher plasma protein binding of ibandronate as compared to zoledronic acid may contribute to differences in the renal safety of the two drugs (Bergner *et al*., 2007; Body *et al*., 2005). The reported concentration of ^{14}C-ibandronate in the rat kidney, 0.3% of 0.10 mg/kg dose 24 h after drug administration (Bauss *et al*., 2004), or approximately 118 pmol/g assuming 250 g/rat and 2g/rat kidney (Davis & Morris, 1993), is proportional to the observed zoledronic acid 24-h kidney concentration of 184 pmol/g per 0.15 mg/kg

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dose, indicating no difference in kidney retention between the two drugs. Plasma protein binding of zoledronic acid was observed to be dependent on plasma free calcium levels and pH, as has been reported previously for alendronate (Lin, 1996; Lin *et al*. 1993). Slight shifts in the pH of plasma during *in vitro* incubations (e.g. due to loss of carbon dioxide) may contribute, together with other factors, to inter-study variability of plasma protein binding, possibly leading to the wide differences in reported values for ibandronate (Barrett *et al*., 2004; Dooley & Balfour, 1999). Under rigorous testing conditions, the protein binding of ibandronate in human plasma was found to be slightly lower than that of zoledronic acid. Both drugs showed a qualitatively similar binding pattern in plasma of the tested species (rat > dog > human, Table 2) and a slight concentration dependency in dog and human plasma. These *in vitro* findings are in line with the available data reported for other bisphosphonates: for alendronate, plasma protein binding in the rat is higher than in dog and human (Lin *et al*., 1999) and alendronate and etidronate show a concentration dependency of plasma protein binding (Lin, 1996). The renal clearance of zoledronic acid, 60 mL/min (Skerjanec et al., 2003) is identical to that reported for ibandronate (Barrett et al., 2004), consistent with our finding of comparable plasma protein binding of the two drugs.

The biodistribution results suggest that zoledronic acid disposition in non-calcified tissue is governed by the extensive uptake into and slow release from bone, as generally observed for bisphosphonate drugs. The findings that zoledronic acid and ibandronate show similar dose normalized levels in the rat kidney, have comparable elimination half-lives in rat kidney, and do not appreciably differ in their plasma protein binding across rat, dog, and human, counter the reported claims of pharmacokinetic and biodistribution

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differences providing the basis for potential renal safety differences in animals and humans.

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Legends for Figures

Figure 1: Chemical structure of ^{14}C labeled zoledronic acid (A) and ibandronate (B).

Figure 2: Tissue concentrations vs. time of ^{14}C-zoledronic acid related radioactivity (mean ± SD, n = 3 rats) after intravenous once daily dosing for 16 consecutive days to rats (0.15 mg/kg/day; A: calcified tissues, inset: day 1; B: non-calcified tissues, inset: plasma enlarged). Concentrations are based on measurement of total ^{14}C-radioactivty after dissection; for clarity standard deviation were excluded for the non-calcified tissues. Concentrations are shown for the following times: 24 h after the first dose, 24 h after the 8th dose, then 1, 16, 31, 64, 128, and 240 days after the 16th dose.

Figure 3: Selected whole-body autoradiogram at 5 min (A) and 1 month (B) after an intravenous dose of 0.15 mg/kg ^{14}C-zoledronic acid to male rats; whiter areas correspond to higher radioactivity level.

Figure 4: Tissue concentrations (mean ± SD, n = 3 dogs) of ^{14}C-zoledronic acid related radioactivity 96 h after a single 0.15 mg/kg intravenous infusion to dogs (A: calcified tissues, B: non-calcified tissues). Concentrations are based on measurement of total ^{14}C-radioactivty after dissection; asterisks indicate concentrations below the LOQ.

Figure 5: Autoradioluminographs of the tibia (A: longitudinal section), and a vertebra (B: horizontal section, vertebra lumbalis) at 96 h after a 15-min intravenous infusion of 0.15

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mg/kg ^{14}C-zoledronic acid to skeletally mature adult male dogs; whiter areas correspond to higher levels of radioactivity.

Figure 6: Excretion of ^{14}C-zoledronic acid related radioactivity in rats and dogs. A:) Mean cumulative recovery of radioactivity in male rats (n = 3) after administration of 0.15 mg/kg ^{14}C-zoledronic acid given as a single intravenous bolus dose; B:) Mean cumulative recovery of radioactivity after administration of 0.15 mg/kg ^{14}C-zoledronic acid given as a single intravenous infusion to dogs.

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Table 1. Tissue exposure to zoledronic acid related radioactivity in rats after repeated dosing.

Mean zoledronic acid $AUC_{0\text{-}256\ d}$ and tissue to plasma AUC ratios, of 16 daily 0.15 mg/kg doses given intravenously to rats. Tissues were harvested by dissection at 5 min, 4 h, and 24 h after the first dose, 24 h after the 8th dose, then 1, 16, 31, 64, 128, and 240 days after the 16th daily dose (n=3 rats/time point). Concentrations are based on measurement of total ^{14}C-radioactivty.

