Comprehensive and Quality Analysis of Freeze-Dried Methylene Diphosphonate Labelled with Technetium-99m: An Extensive Comparison of Biodistribution Study with Commercial Standards

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ABSTRACT

Technetium-99m (99mTc) is an important radionuclide used for the prognosis of bone scintigraphy labeled with a suitable kit. The present investigations focus on the aseptic preparation and application of the methylene diphosphoni acid (MDP) kit on bio-organ after labeling with 99mTc collected from a ^{99}Mo/^{99m}Tc generator. Before going to the bio-application of the labeled kit, radiochemical purity was determined by paper chromatography using the high-purity germanium (HPGe) detector, and the radiochemical purity of ^{99m}Tc-MDP was found to be 99%. Furthermore, negative results were found for pyrogen and bacterial endotoxin tests; fungal growth was also absent. Radiation dose uptake in different organs of Wistar rats was measured by a dose calibrator, and observed results indicate the highest accumulation of ^{99m}Tc-MDP in the skull and skeletal part. This study also performed a comparative study model between laboratory and commercially prepared Monrol MDP kits. This study was performed as a bio-application of laboratory-prepared ^{99m}Tc-MDP prior to commercial production of cold kits at the radioisotope production division of the Bangladesh Atomic Energy Commission (BAEC). Overall, the observations from this study are within the accepted range and provide prospects for commercial production of the kits for human administration.

Keywords: MDP, ^{99m}Tc, Endotoxin, Iodometry, Hypercalcemia, Hydroxyapatite.

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INTRODUCTION

Nuclear medicine has long been employed in diagnostic, therapeutic, and preventive sectors, with diagnostics being the most prominent use (1). Nuclear medicine employs molecular imaging and radiopharmaceuticals to aid physicians in diagnosing and treating a variety of diseases, especially tumors and cancers.(2). Radiopharmaceuticals are explained as drugs that are radioactive and have a pharmaceutical effect used for diagnosis or therapy without causing any alteration in the patient's physiological response (3). A radiopharmaceutical can be a radioactive element, such as ^{133}Xe, or a labeled compound, such as ^{99m}Tc-labeled compounds (4). Technetium-99m, the most frequently used radioisotope for labeling radiopharmaceuticals, is generally obtained from a 99Mo/99mTc generator through the elution of ^{99m}TcO4 (2-3). Radiopharmaceuticals can be either radioactive elements, like ^{133}Xe, or compounds that are labeled with radioactive isotopes, such as ^{99m}Tc (5). For a long time, diagnostic needs have been met using freeze-dried kits (cold radiopharmaceuticals) that are labeled with 99mTc (4). The production of these kits has been carried out under aseptic conditions (3). It is crucial to meticulously delineate the manufacturing procedures to avert microbiological contamination throughout the entire production process. ^{99m}Tc-labeled complexes have long been utilized as bone imaging agents (6). In particular, ^{99m}Tc labelled with Methylene Diphosphonate (MDP) reveals the entrapping power of hydroxyapatite crystals in the bone's mineral phase due to the diphosphonate compounds (7). To ensure the quality of formulated kits, a range of analytical

techniques are employed, including paper chromatography, which assesses radiochemical purity and kit efficiency. Furthermore, bacteria endotoxin and fungal growth tests must be performed within specif microbiological parameters to confirm the sterility of ^{99m}Tc-MDP kits. Bio-distribution studies identify target organs and predict safety and efficacy, which includes pharmacology and safety studies. Subsequently, the present study conducted initial in-vivo experiments on rats and assessed the outcomes against a control and reference group. The aim of this investigation is the preparation of freeze-dried methylene diphosphonate (MDP), its quality control procedures, the biodistribution study of Technetium-99m (^{99m}Tc) labelled with MDP (^{99m}Tc-MDP), and subsequent comparison with a reference Monrol MDP kit.

EXPERIMENT METHODOLOGY

Materials: All chemicals, reagents, and solvents employed in this study were of laboratory grade and sourced from Sigma Aldrich. The research was conducted on MDP kits which were synthesized by the freeze-drying method. 99mTcO_4^- was obtained from the 99Mo-^{99m}Tc generator generated at the Radioisotope Production Division of the BAEC.

