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Using μX-Ray CT to observe postmortem diffusion from the stomach in a rat model.

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Highlights

- Postmortem diffusion of drugs from stomach can hinder interpretation
- X-RAY CT can visualise postmortem diffusion from the stomach in a rat model
- Body position influences direction of diffusion from stomach
- Most diffusion from the stomach occurs within the first 24 hours

Abstract

The stomach has long been recognised as a depot for postmortem diffusion. A better understanding of the phenomena of postmortem diffusion would aid forensic practitioners in their interpretation of toxicological results. A limitation of previous stomach diffusion studies was the lack of ability to visualise postmortem diffusion in real time, the use of μX-ray Computed Tomography (CT) could overcome this problem. We utilised CT to track the diffusion of the contrast medium caesium ions (Cs^+) (administered by oral gavage) from the rat stomach over 6 days. We investigated the influence of temperature (4°C and 20°C) and body position (horizontal and vertical). The results show that the a) diffusion of Cs^+ from the stomach can be visualised over 6 days, over which a significant amount (~50 %) of the diffusion occurs in the first 24 h following administration; b) storing the rat at 4°C reduces the distance of diffusion from the stomach by ~ 66%; c) body position influences the route of diffusion and d) in 2 of the 16 rats studied Cs^+ was found in the right lobe of the liver. Overall these

results show that CT using Cs^+ is a good model to visualise postmortem diffusion and that bodies show significant variation in postmortem diffusion.It is also clear that bodies should be refrigerated and postmortem samples should be taken as soon as possible to minimise the influences of postmortem diffusion from the stomach.

KEYWORDS: Postmortem redistribution, temperature, body position, μX-Ray CT, stomach, forensic toxicology, interpretation.

1. Introduction

In unexplained death investigations forensic toxicologists are required to determine if drugs may have played a role in the death, and if so to what extent. It is currently well understood that the concentration of drug determined postmortem might not accurately reflect any drug concentration in that sample at the time of death. The main reason for this is thought to be the "toxicological nightmare" of postmortem redistribution (PMR) [1]. PMR is well-recognised phenomena that involves the increases or decreases of drug concentration at a specific site(s) in the body between the time of death and the time of a postmortem sample being taken. The factors that can contribute to PMR include the postmortem interval (time elapsed since time of death (TOD) to autopsy) [2,3], postmortem circulation [4] and the putrefactive process [5]. The main mechanism for PMR is thought to be via passive diffusion [6]. The organs in which drugs concentrate during life (such as the lungs and the heart), can act as drug depots that contain high drug concentrations and produce a locus from which a drug can diffuse into the surrounding areas [7]. This makes it important to sample from sites (such as the femoral vein and popliteal vein) that are not subject to large influences from PMR [1,8]. At the time of death the stomach can contain substantial quantities of unabsorbed drugs [1]. If death occurs before the drug has time to fully absorb this would give a locus for postmortem diffusion of drugs. Drugs such as barbiturates and tricyclic antidepressants, that inhibit gastric emptying may also lead to decreased absorption from the upper small intestine antemortem, especially in overdose cases [9]. These factors make the stomach a potential depot for PMR, depending on the individual case.

Although there have been numerous animal [6,10–14] and cadaveric models [15–17] that have investigated postmortem diffusion from the stomach, to date there have been no studies that have been able to investigate diffusional routes in the same animal at multiple time points, or to visualise the routes of postmortem diffusion. The use of μX-Ray Computed Tomography (CT) allows a series of X-rays images to be combined to produce a 3-dimensional (3D) image [18]. This technique may allow the visualisation of postmortem diffusion hence giving a greater understanding of the phenomena, leading to improved interpretation of forensic toxicology results.

The aim of this study was to validate the use of CT as a tool to observe diffusion routes and distance of the contrast agent caesium ions (Cs^+) from the stomach of a rat over 6 days at 4°C and 20°C. We also investigated the influence of the body position (horizontal, to mimic lying down and vertical to mimic the sitting position or hanging) on diffusion of Cs^+ from the stomach.

2. Materials and Methods

2.1 Chemicals and Reagents

An aqueous solution of caesium chloride (2.0 $mol.L^{-1}$) (Bethida Research Laboratories Ltd. Gaithersburg, Maryland, USA) was found to be suitable for CT visualisation based on previous studies [19].

2.2 Study Design

Wistar Rat (*Rattus Rattus*) frozen cadavers with a mean weight (± standard deviation (SD)) of 191.5 ± 6.57 g were sourced from a local pet store and were defrosted overnight at 4°C prior to any experimentation. The variables for the study were 4 °C (horizontal and vertical; groups H4 and V4) and 20 °C (horizontal and vertical; groups H20 and V20). Accordingly, a power calculation was carried out to determine the number of rats that would needed for each variable of the study (MiniTab® 18, (Minitab Inc)). This analysis determined that 4 rats were suitable for each study variable with a power of 0.86, hence 16 rats in total were utilised. (See supplementary material S1).

The horizontal rats were placed in a supine position for CT scanning before being returned to a horizontal position as the scan area was not large enough to scan the rat horizontally.

Each rat was weighed and administered a volume of Cs^+ solution in the supine position by oral gavage using a 2 ml syringe with flexible catheter tubing (Steriseal, UK). The volume of Cs^+ solution administered was dependent on the weight of the rat to ensure the stomach was not overfilled. 1 mL of Cs^+ solution (2.0 $mol.L^{-1}$) was administered for rats weighing less than or equal to 200 g and 1.5 mL was administered for rats weighing greater than 200 g. For groups H20 and V20, each rat was allowed to equilibrate to room temperature before the administration of Cs^+. In order to confirm the administration of the caesium to the stomach the individual rats were CT scanned as soon as possible (within 30 minutes following administration). Each rats were then scanned at time intervals of 1, 2, 3 and 6 days; (24, 48, 72 and 144 h).