	$AUC_{0\text{-}256d}$, nmol h/g	Tissue to plasma ratio
Plasma	23.1	1
Cranium	95501	4127
Tibia	298770	12911
Vertebrae thoracales	146963	6351
Adrenal	565	24.4
Aorta	193	8.33
Kidney	934	40.4
Liver	411	17.7
Small intestine	382	16.5
Spleen	912	39.4
Stomach	313	13.5
Thyroid	250	10.8

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Table 2: Plasma protein binding of zoledronic acid and ibandronate.

Unbound fractions (mean ± SD, expressed in %) of ^{14}C-zoledronic acid and ^{14}C-ibandronate in pooled plasma from rat and dog and three individual human plasma samples. Concentrations are based on measurement of total ^{14}C-radioactivity.

	Nominal concentration (ng/mL)			
	2	20	200	2000
	Zoledronic acid			
Rat plasma	19.6±1.4	13.1±0.6	12.1±0.8	16.4±0.6
Dog plasma	50.9±2.1	54.7±1.1	62.5±1.7	64.0±0.7
Human plasma	59.6±2.4	65.4±2.0	71.7±0.2	76.9±1.9
	Ibandronate			
Rat plasma	28.9±1.4	24.2±2.6	32.4±1.3	48.5±1.7
Dog plasma	59.3±3.8	64.7±1.3	71.6±0.6	75.1±1.5
Human plasma	68.9±13.3	73.8±1.0	76.3±1.1	78.6±1.0