Preparation of MDP cold kits

MDP can be identified by the general formula H2O3P-X-PO3H2, where X = -CH2 forMDP (8). P(8). Irrespective of the substituents, phosphonates exhibit a propensity to form oligomers, which can be avoided by adding antioxidants such as ascorbic acid. The cold kit formulation of MDP was meticulously prepared with sterilized equipment under stringent aseptic conditions in a laminar airflw hood. The pH was adjusted to 6.0; each vial contains 10 mg MDP, 2 mg ascorbic acid, and 1 mg stannous chloride. hydrate following the International Atomic Energy Agency's (IAEA) guideline narrated in the technical report (IAEA series no-466). IAEA has established guidelines for the preparation of MDP cold kits to ensure their sterility, quality, and safety. Freeze-drying is a drying technique that includes three steps: freezing, primary drying, and secondary drying; moreover, this method is used for thermo-labile products (9). Liquid formulation of MDP has some decomposition issues due to temperature and heat; that's why it needs to be lyophilized by using the freeze-drying technique (10).

Quality control

Quality control is paramount in the preparation of methylene diphosphonate's lyophilized kits. Ensuring rigorous quality standards not only guarantees the reliability and accuracy of the kits but also safeguards user safety. Each of the lyophilized preparations was subjected to quality control testing, which included microbiological testing (e.g., bacterial and fungal growth tests) and percentage of radiochemical purity (%RCP) (11).

Microbiological Testing

Bacterial endotoxin test

To ascertain the presence of bacterial endotoxin, commonly known as pyrogen, a TAL (Tachypleus Amebocyte Lysate) reagent with a sensitivity threshold of 0.5 EU/mL was employed. For this purpose, TAL reagent was reconstituted following the manufacturer's instructions, adding a precise volume of pyrogen-free water. An exact quantity of reconstituted TAL reagent (0.2 ml) was aliquoted into each of the test ampoules, to which an equivalent volume of test samples was subsequently introduced. A positive control sample containing endotoxin, as well as a negative control without endotoxin, were included to validate the test. The ampules containing the mixture were incubated at 37°C for 1 hour, and the gel formation or color change was observed and compared with positive and negative controls for indicating the presence of endotoxin.

Fungal test

Fungal contamination was tested using potato dextrose agar (PDA), which is specifically designed to support fungal growth. The PDA media were prepared using the manufacturer's instructions and sterilized by autoclaving at 121°C and 15 psi pressure for 15 min. Next, sterilized PDA plates were prepared by dispensing 20 ml of PDA media in each petri dish and allowing them to solidify for 30 minutes. The process was conducted in the sterle conditions (by UV radiation) under a laminar air flow hood. The solid agar media plates were inoculated with a small portion of the sample (0.1 ml), which was spread over the surface of the PDA plate using a sterile inoculation loop. Similarly, three other media plates were inoculated with 0.1 ml fungal spore for positive control. Three media plates were kept as media control, and three media plates were kept as bench control. Then, the inoculated media plates were incubated at 25°C for 3 to 7 days for fungal growth.

Radio-labeling and Radiochemical Purity Determination

For radio-labeling, a specified amount of sodium pertechnetate ($Na^{99m}TcO4$) with an activity of 250 mCi in a 2-3 mL elution from the $99Mo/^{99m}Tc$ generator was introduced into a vial containing the MDP kit. The solution was gently agitated and then allowed to rest at room temperature for 20 minutes for obtaining 99mTc-MDP radiopharmaceutical. The radiochemical purity of ^{99m}Tc-MDP was assessed by using paper chromatography systems (12,Whatman 3 Whatman 3mm chromatography paper was served as the stationary phase while acetone and 0.9% sodium chloride solution were used as themobile phase to separate $reduce^{99m}TcO_2c$ ($^{99m}TcO2$) and 99mTc-MDP from $pert99mTcO_4ate$ on (99mTcO4 -). A droplet was spotted on the bottom point of stripof Whatman paper. The strips were promptly placed in a test tube, to which approximately 1 ml of acetone was added and developed until the solvent front had drifted to the top point of the paper. After that, the strips were dried and cut into equal segments: top and bottom. Each portion was then counted for gamma radiation. The same procedures were replicated using the saline solution.