2.3 μX-Ray CT Scanning

A Nikon Metrology HMX 225 μCT scanner (Nikon, UK) with maximum energy of 225 kV and maximum current of 1000 µA, a 5 µm focal size and a tungsten target was used for data collection. The scan parameters were set to; 70 kV, 89 µA with a 1 mm aluminium filter and 700 projections were collected per scan. All rats were scanned in the vertical position. Projections were converted to volume files by CTPro (Nikon, UK)

2.4 Data and Statistical Analysis

VGStudio Max Version 3.1, (Volume Graphics, GmbH Germany) was used to analyse the volume files produced. The diffusional distance of Cs^+ was measured using the surface determination and distance tools. Diffusion distance was measured in six different trajectories: caudal, rostral, ventral, dorsal, lateral (right) and lateral (left) see Figure 1.

IBM SPSS v24 (IBM Corp©, USA) was utilised to analyse the data produced from the diffusion measurements. A Linear Mixed Model (LMM) was chosen to determine significant difference for each factor (temperature, body position, time) at each trajectory. This model was chosen as the study used a repeated measure, i.e. rats measured at different time points, with position and temperature as fixed factors and time as a covariate. The rat number was treated as a random factor (see supplementary material S2). To satisfy the assumptions of the LMM, histogram and normal Q-Q plots were produced to confirm residuals were normally distributed (see supplementary material S3). Statistical significance (α) was set at ≤ 0.05 for all tests. F is the variation between sample means/ variation within the samples [20].

3. Results and Discussion

The aim of this study was to validate the use of CT to visualise diffusion of Cs^+ (as a substitute for drugs/alcohol) from the stomach using a rat model. The rats were scanned at 1, 2, 3- and 6-days post caesium administration and were stored at either 4 °C or 20 °C in horizontal (supine) or vertical positioning.

3.1 Statistical Analysis

All residuals of each model were found to be normally distributed according to the normal Q-Q plot and histograms. There was a linear relationship between the predicted values of the model and the dependent variable, the trajectory. The residuals and the predicted values were independent, so all assumptions of the LMM were satisfied for each trajectory test. For caudal, rostral and lateral trajectories, there are significant interactions between position and temperature, position and time and temperature and time. It is important to note that significant main effects are difficult to interpret where there are significant interactions. All statistical output for the LMM can be seen in supplementary material S2.

3.2 Visualisation of caesium diffusion from the stomach using CT

As can be seen in figures 2 - 5 CT was able to visualise the diffusion of Cs^+ from the stomach over a time period of 6 days. Visually it can be seen that for H20 and V20 groups the volume Cs^+ diffused into was greater than (Fig 2 and 3) that for H4 and V4 groups (Figure 4 and 5). It was also observed that for H4 and V4 groups the Cs^+ moved more as a single mass and did not exhibit as widespread a dispersion as the H20 and V20 groups. A recurring observation within this study was the natural putrefactive process that occurred over 6 days (data not shown). This took the form of discrete pockets of gas encapsulating the organs (specifically in the torso). These were mostly visible in groups H20 and V20. The swelling of these gas pockets over time could potentially aid mixing of blood and other body fluids and therefore change the concentration of drugs from site to site. These results are supported by previous work that has shown the influence of putrefaction on postmortem blood circulation [4].

The CT visualisation also highlighted differences between individual rats. In 3 of the 16 rats some of the Cs^+ was observed to have moved into the small intestine (example in Fig 6). The movement of stomach contents to the small intestine was previously observed in human cadaveric studies of alcohol and drug diffusion from the stomach [15,17]. Together these previous studies and the current study show that postmortem diffusion may occur from both the stomach and the small intestine. This differing sites of diffusion could potentially lead to significant case to case variation in PMR and would further complicate any interpretation of postmortem drug concentrations. The individual case variation we observed was further illustrated by the CT visualisation of Cs^+ diffusion in and around the liver. Deep within the right lobe of the liver is considered to be the best postmortem liver sample for the investigation of drug concentrations due to the right lobe of the liver being furthest away from the stomach and considered to be the least likely to be influenced by PMR from the stomach [15–17]. However the right lobe is not as immune to PMR as might be expected (example Figure 7) as there was Cs^+ detected around the right lobe of the liver in 2 of 16 rats. Further investigation showed Cs^+ was present throughout the end right lobe of the liver (data not shown). This is not unexpected as previous studies using human cadaver models of diffusion from the stomach (using both drugs (amitriptyline, acetaminophen and lithium) and volatile solvents [15,16]) and animal studies (using

ethanol-d6 [21]) showed that these substances were detected in the right lobe at the liver although at much lower concentrations and a later time than found in the left lobe. Our work does however reiterate that PMR can be unpredictable and can vary significantly from case to case, but that deep within the right lobe is still the best position for a postmortem liver sampling.