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Figure 1

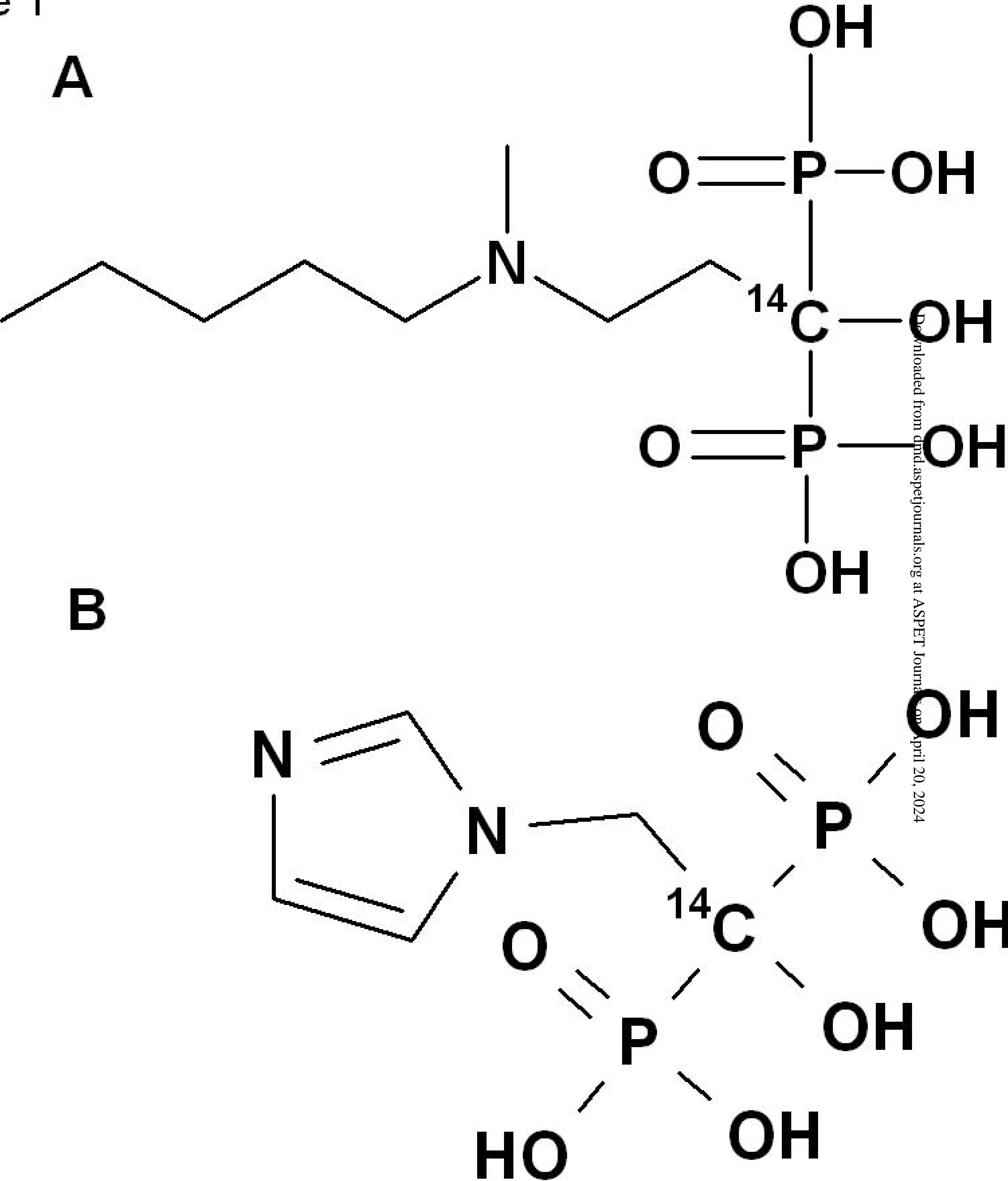

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Figure 2

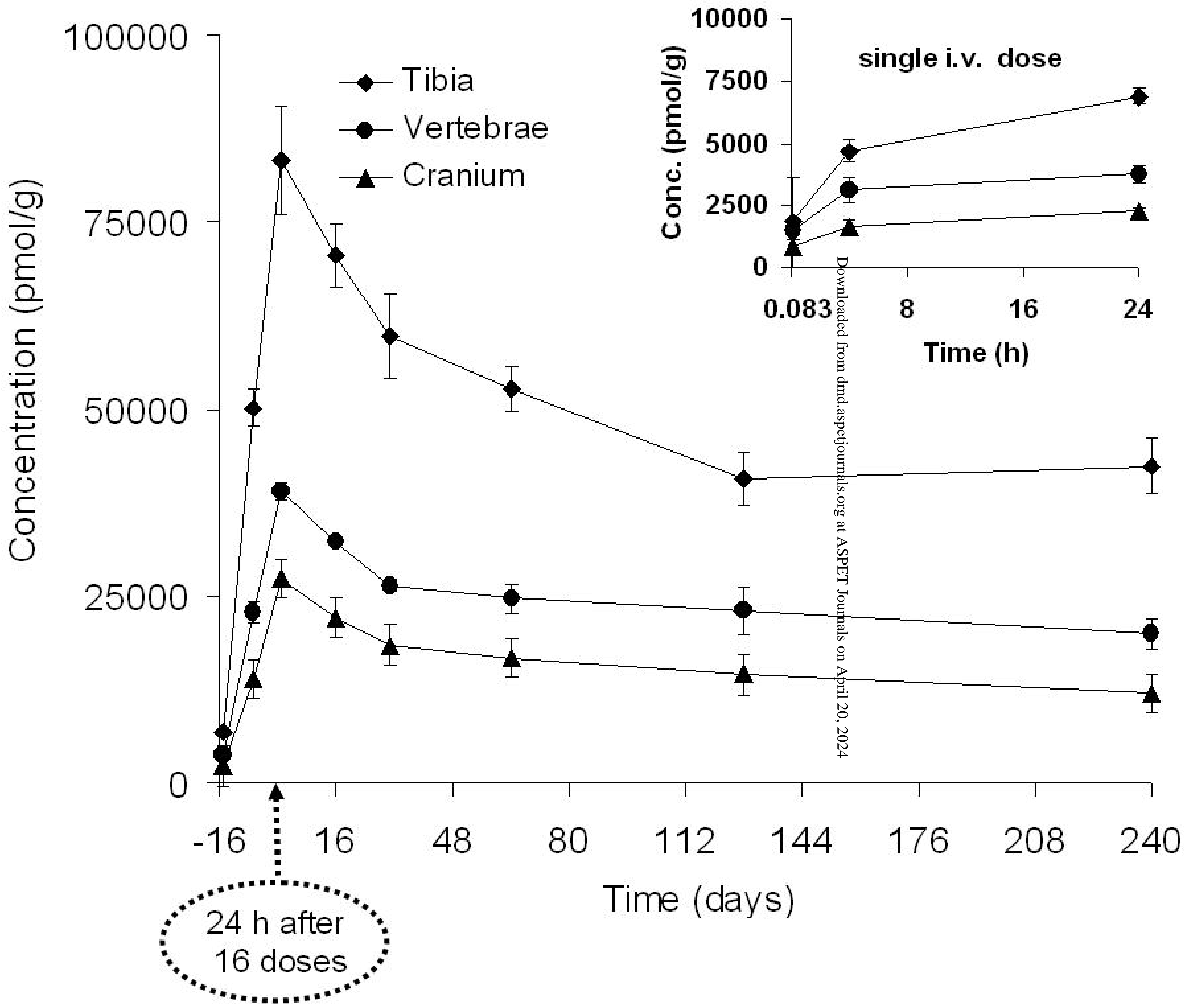