Biodistribution studies

Biodistribution studies were conducted on Wistar rats, each averaging 250 gm in body weight. A 0.2 ml dose of radiolabeled ^{99m}Tc-MDP, approximately 2.0 mCi, was administered via injection into the tail vein (caudal vein) of each rat. Experiments were performed also with lower doses, and animals were anesthetized prior to being sacrificed two hours after dosage administration. Targeted organs, such as the skull (brain), bones (femur, humerus, spines), and various soft tissues, were then carefully dissected and isolated for further analysis. Radiation dose uptake was detected by a dose calibrator (Veenstra Instrumentation 404). Animals taken for experiments were grouped into three. The first group was the 'Control' group; in this group of animals, only Tc-99m was injected. The second group was the 'Standard' group; in this group, MON. MDP KIT (10 mg lyophilized powder) from 'Monrol' was used for labeling with Tc-99m and then injected into animals. The third group belonged to this study, 'MDP'; in this group, prepared MDP was used for labeling and then injected into animals. For 'MDP,' there were two groups for study, one is 'MDP 1' and 'MDP 2.'. This lyophilized MDP was prepared in a single batch and just injected into animals in two different dosage vials.

RESULT AND DISCUSSION

The results of the tests for bacterial endotoxin and fungal presence have both returned negative outcomes. In the bacterial endotoxin test, no detectable endotoxins were observed, indicating the sample was free from bacterial byproducts that could pose a potential risk. Similarly, the fungal test yielded a negative result, confirming the absence of fungal elements that could compromise the sample's integrity.

The radiochemical purity (13) of the complex was determined by the separation of radioactivity into complexed (^{99m}Tc-MDPcomplex), free ($^{99m}TcO4$-), and reduced hydrolyzed technetium($^{99m}TcO2$) in paper chromatography.

$$\text{From acetone, \% of free } {}^{99m}TcO_4^- = \frac{\textit{HPGe count for the top part}}{\textit{HPGe count for top and bottom part}} \text{x100}$$

$$\text{From saline, \% of reduced -hydrolyzed } {}^{99m}TcO_2 = \frac{\textit{HPGe count for bottom part}}{\textit{HPGe count for top and bottom part}} 100$$

Percentage of Radiochemical purity = 100- (% of free +percentage of $^{99m}TcO2$)

Table-1: The radiochemical purity of the prepared kits

Sample No.	$\%^{99m}Tc$-MDP (RCP)	$\%\ ^{99m}TcO_4^-$	$\%TcO_2$
1	99.93%	0.001	0.07
2	99.98%	0.0004	0.013
3	99.95%	0.003	0.038

Biodistribution is fundamental for identifying target organs, assessing safety and efficacy, and conducting pharmacological studies; it also it plays a crucial role in predicting outcomes based on the specific characteristics of vectors and constructs (4). Radiopharmaceuticals typically consist of two primary components: a radioactive element (radionuclide), which enables external imaging, and a non-radioactive element. The other crucial component involves a biologically active molecule, which could be a drug or a cell (such as red and white blood cells labelled with a radionuclide). This molecule serves as a carrier or ligand, directing the radionuclide to a specific organ (15). Because of this binding action, the radiation dose uptake was concentrated in the tissues, brain, and skeleton of Wistar rats, which was measured by a dose calibrator.

