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Bioevaluation of ^{99m}Tc-Pipazethate as a Potential Guide Tracer for Malignant Tumor Imaging

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ABSTRACT

Diagnosis of tumor is one of the most important stages in the treatment, as good quality imaging and accurate analysis means to choose the right and more effective tracer. Therefore, the study of the ability of new radiopharmaceutical compound to track solid tumor became a pioneering field. Thus, the study of pipazethate as a possible solid tumor marker was in our scope. It was labeled with technetium reaching a (RCY) radiochemical yield of 96.5%, using $SnCl_2.2H_2O$ as a reducing agent. A biodistribution study of ^{99m}Tc-Pip in tumored mice was performed which revealed the capability of the complex to image solid tumor as it was accumulated in the tumor with a concentration of 13% at 30min (p.i) post injection T/NT ratio was considered, the ratio reached 2.9 at 30 min post-injection, and this value confirmed the high sensitivity and selectivity of ^{99m}Tc-Pip to tumor. ^{99m}Tc-Pip passed the (BBB) blood brain barrier reaching the brain in a concentration of 5.8g/tissue at 5 p.i in normal mice while it reached 5.5 g/tissue at 5 p.i in tumored mice. Finally, molecular modeling was applied for prediction of the most energetically stable structure of ^{99m}Tc-pip.

INTRODUCTION

Pipazethate HCl - represented in Fig. (1) is 2-(2-piperidinoethoxy) ethyl 10H-pyrido [3, 2-b] [1, 4] benzothiadiazine-10-carboxylate hydrochloride [1]. It is a non-narcotic antitussive drug which performs centrally on the medullary cough center [2], it binds to the sigma-1 receptor with an IC50 value of 190 nM [3].

The sigma-1 receptor has an essential role in many pathological disorders in the central nervous system (CNS), concerned in various neuropsychiatric syndromes. Positron emission tomography (PET) is widely used for the imaging of sigma-1 receptor present in brain as it is considered as a noninvasively instrument for the study of the pathophysiological disease [4].

The use of radioisotopes such as ^{18}F, and ^{123}I is extensively used with PET agent, which was approved for the diagnosis of many types of cancers. The radioisotope ^{18}F is used in the form of [^{18}F]fluorodeoxyglucose (FDG). Its choice was because of its similarity to glucose, which is highly consumed by cancer cells [5]. Though, its short $t_{1/2}$ of about 1.83 h and its high manufacturing cost caused a limitation for its medical application. Therefore, Technetium-99m has been the most utilized radionuclide in nuclear medicine due to its optimal physical characteristics (half-life time of 6 h, low emitting gamma scintillation energy of 140 keV, and minimal doses to the patients). Moreover, it is conveniently available from the ^{99} Mo / ^{99m} Tc generator [6-7].

Herein, pipazethate was radiolabeled with ^{99m}Tc to be used as a radiopharmaceutical for brain imaging. Molecular modeling was performed for prediction of the more stable structure of ^{99m}Tc-pip. Finally, the ability of our tracer to image tumors such as brain cancer was studied by inducing a solid tumor in Swiss albino mice using Ehrlich carcinoma. The choice of using Ehrlich ascites carcinoma (EAC) depended on its relation to the human tumors for its indistinguishable feature and rapid proliferation rate [8-9].A preclinical T/NT ratio and brain blood ratio were calculated.

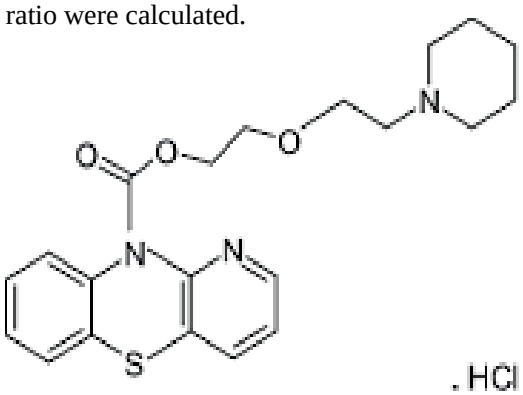

Fig. (1): Pipazethate HCL structure

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MATERIALS AND TOOLS

1-Materials

All materials used were of analytical grade. Pipazethate was kindly obtained from EIPICO Company, Egypt.

2-Equipment

The gamma counter (Nucleus Model 2010) was used to measure the radioactivity using a well type NaI (Tl) crystal.