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Figure 2

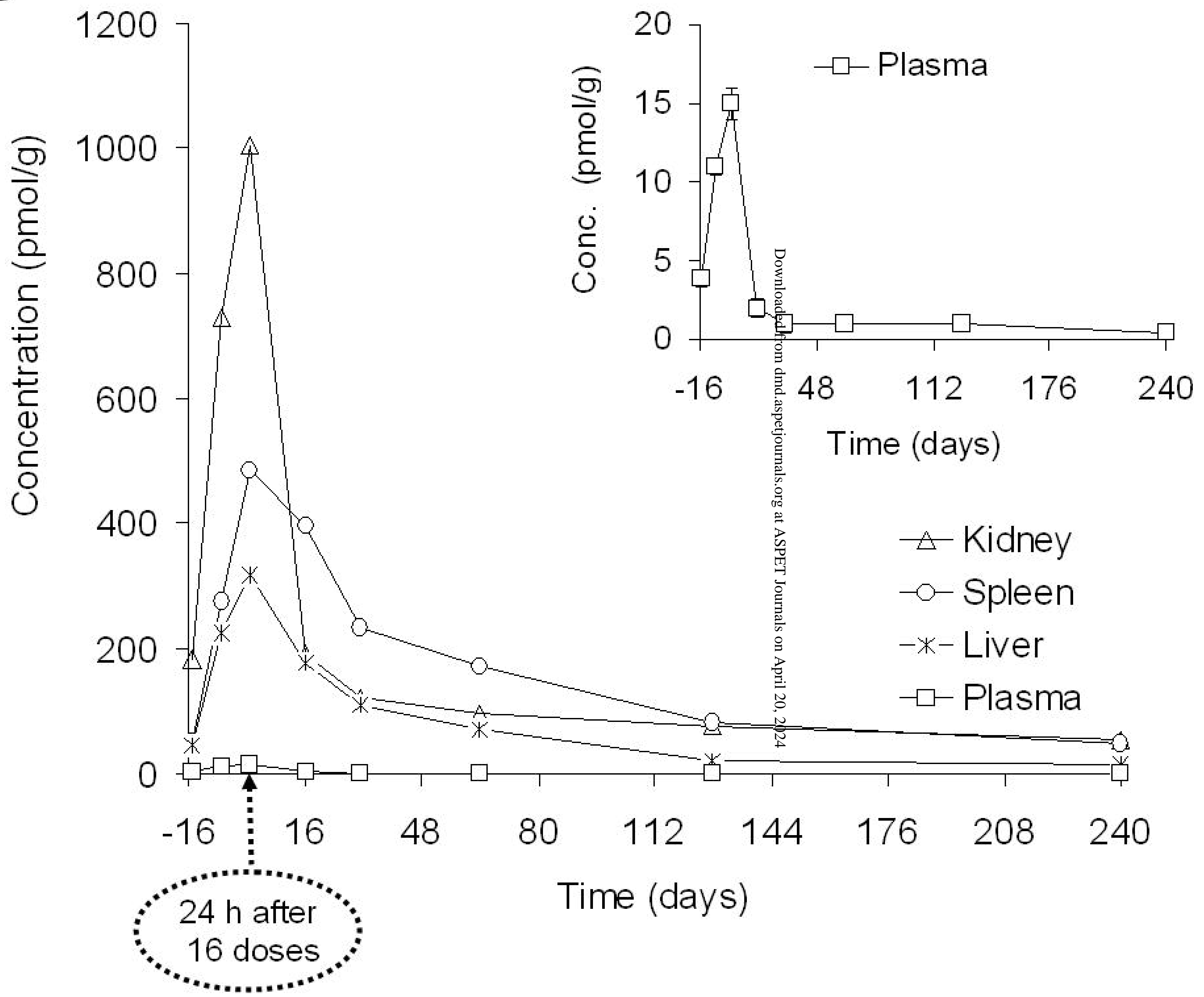

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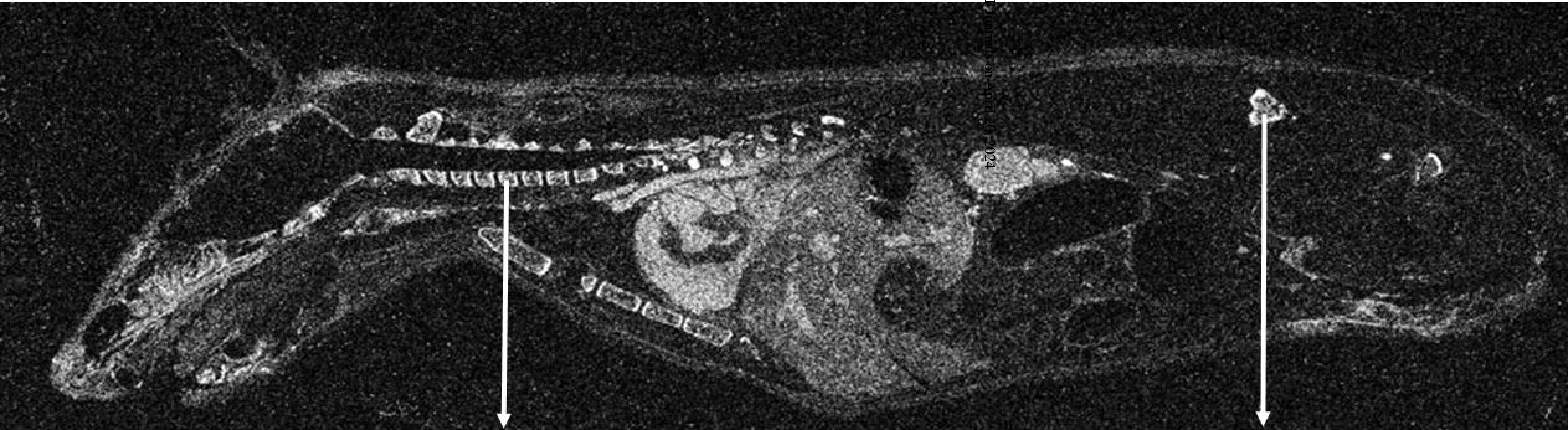
Figure 3