3.3 Trajectories of diffusion of Cs^+ from the stomach.

3.3.1 Influence of body position

Previous work investigating the diffusion of drugs and alcohol from the stomach have only studied diffusion when the cadaver has been left in a supine position [15, 17]. We studied the influence of body position on the diffusion of Cs^+ from the stomach, particularly as it is well known that the postmortem blood lividity and hypostasis is influenced by gravitational pull [22]. The vertical body positioning in this study simulates a death that occurred in an upright position (i.e. a hanging or seated) and then the individual was not discovered for a few days. In both the supine (groups H4 and H20) and vertical (groups V4 and V20) positional studies an increase in diffusional distance was observed over time (figure 8 and 9). Statistical analysis showed that body position had a significant influence on diffusion in the caudal, dorsal, lateral (L) and ventral trajectories (see supplementary material S2). The largest amount of diffusion from the stomach was in the caudal (towards the tail; 49 ± 6 mm) and the lateral (L) directions (left of the stomach; 27 ± 7 mm) after 6 days in H20 group (figure 8A). These results mirror previous rat and human cadaveric studies where drug/alcohol concentrations were observed to be greater in the left kidney, compared to the right [15,16,21]. As the intestinal tissue or peritoneal fluid are not common samples in forensic studies the diffusion of substances has not previously been investigated in these areas, so this study is the first to visualise and measure diffusion into the abdominal cavity. Tissue permeability and differences between tissue and organ permeability, may explain the routes of postmortem diffusion of Cs^+ [23]. As diffusion into the abdominal cavity around the intestine would offer the "path of least resistance", it is not unexpected that the largest amount of diffusion observed was in the abdominal cavity. The diffusion in certain

directions (such as dorsal (downwards)) would of course have been limited due to lack of anatomical space in supine rats.

In the V20 group the most extensive diffusion route of caesium ions over 6 days was in the caudal (downwards) direction and also in the lateral (left) direction with a diffusion of 38 ± 4 mm and 23 ± 2 mm respectively after 6 days (Figure 9). This was to be expected as it follows the gravitational pull and also follows the route of "least resistance". The diffusion in the caudal direction (down in vertical; towards tail in supine) was smaller in the V20 group compared to the H20 group, with diffusional distances of 38 ± 4 mm and 49 ± 6 mm respectively. These differences may be due to variations between each rat, pressure of the abdominal wall on the abdominal area or the possible distribution of Cs^+ caused by the movement of the supine rat to the vertical position for scanning. The differences in diffusion mirrors the study of Parker *et al.* where the changes in tissue concentrations of secobarbital were investigated in rats after oral administration. Although differences in liver concentration of secobarbital were observed they were not significantly different [24], but they do illustrate that body position may influence PMR after death. The results in this study show that further work should be carried out to determine if there is increased diffusion of drugs to specific anatomical sites solely due to body position, particularly where individuals have died by hanging and that body position and postmortem manipulation of a body may need to be taken into account when interpreting drug concentrations in death cases.

3.3.2 Influence of temperature

Fickian diffusional theory [25] and previous experimental work [17] have demonstrated that the cooling of cadavers reduces the rate of diffusion and will limit the influence of PMR between the arrival of a cadaver at a mortuary and the commencement of post-mortem sampling. We investigated the influence of the diffusion of Cs^+ from the stomach when the rat was stored at 4 °C in the supine position (V4 group). Temperature was shown to have a significant influence on diffusion in the caudal, rostral, ventral, lateral (L) and lateral (R) diffusion (see supplementary material S2). There

was no influence of temperature on dorsal diffusion between groups V4 and V20 as expected due to the limited anatomical space available for diffusion. In the direction of the greatest amount of diffusion (caudal), it can be seen (Figure 3B) that the diffusional distance of Cs^+ was reduced by ~66 % over 6 days in the V4 group compared to the V20 group. Thus illustrating how important it is to cool cadavers to reduce postmortem diffusion from drug depots. This study did reveal that diffusion of Cs^+ for rats in both V20 and H20 groups, was extremely widespread through the trunk of the body, as can be seen in Figure 2 and 3, whereas V4 and H4 showed the Cs^+ moving more as a single mass and did not exhibit the same spreading (Figure 4 and 5). The work in this study re-enforces the guidance that cadavers should be stored at fridge temperature as soon as possible after being found.

3.4 Speed of postmortem redistribution

The speed of PMR is often not appreciated even though studies have shown how rapid it can be with drug concentrations changing in minutes [26,27]. In this study figures 8 and 9 illustrate that within the first 24 h for 3 out of 6 trajectories, time (h) had a significant influence on the diffusion trajectory (caudal: $p < 0.001$, lateral: $p = 0.001$, ventral: $p < 0.002$). Overall 50% of the total diffusion of Cs^+ (based on distance) occurs within the 1st 24 h of the study (supplementary material S2). This is expected as it follows Ficks law of diffusion where major diffusional flux would be expected to happen rapidly and then decrease as time increases [25]. The timing of the individual directional diffusions would depend on the specific circumstances. Previous studies have also shown that the maximum amount of diffusion is within 24 h [15,17,21,28] but with some changes, such as blood drug concentrations and this can alter significantly within minutes, specifically in animal models [26,27]. It is likely that the rapidity and the magnitude of the changes will depend not only on the concentration gradient present but also the diffusional distance over which the changes are being measured. These results demonstrate that any postmortem sampling, particularly from the femoral vein, should be taken as soon as possible from a cadaver to minimise any potential PMR. The most logical time would be the arrival of the cadaver at the mortuary, as is carried out in some jurisdictions [3], but this may not be possible in all jurisdictions due to local or national legislation.

3.5 Limitations of the study

This study has validated Cs^+ coupled with CT measurements allows the effective visualisation of diffusion routes and that it behaves in a similar way as ethanol and lithium does in human models [15,17]. However the model does suffer from a number of limitations. A) As a small animal model this would not be directly comparable to the diffusion seen in humans due the very different surface area to volume ratios and the diffusion distances. Ideally this model would need to be validated in larger animals (such as pigs) or humans to allow direct comparisons to humans. B) The use of animals that had have been frozen is likely to lead to changes in membrane permeability that would not be observed in unfrozen animals, potentially leading to different diffusion routes/speed than that which would be observed in a freshly deceased animal/human. C) As Cs^+ is not an organic drug molecule it would not exhibit the same physiochemical properties a drug molecule would (such as changing ionisation at different pH and lipophilicity) and could potentially diffuse across membranes differently [29], in further studies the use of radiopaque molecules that display similar properties to drug molecules would be favourable. For this study however, Cs^+ is a suitable contrast agent where our prime aim is to visualise postmortem diffusion routes from the stomach. D) The limit of visualisation using CT and Cs^+ is that when the Cs^+ concentration drops below a certain level it is insufficiently radio opaque and becomes indistinguishable from the background model. Our CT methodology in this model could however be extended and improved by independently measuring and mapping Cs^+ concentrations at relevant toxicological sampling sites against the CT generated visualisations, thus increasing the limit of detection of Cs^+ and confirming its route of diffusion.