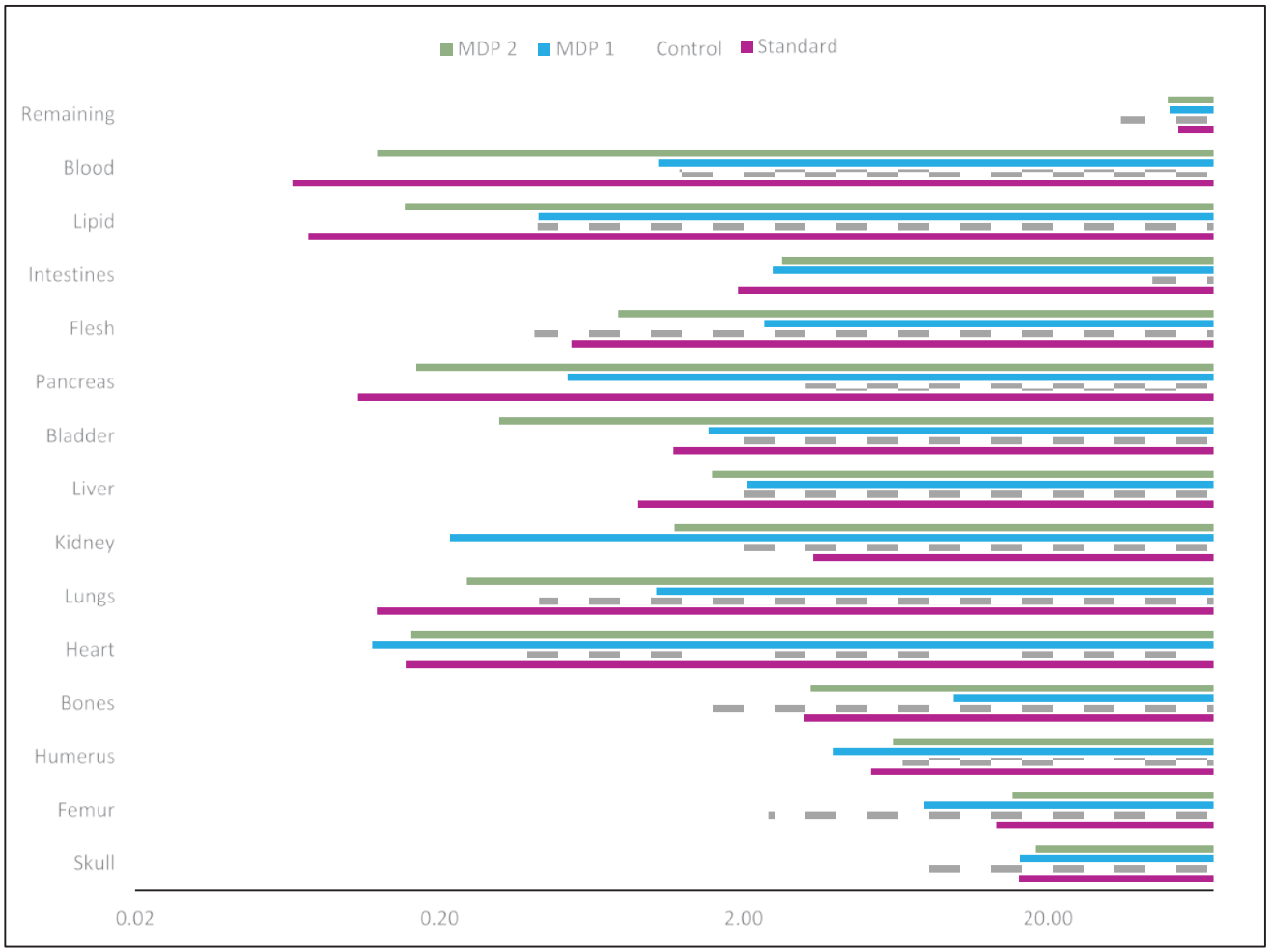

Figure 1: Graphical representation of Biodistribution in Wistar rats. Accumulation of ^{99m}Tc-MDP dose according to its percentage with whole-body activity (percentage of dose uptake are shown in logarithmic scale according to organ); the Black broken line is presented control group, MDP 2 is suspected to be highest distribution towards skeletal part.

Less accumulation was observed in the control group compared to the MDP group. The highest uptake was measured in MDP 2, standard, MDP 1, and control groups, respectively, for the skull (18.23, 16.04, 16.17, and 7.23%) and femur (15.28, 13.52, 7.84, and 2.41%) but not in skeletal muscle (in figure 1, percentage of flesh). Other groups can be summarized as having a high degree of maximum similarity in the accumulation of 99mTc-MDP (Figure 1). Accumulation in the femur for the standard group exceeds 13%, while for MDP 1, it is approximately 8%, and for MDP 2, it reaches up to 15%. In contrast, the control group shows femoral accumulation of over 2% (Figure 2).

The obtained results indicated the highest accumulation of 99mTc-MDP in the brain and skeleton likely due to its binding affinity for hydroxyapatite crystals and calcium salts. Additionally, uptake in soft tissue such as lungs, kidney, liver, pancreas, and intestine (particularly in the control group) occurred may be attributed to underlying pathological condtions such as hypercalcemia (16). The assembling mechanisms of technetium-99m with methylene diphosphonate (99mTc-MDP) were scrutinized using hydroxyapatite powder and various phosphates in bone tissue (17).

Skeletal part ingestion is partially delineated in Figure (2), in which it is observed that the maximum dose accumulated in the skull (brain part) and femur exceeded 16%. Several studies have summarized that approximately 50% of the administered radiation dose is retained within the skeletal system, while the remainder is distributed across body parts (18).