A



Vertebra **Tibia**

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Figure 3

B

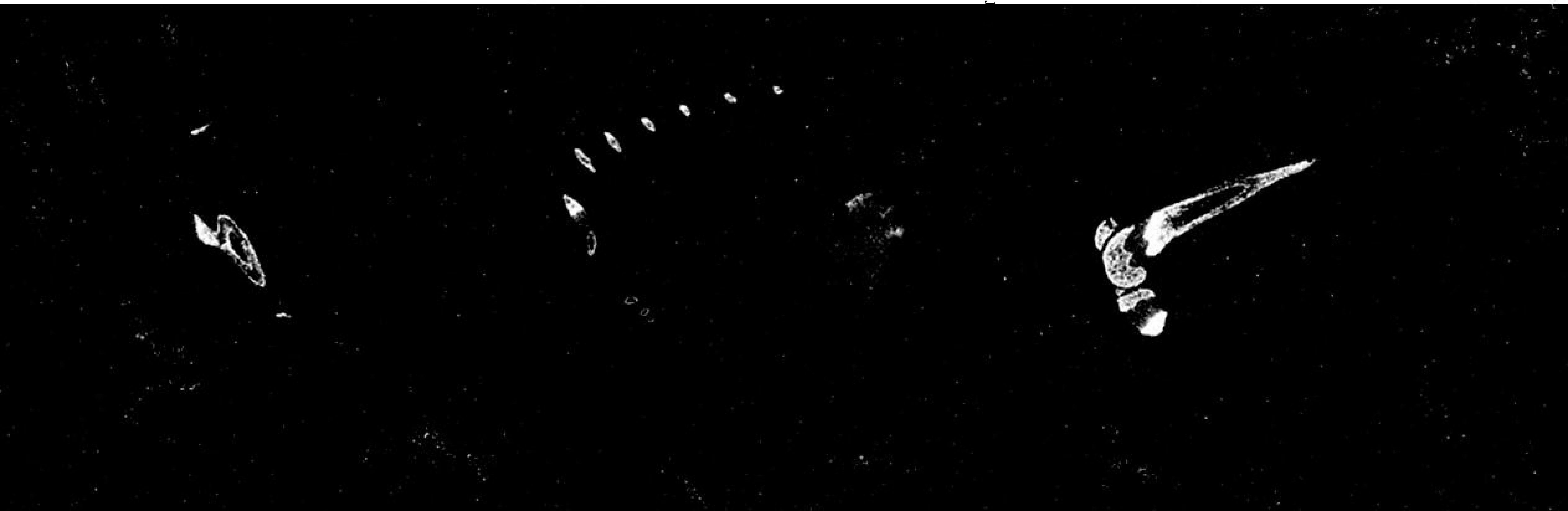

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Figure 4