4. Conclusion

We successfully utilised CT to investigate the influence of temperature (4°C and 20°C) and body position (horizontal and vertical) on PMR using Cs^+ as a contrast agent. Our results show that the diffusion of Cs^+ from the stomach can easily be visualised during a 6 day period. It was discovered that majority significant amount (~50 %) of the diffusion occurs in the first 24 h following

administration and that storing the rat at 4°C reduces the distance of diffusion from the stomach by ~ 66%. Additionally, body position influences the route of diffusion and in 2 of the 16 rats studied Cs^+ was found in the right lobe of the liver. Overall these results show that CT using Cs^+ as contrast agent is a good model for the visualisation of post-mortem diffusion and that bodies show significant variation in PMR. From the work it is apparent that bodies should be refrigerated and post-mortem samples should be taken as soon as possible to minimise the influences of PMR.

Author Contributions (CRediT Statement; https://casrai.org/credit/)

Eve Dryburgh	Investigation, Methodology, Formal Analysis, Writing – Original Draft, Writing - Review & Editing
Llinos Honeybun	Investigation, Methodology, Formal Analysis, Writing - Review & Editing
Keith Sturrock	Conceptualization, Methodology, Formal Analysis, Visualization, Writing - Review & Editing, Project Administration, Resources, Supervision
Anne Savage	Formal Analysis, Methodology, Validation, Writing - Review & Editing
Peter D Maskell	Conceptualization, Methodology, Formal Analysis, Project Administration, Validation, Visualisation, Resources, Writing - Review & Editing, Supervision

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Figure 1: Illustration of the anatomical directions in a rat. Arrows show diffusion routes measured from the stomach for each rat.

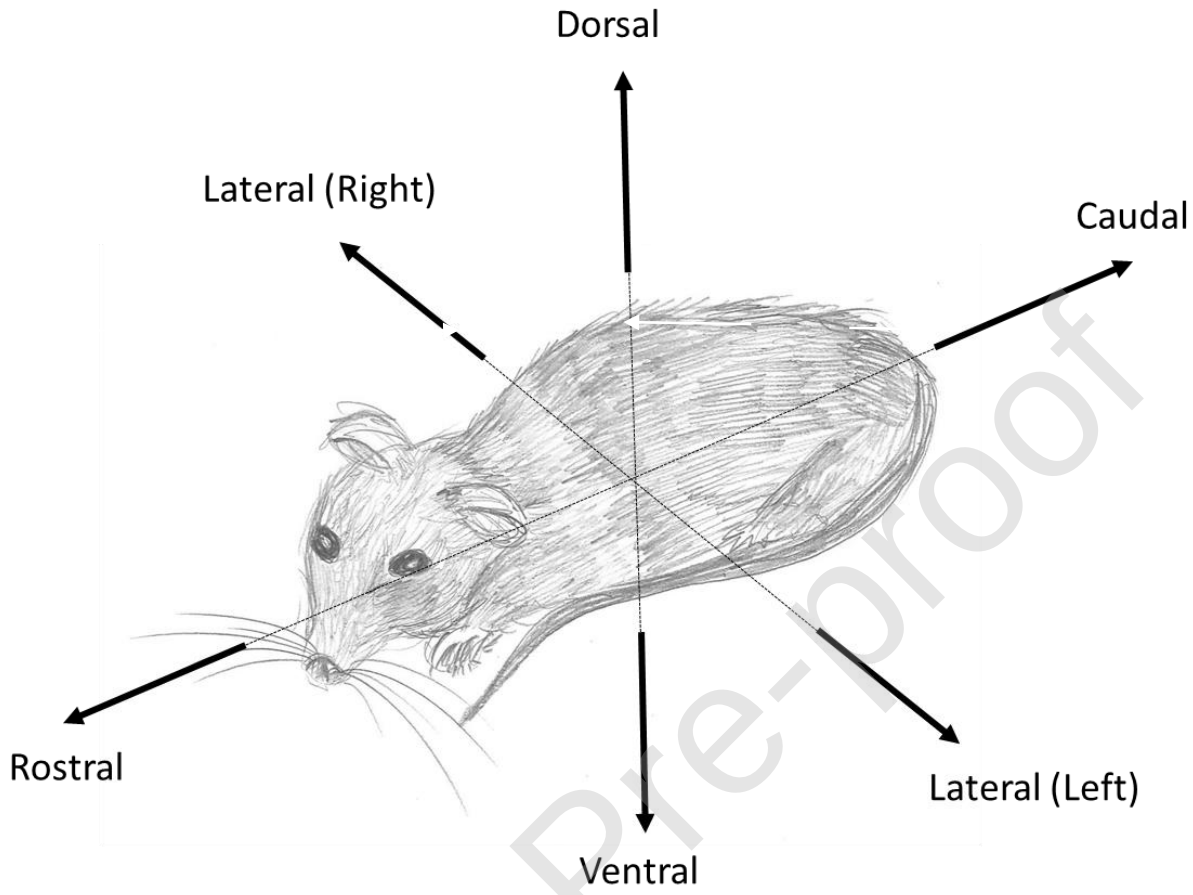


Figure 2: Reconstructed images showing time lapsed scans at a) 1, b) 2, c) 3 and d) 6 days for a 20°C rat kept in the supine position.