HPLC [Merck-Hitachi Model], with L- 6000 pump and L-4000 UV Spectrophotometric Detector Merck, stationary phase including a reversed phase C18 column (250mm x 4.5 mm, 5 µm).

3-Animals

Male albino mice (weighing 25-35g) were obtained from Animal House of Nile Pharmaceutical Company, Cairo, Egypt. The animals were sustained under controlled conditions by providing standard food pellets, clean water and stable climate. All biological experiments were executed in agreement with guidelines recognized by Animal Ethics Committee of Egyptian Atomic Energy Authority (ethical approval EAEA/2019/188).

Experimental

1-Labeling of Pipazethate with ^{99m}Tc

^{99m}Tc was used for labeling Pipazethate through a reduction reaction using $SnCl_2 .2H_2O$ [10-11].The reaction was performed in a clean evacuated rubber stoppered vial for the study of the optimal condition to attain the highest radiochemical yield of ^{99m}Tc-Pip. Different amounts of Pipazethate (50-300µg) were used, followed by variant amounts of $SnCl_2$ (50-300µg), then the reaction medium was adjusted by adding 0.1ml of different pH media. At last, 0.1ml (7.2MBq) of ^{99m}Tc-pertechnetate freshly eluted from ^{99}Mo/^{99m}Tc generator was added. The reaction mixture was incubated for different time intervals at different temperatures.

2-Radiochemical Purity and Yield

They were determined using paper chromatography and HPLC.

2.1- Paper Chromatography

RCY was calculated by dropping a spot of the reaction mixture on a Whatman No.1 paper chromatography. The RCY of ^{99m}Tc-Pipazethate was determined by ascending chromatography using acetone to separate free pertechnetate from the complex and the reduced colloids, both remained at the spotting point. While a mixture of water, ethanol and ammonia was used at a ratio 5:2:1 to distinct the reduced colloids which remained at the spotting point, the complex was moved to the front with free pertechnetate. Finally by outlining the free pertechnetate and the reduced colloids, the amount of the complex was calculated from the difference [12].

2.2-HPLC analysis

The radiochemical purity (RCP) of radiolabeled pipazethate was managed by HPLC. The mobile phase comprised water and methanol (40:60, v/v), and the flow rate was adjusted to 1.0 ml/min, the λ was attuned at 230 nm [13]. Fractions were collected and counted using a γ-counter.

3- The determination of the partition coefficient of ^{99m}Tc-Pip

To determine the partition coefficient (P), the ^{99m}Tc-Pip (attained by HPLC separation) was mingled with equal volumes of 1-octanol (the organic layer) and 0.025M phosphate buffer of pH 7.4 (as an aqueous layer) in a test tube. The mixture was vortexed at ambient temperature for 2 min., followed by centrifugation for 5 min. at 5000 rpm. Consequently, samples from the organic and aqueous layers were transmitted to new a test tube. Each tube was counted using a gamma counter. The partition coefficient value was presented as $\log P_{octanol/buffer}$ for measuring the lipophilicity of ^{99m}Tc-Pip.

4-Biological distribution of ^{99m}Tc-Pip in normal and tumored mice

The tracer was introduced to normal mice via intravenous route to evaluate its in-vivo stability. For each experiment, 4 mice were used. The mice were sacrificed at specific times after injection. The organs were disected, weighted and measured using a gamma counter. The injected dose was considered to be per gram (ID/gram) . The blood, bone, and muscles were predicted to be 7%, 10%, and 40% from the total body weight, respectively [14- 15].

The bioevaluation of ^{99m}Tc-Pip tracer in tumored mice was performed and the sets were prepared in the same pattern of the normal mice. The solid tumor was provoked by injecting the right thigh with an 0.2ml parent tumor line solution (Ehrlich ascites carcinoma) which was diluted with a sterile physiological saline, however the left thigh muscle was considered as control[16], the animals were kept for 14 days under organized condition till the tumor was provoked. Finally, the tracer was injected in the tumored mice through IV injection. After specific intervals of time, the animals were sacrificed, T/NT ratio was calculated to

assume the selectivity of the tracer to the provoked tumor. The brain to blood ratio was estimated for both normal and tumored mice to evaluate the investigated complex as brain imaging marker.

5- ^{99m}Tc-Pip molecular modeling

Molecular modeling was applied to expect the most promising complex of ^{99m}Tc-Pip and to confirm that the new complex obtained will not disturb the targeting of pipazethate to its target organ. Spartan software for molecular modeling was used to produce, calculate and predict the most stable molecular structures of pipazethate with ^{99m}Tc [17].