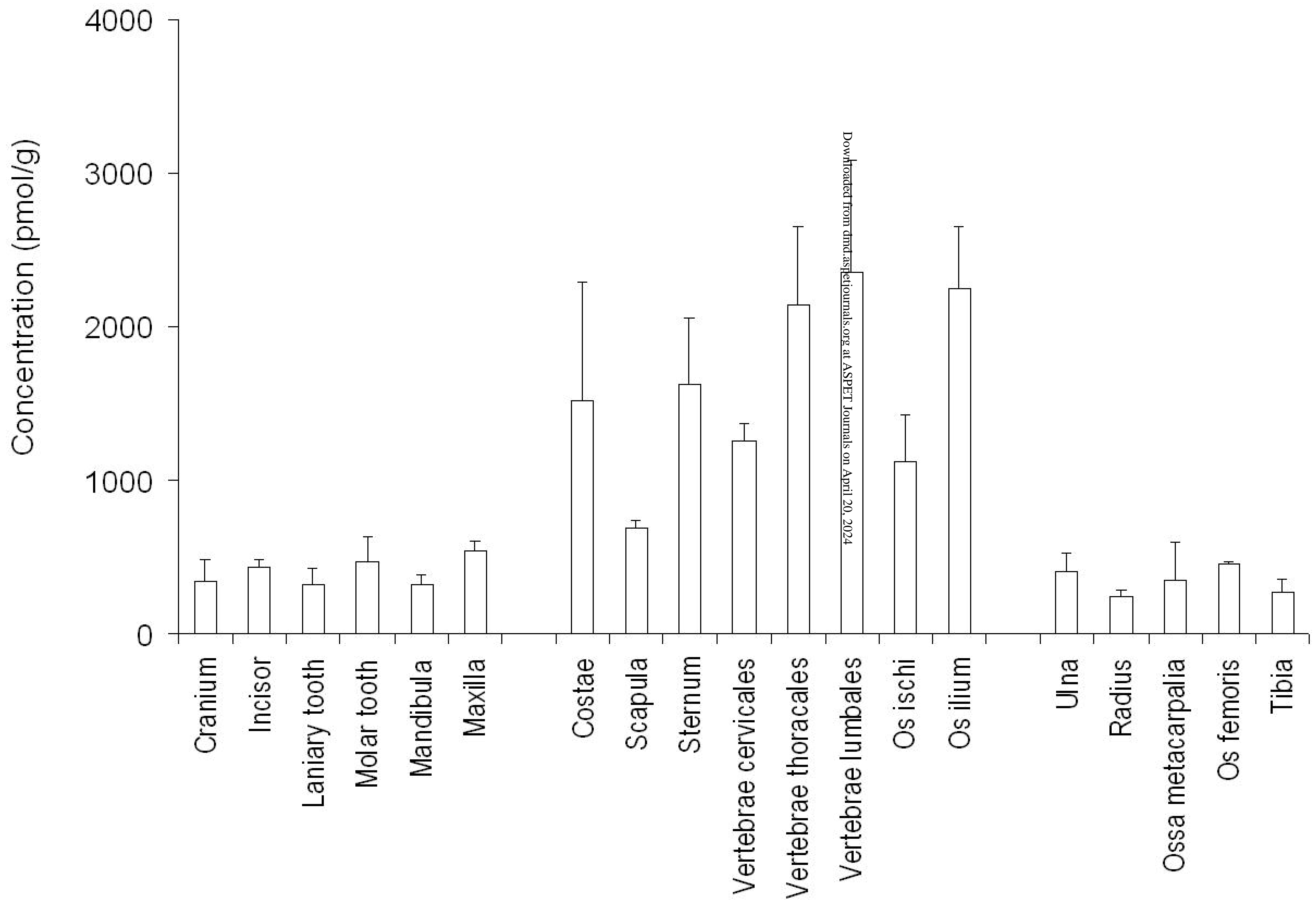

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Figure 4

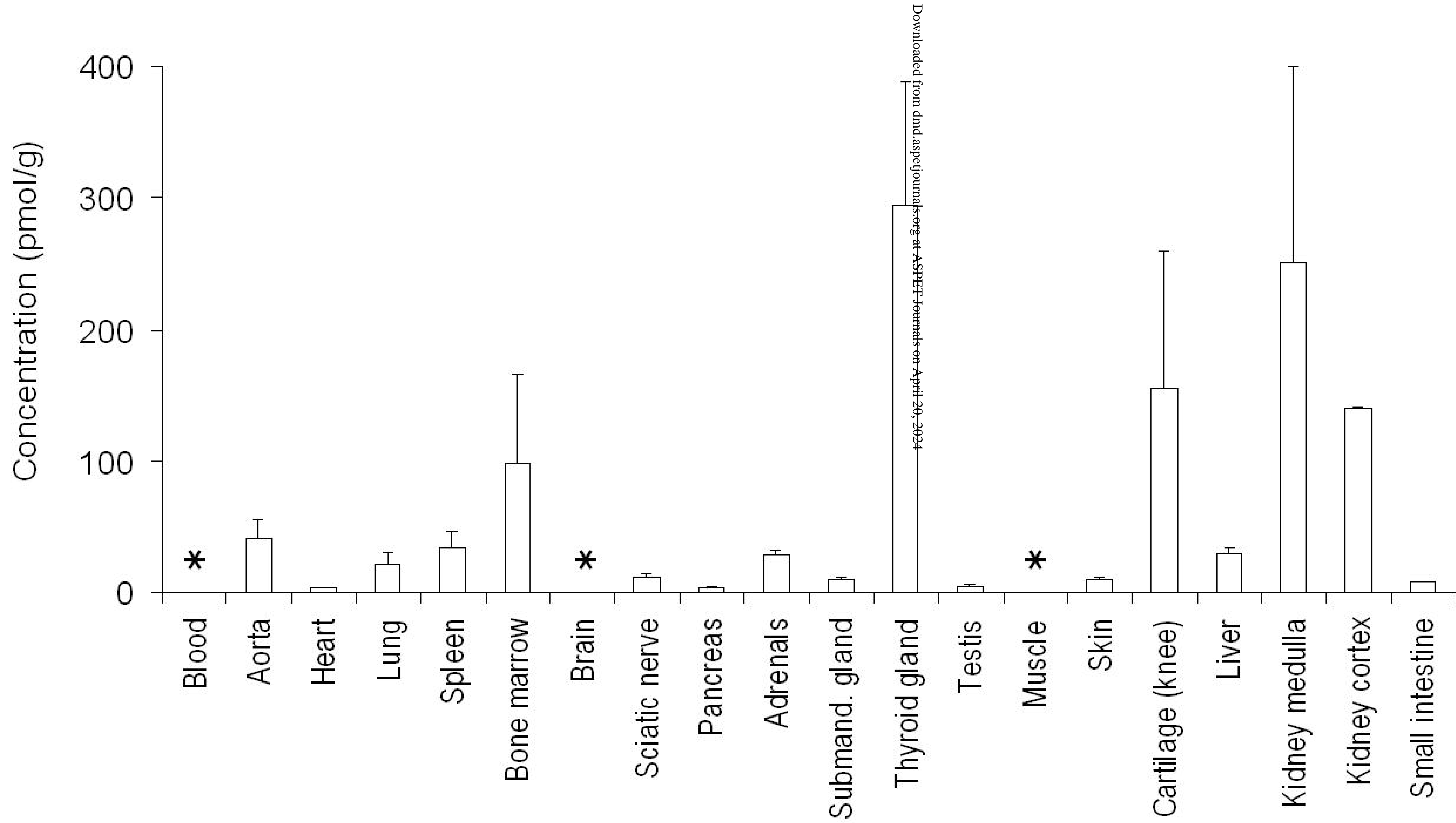