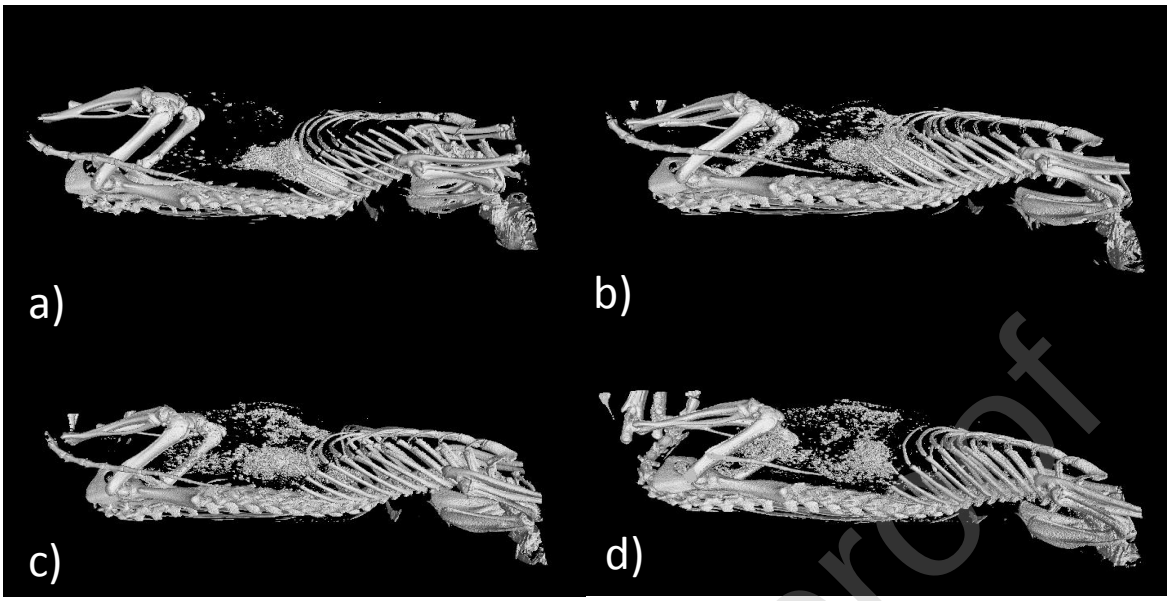


Figure 3: Reconstructed images showing time lapsed scans at a) 1, b) 2, c) 3 and d) 6 days for a 20°C rat kept in the vertical position.