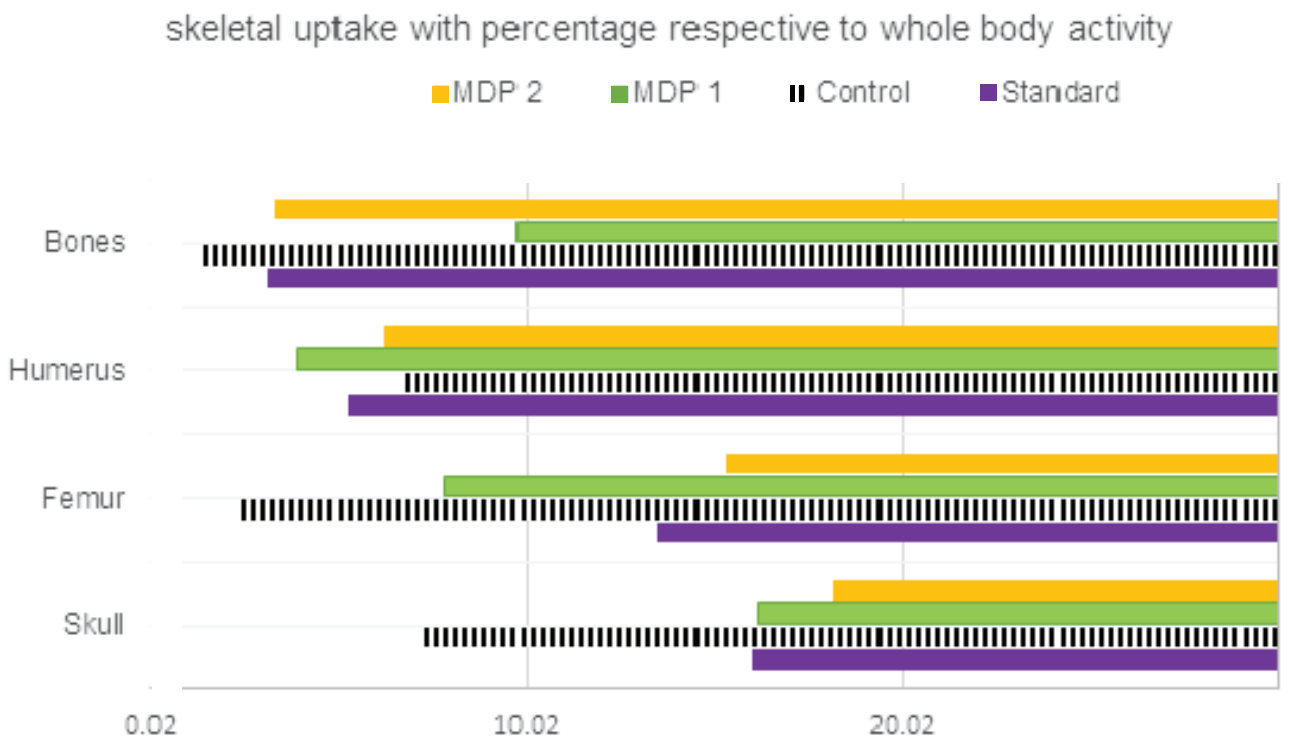

Figure 2: Representation of skeletal uptake of various groups in distinct organs (Marked as Control, Standard, MDP 1 and MDP 2); only skeletal parts are presented like skull, femur, humerus, bones.

This research yielded similar results, which illustrate maximum accumulation in skeletal parts. The higher uptake of 99mTc-MDP in the skull can be attributed to the presence of small bones within the nervous system, which provide an increased surface area (19), leading to enhanced absorption. This finding, as observed on bone scintigraphy, could have influential execution in the diagnostic arena and the clinical assessment of the patient (20). Furthermore, 99mTc labelled MDP and other compounds are now commonly used as imaging agents for the central nervous system (21). Another technique, the radiographic skeletal survey, is more sensitive than bone scintigraphy because it can reveal old fractures that have healed. However, detecting such fractures with bone scintigraphy is challenging, so skeletal surveys are typically reserved for rare cases where detailed imaging is necessary (22). The correlation between organs and the percentage of injected dose per organ is not yet perfectly defined. However, numerous studies have investigated the relationship between radiosensitivity and the weight of different organs. An organ's radiosensitivity is influenced by factors such as the type of organ, its physiological condition, and its size and weight. Stem cell populations within these organs, which may or may not may or may not be actively replicating or proliferating, contribute to the organ's radiation-induced sensitivity. This sensitivity is further impacted by the organ's weight, as organs with varying cellular characteristics may show different responses to radiation. Notable, an increase in organ weight is detectable and carries significant clinical implications (20).