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Figure 5

A

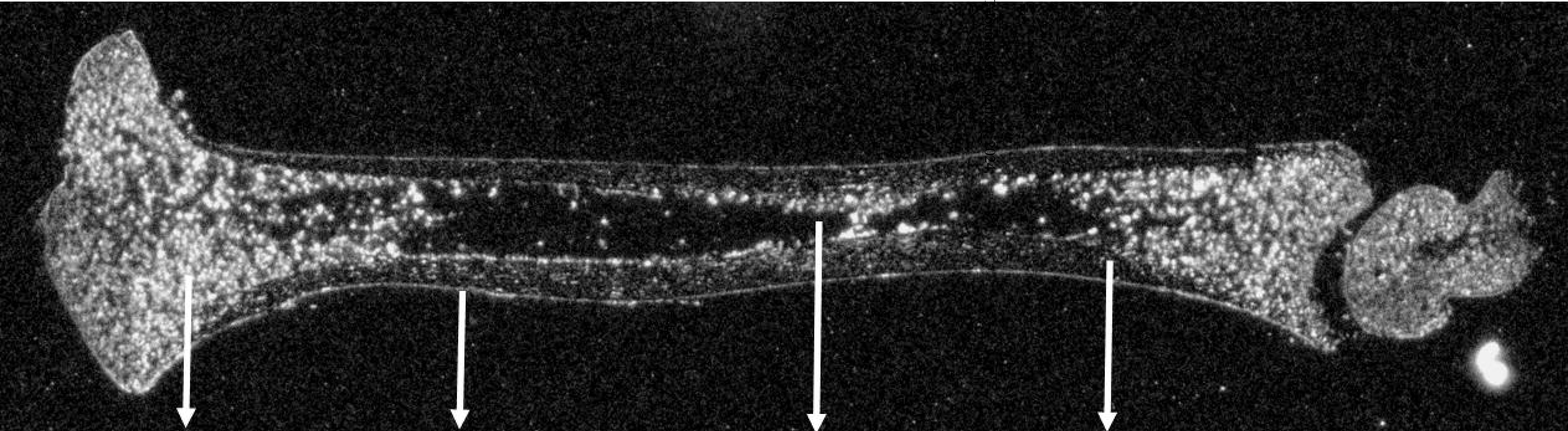

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Figure 5

B

Spinal chord