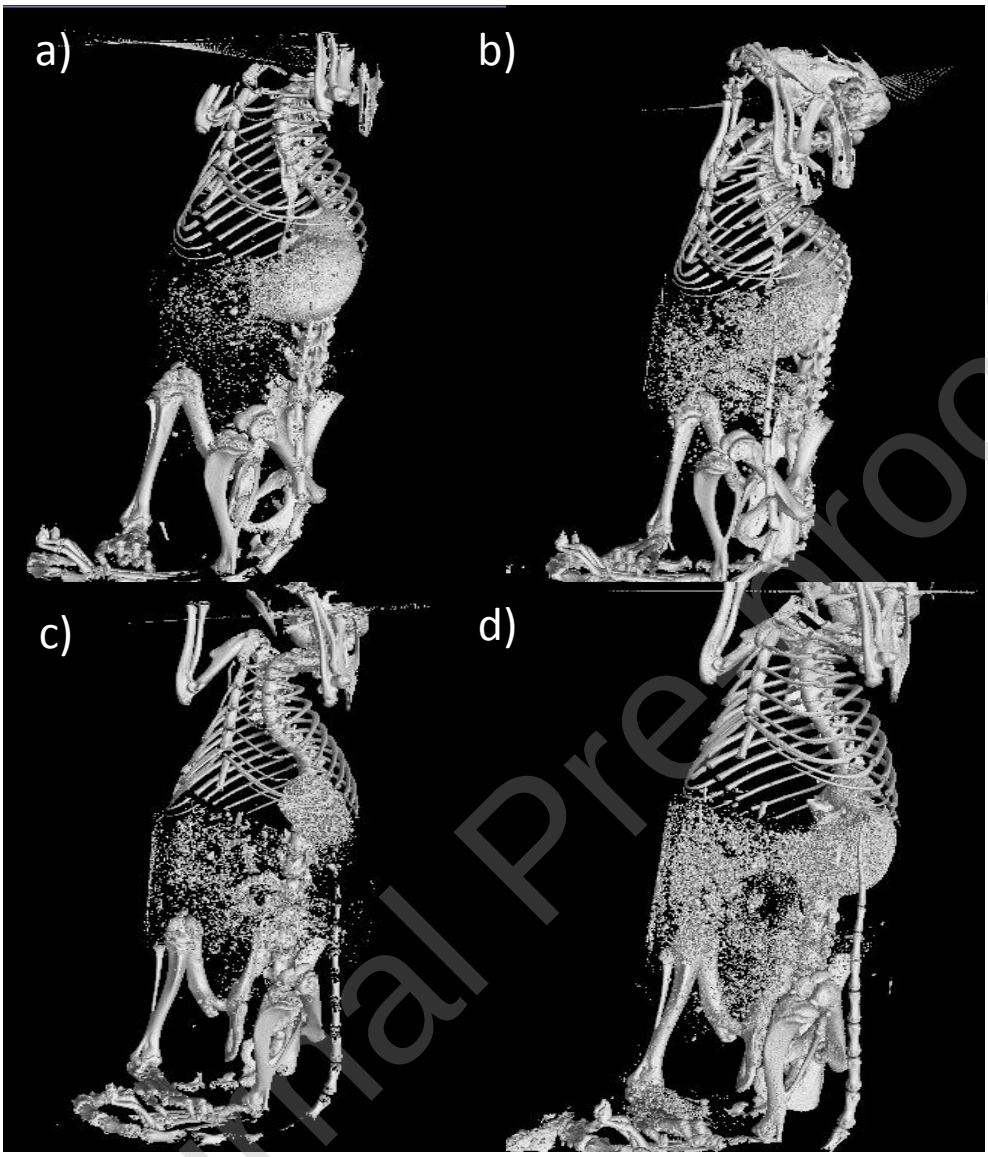


Figure 4: Reconstructed images showing time lapsed scans at a) 1, b) 2, c) 3 and d)6 days for rats stored at 4°C in caudal position.

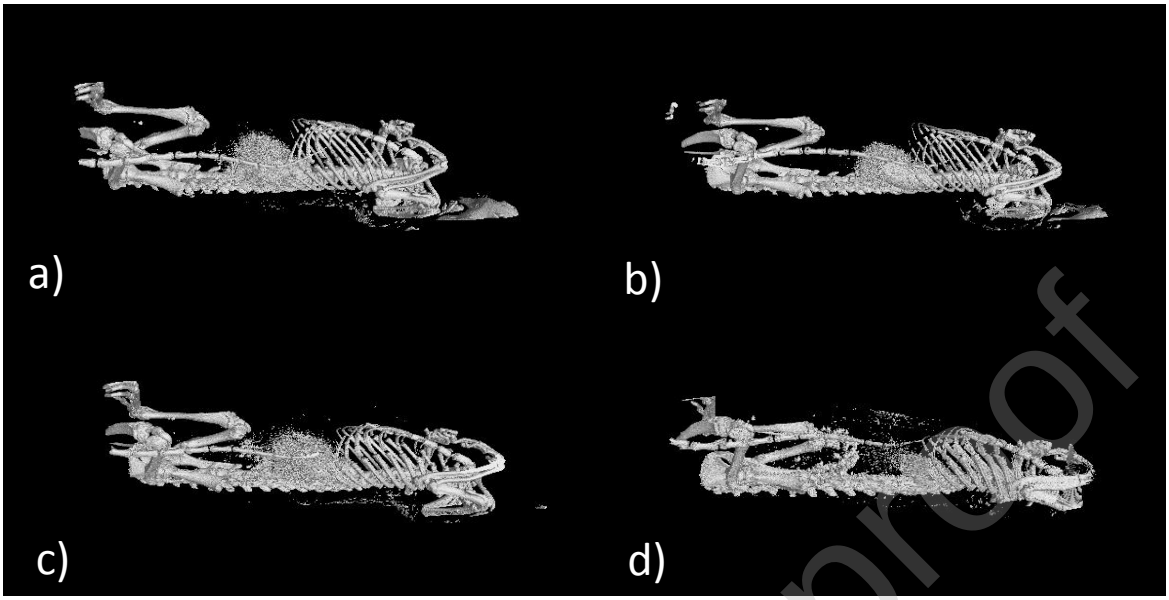


Figure 5: Reconstructed images showing time lapsed scans at a) 1, b) 2, c) 3 and d) 6 days for a rat stored at 4°C in vertic

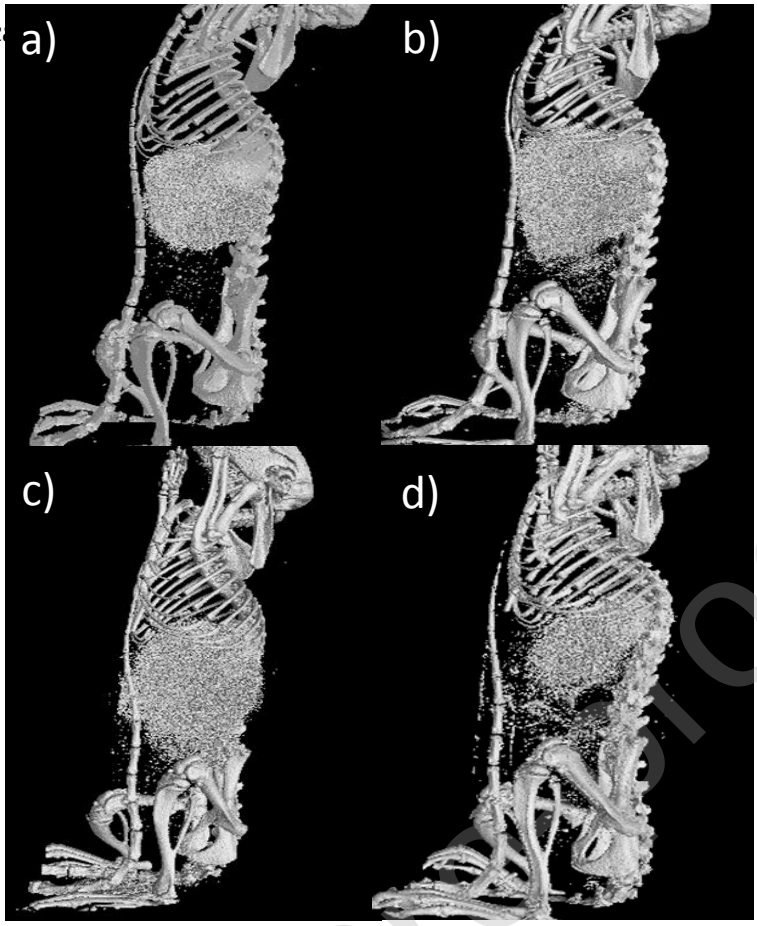


Figure 6: Reconstructed image illustrating caesium in the small intestine (circled) after passing through the pyloric sphincter.