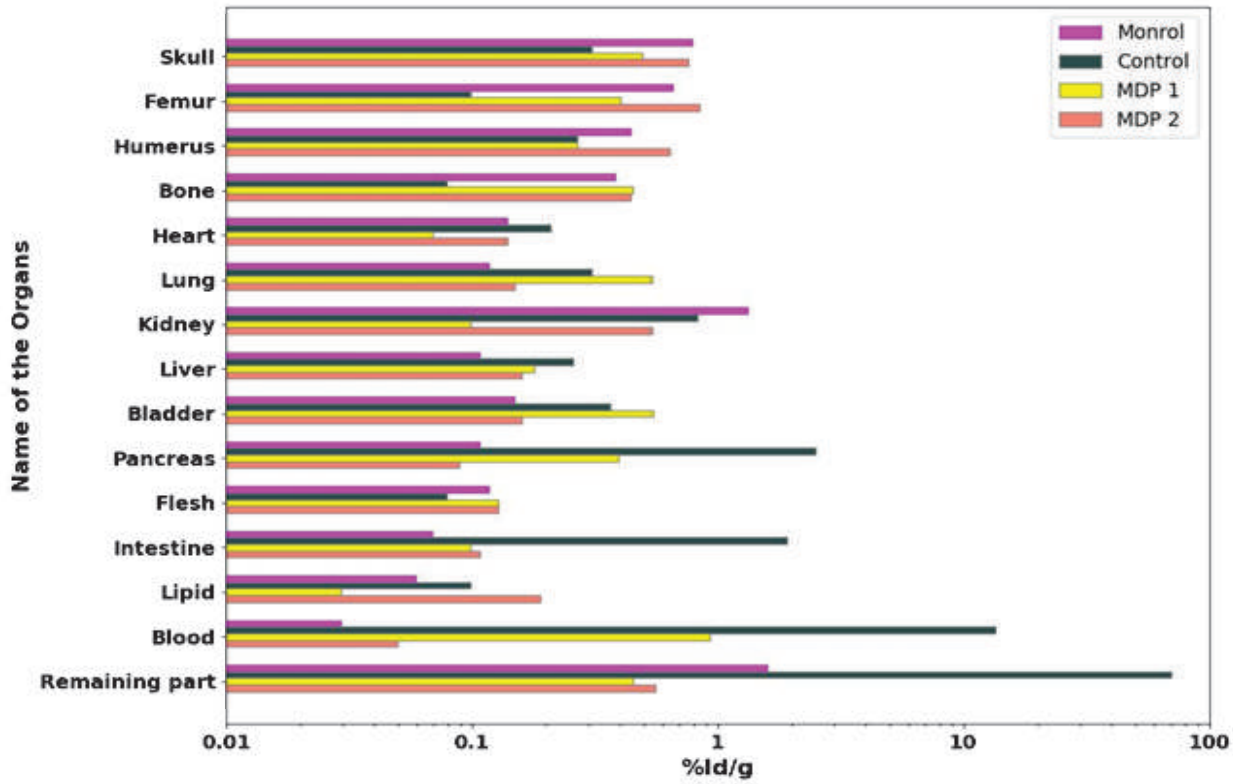

Figure 3: Schematic representation of the percentage of injected dose per organ (percentage of injected dose per organ is calculated by percentage respective to whole body per body weight in grams)

According to that statement, it was observed that the amount of persuaded dose is related to the weight of organs, and that is delineated in the above picture (Figure 3); the interrelationship between the weight of various organs and the percentage of corresponding induced dosage.