Vertebra body

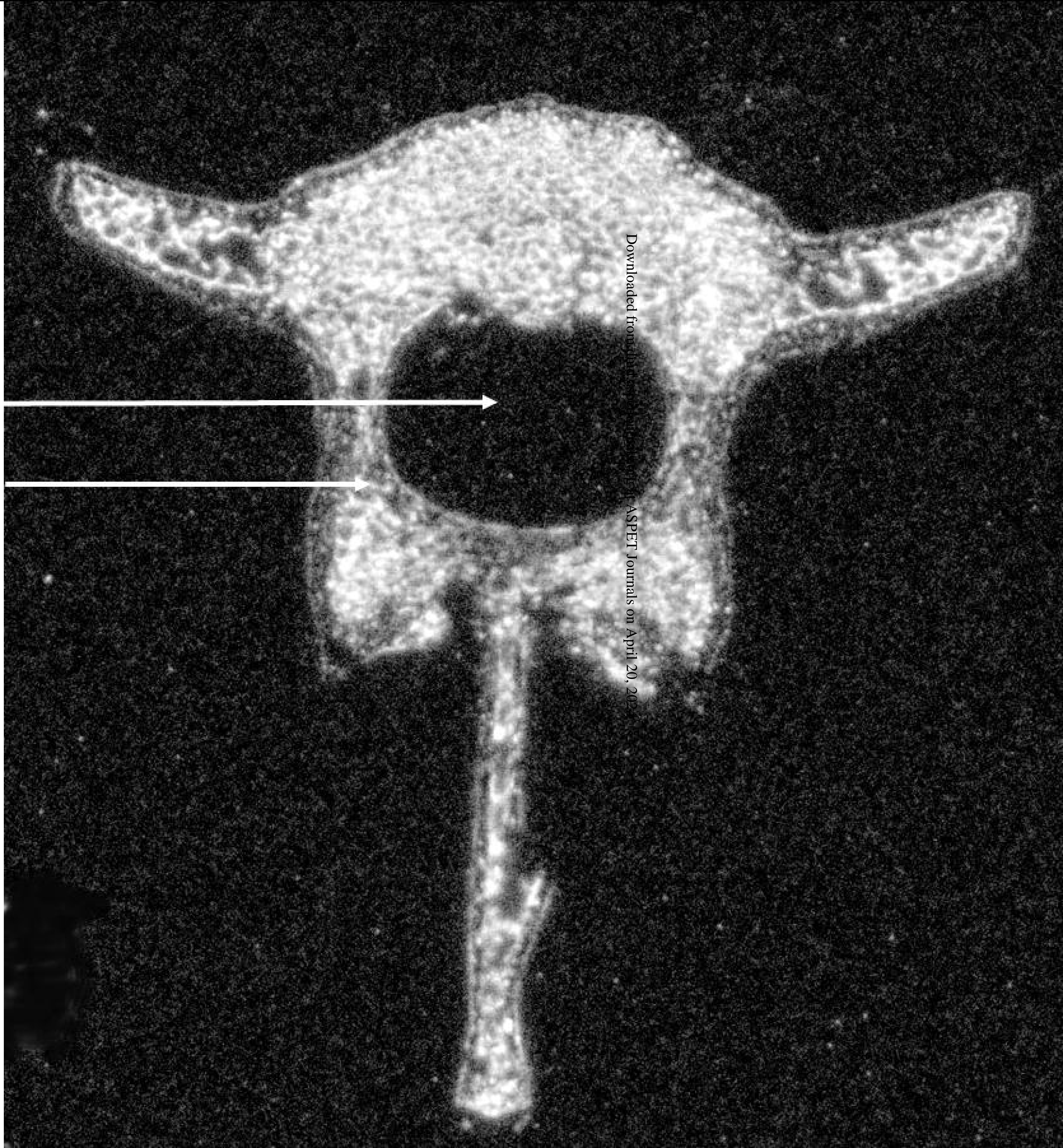

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Figure 6

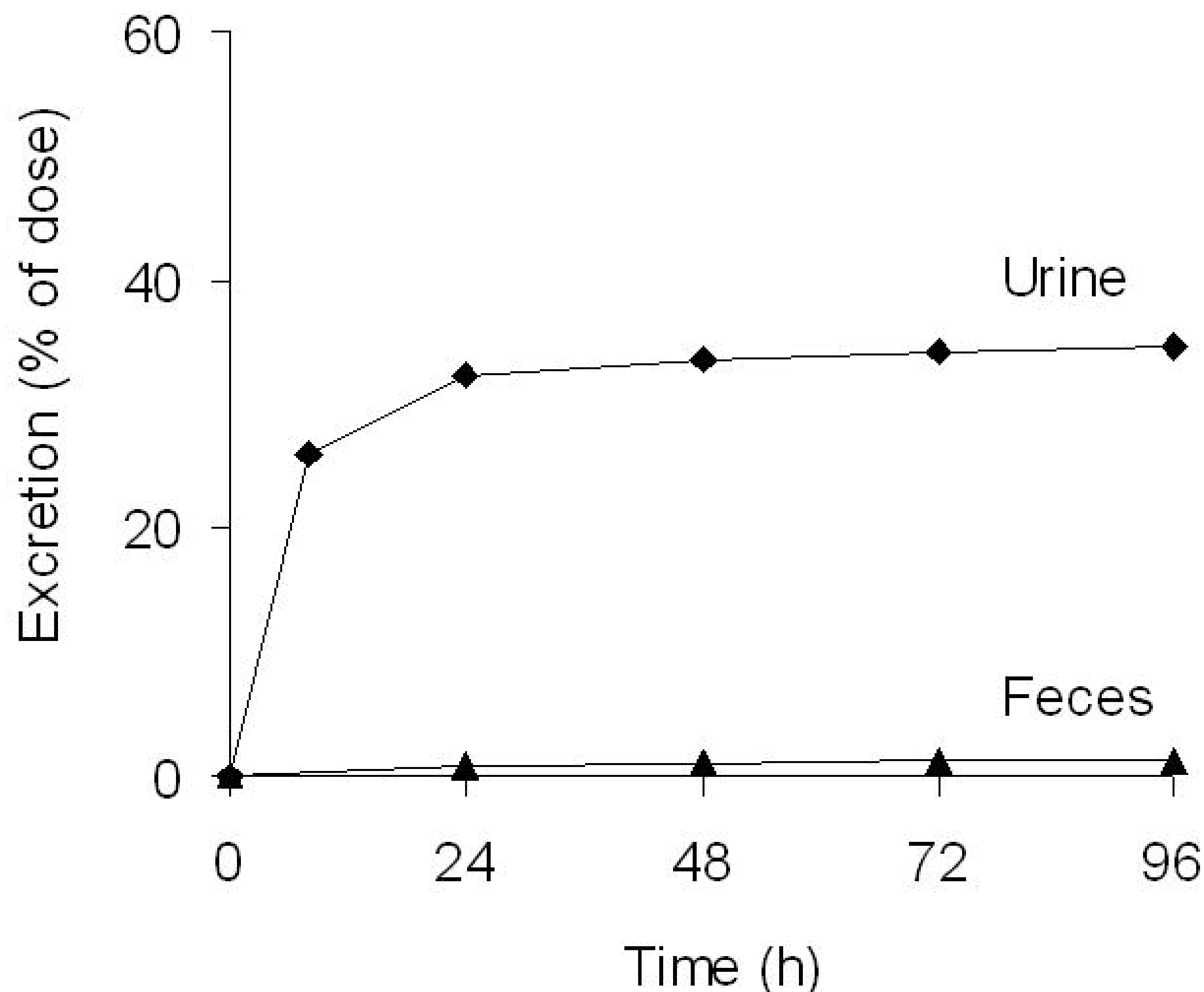

Figure 6