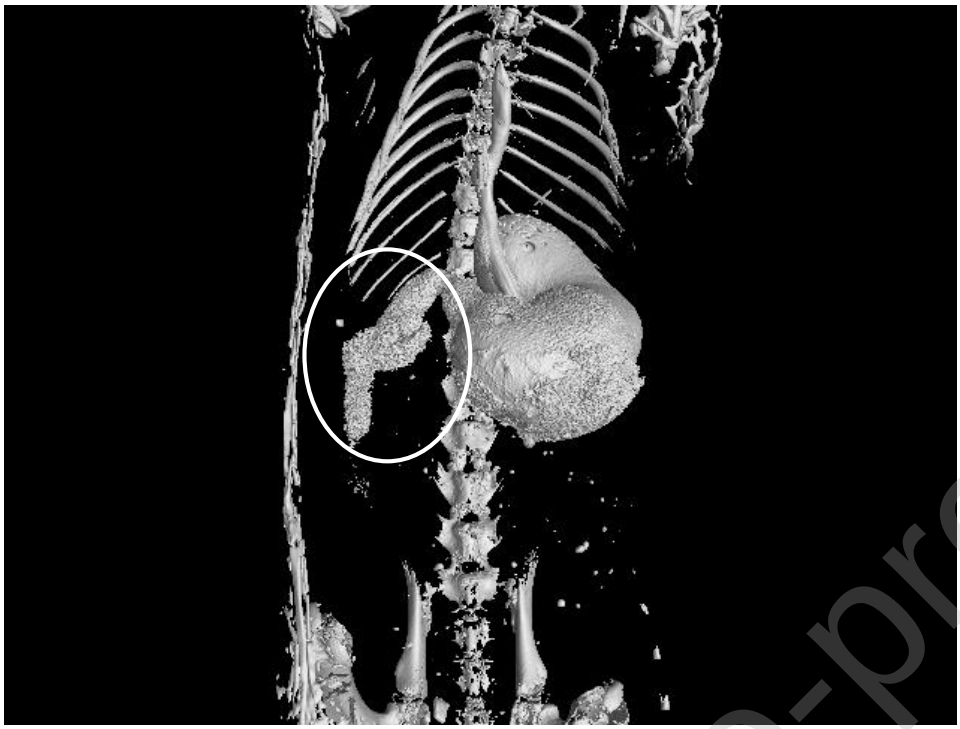


Figure 7: Reconstructed image of vertical body position study at 144 hrs post Caesium administration. Caudal diffusion and lateral diffusion most extensive. Pooling is showing in the 3-D reconstruction in the liver (circled).

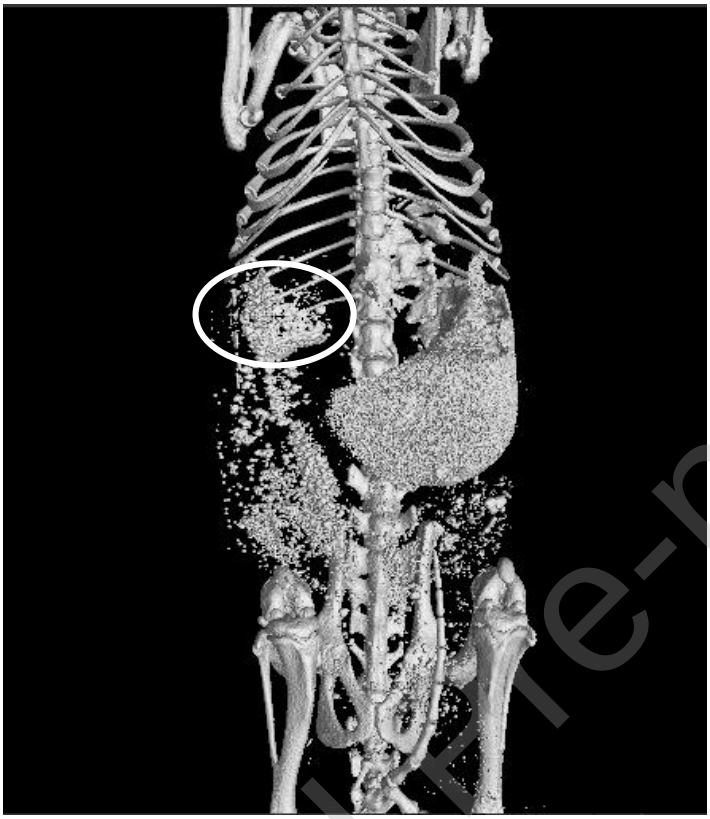
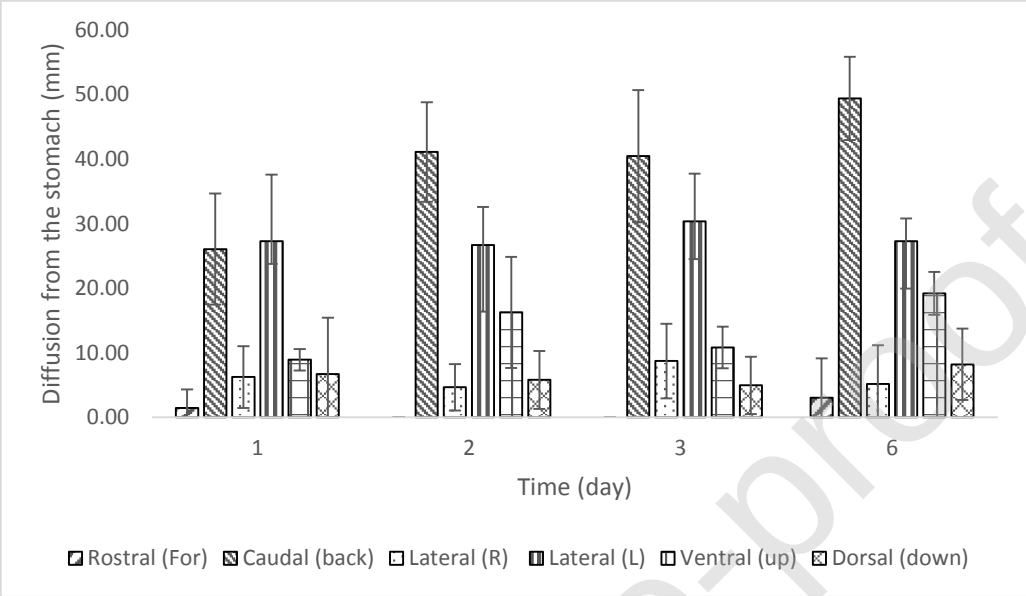