The key approach was taking in concern the energy of the created complex. The diminution in the energy of the complex revealed the great stability of such complex, therefore it will increase the possibility of its formation.

RESULTS AND DISCUSSION

1-Pipazethate labeling with ^{99m}Tc

1.1-The effect of the pH on the RCY

The pH of the reaction is a factor affecting the RCY intensively, as shown in Fig. (2). A maximum yield of 96.5% was reached at pH value of 4.By shifting the pH of the reaction towards the neutral area, the yield decreased to 85%, while further shifting the pH to the alkaline medium, the RCY declined dramatically to 77%.

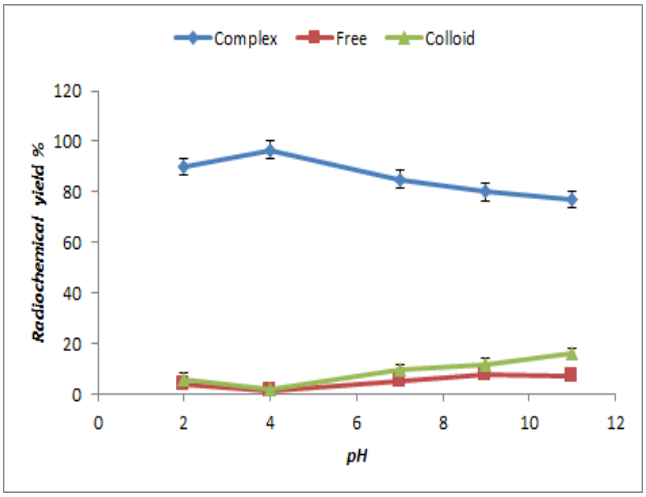


Fig. (2): Variation of radiochemical yields of ^{99m}Tc -Pip.as a function of pH [300 µg Pip +100 µL buffer at different pH+200 µg $SnCl_2.2H_2O$] at room temperature within 30 min.

1.2. The effect of tin content on the RCY.

Tin(II) chloride solution ($SnCl_2.2H_2O$, 1mg/ml) was applied as a reducing agent to transform $^{99m}TcO_4^-$ (in which Tc oxidation state is +7) to the more active structure $^{99m}Tc{=}O^{3+}$ (in which Tc oxidation state is +5)[18]. The RCY of ^{99m}Tc -pip reached 96.5% by increasing the tin amount up to 200µg as shown in Figure (3). However, increasing the tin amount to 300 µg, the colloid amount increased with a comparable slight fall in the yield of the complex [19].

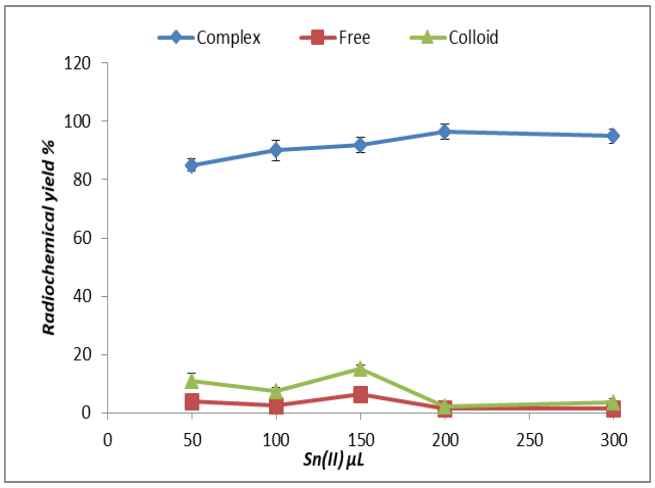


Fig. (3): Variation of radiochemical yield of ^{99m}Tc –Pip [300 µg Pip +100 µL buffer pH4+X µg $SnCl_2.2H_2O$] at room temperature within 30 min.

1.3-The effect of pipazethate amount on the RCY

In this context, it was noticed that at low pipazethate amount, a low yield was obtained as the amount of pipazethate was not enough to make a complex with the available amount of the reduced technetium [20]. An ideal radiochemical yield of 96.5% was achieved when using 200µg of pipazethate. By increasing the pipazethate amount, there was an insignificant increase in the radiochemical yield, as exposed in Fig. (4).