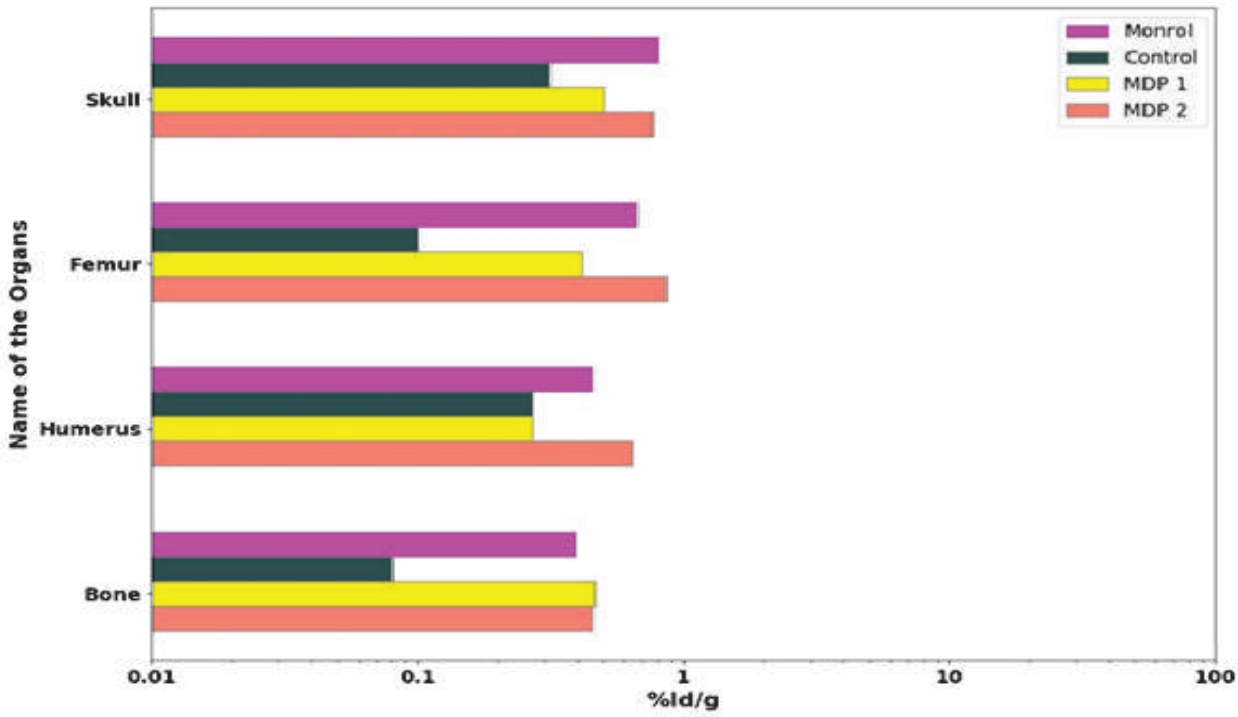

Figure 4: Interrelationship between percentage dose uptake in skeletal part per organ weight (only skeletal parts are shown)

The percentage radiation dose uptake in skeletal parts per organ weight in the present study groups (standard > 0.8, control > 0.5, MDP1 > 8.5, and MDP2 > 0.76 in the skularea) which is manifested in the above picture (Figue 4), such as the femur and other bones (humerus, skull, spines), which had shown maximum ingestion of radiation dose for the MDP 1 group, respectively to other groups. Maximum absorption of radiation dose in bones (femur, humerus, skull) may be explained as bone contains a larger mass of calcium than soft tissue (16) and extended content of bone nutrients as well as the binding to regions of active bone metabolism (23). The background chemistry of these systems is very complicated, and there is no consistent theory that can explain the in-vivo mechanism of action, but it can be inferred that the interaction between diphosphonate ligands and technetium-99 is a possible mode of this action (24). Another study was carried out that referred to the contribution of Sn2+ ionof 99m-Technetium-in the accumulation of 99m-Technetium- MDP, where Sn2+ ions act as reducin agents that permeate the conversion of pertechnetate (99mTcO(99mTcO3-);-) to technetium (III) (99mTcO3-, furthermore, the formation of lipophilic 99mTc-Sn(OH)2+ complexes crosses the blood-brain barrier due to lipid solubility, allowing them into 'calvaria,' which means the top part of the brain (25).

It appears from the above discussion that the percentage of induced dose or uptake of radiation may be dependent on bone infrastructure, number of bones, and organ weight. It is also vital to consider the physiologic and pathologic condition of the patient, disease state, metabolism rate, diffusion of oxygen from the blood to tissue, and functions of various tissues. However, it would be better to conclude the result from human autopsy sampling because the animal model is a better approach to finding a better insight into this present research.

CONCLUSION

The novel formulation, methylene diphosphonate acid and complex 99mTc-MDP, have been successfully prepared. The radiolabeling efficiency of the complex is high, and its stability duration is long enough to allow biodistribution and imaging studies. Compared with the complexes, 99mTc-MDP (MON. MDP KIT 10 mg) displays highly selective uptake in the skeletal system and uptake in the soft tissues (occasionally). These satisfying results lead us to further investigate it as an imaging agent for bone scintigraphy by commercial production.

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