Figure 8: The columns represent the mean diffusion distance of Caesium in mm from rat stomach at 1, 2, 3 and 6 days in each direction for the A) H20 group and B) H4 group. For all time points n = 4. Error bars are standard deviation (SD).

A)



B)

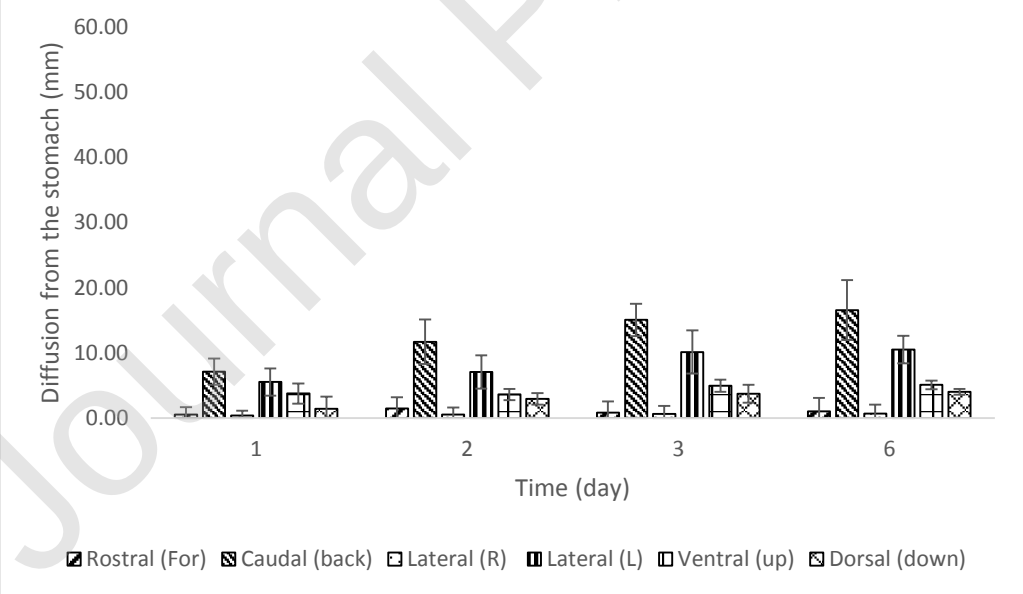
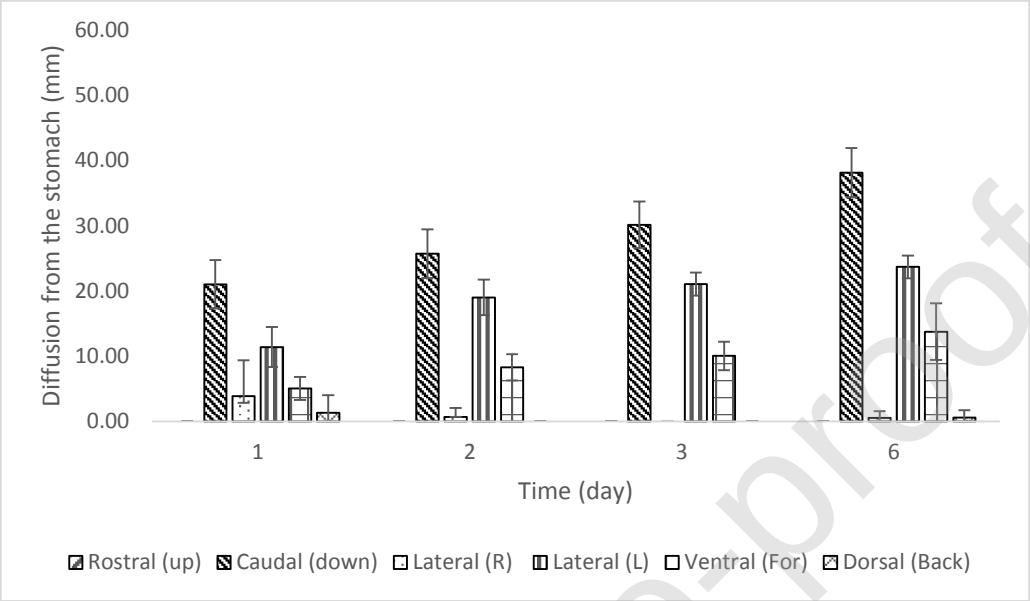


Figure 9: The columns represent the mean diffusion distance of caesium ions in mm from rat stomach at 1, 2, 3 and 6 days in each direction for the A) V20 group and B) V4 group. For all time points n = 4. Error bars are standard deviation (SD).

A)



B)