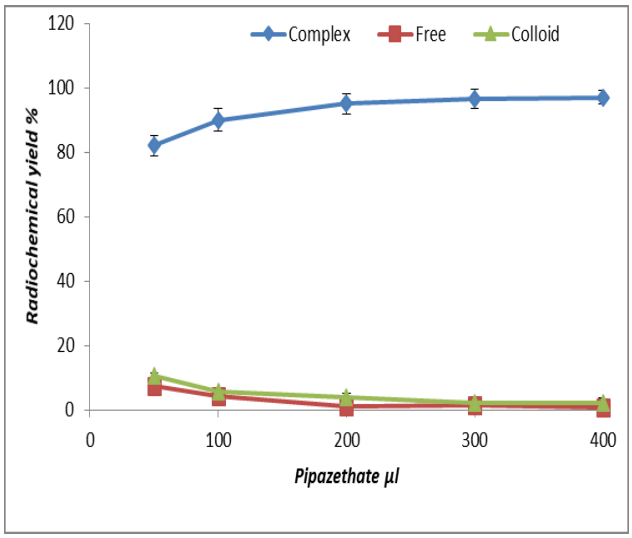


Fig. (4): Variation of radiochemical yield of ^{99m}Tc -Pip [X µg Pip +100 µLbuffer pH4+200 µg $SnCl_2.2H_2O$] at room temperature within 30 min.

1.4-The effect of reaction time on the RCY

The reaction time is a crucial factor that may extremely affect the yield of the labelled pipazethate .At a short reaction time, a very low yield was achieved as it was not enough for the present complex to form [21]. By increasing the reaction time to 30 min, the yield increased up to 96.5 %. Any further escalation of the reaction time had no effect on the radiolabeling yield, as presented in Fig. (5).

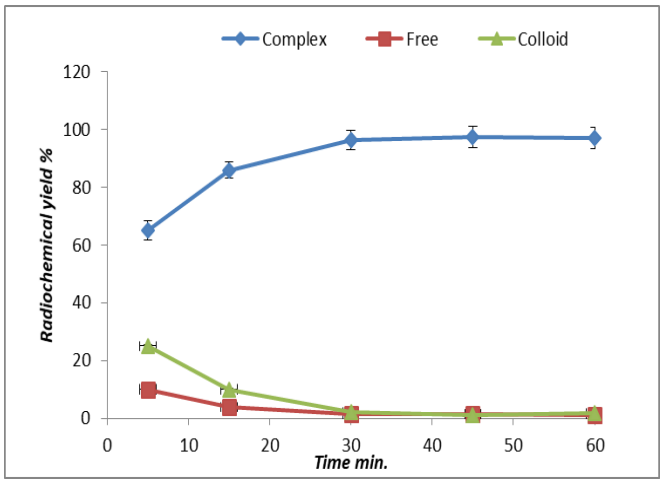


Fig. (5): Variation of radiochemical yield of ^{99m}Tc -Pip [200 µg Pip +100 µL pH4+200 µg $SnCl_2.2H_2O$] at room temperature within X min.

1.5- The effect of the temperature on the RCY

The radiochemical yield of ^{99m}Tc-Pip was slightly affected by changing the reaction temperature as clarified in Fig. (6).At room temperature, an optimal yield was achieved (96.5%). Any increase in temperature till 60 ºC did not cause a considerable change in the yield of the complex, while it was slightly decreased on further temperature increase.

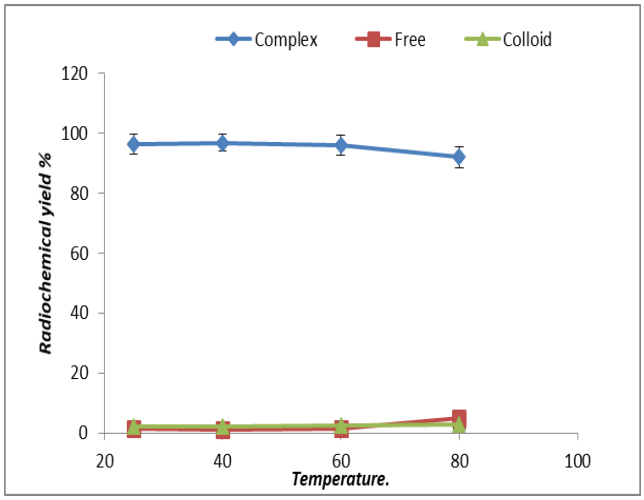


Fig. (6): Variation of radiochemical yield of ^{99m}Tc -Pip [100 µg Pip +100 µL buffer pH4+100 µg $SnCl_2.2H_2O$] at X temperature within Y min.

1.6-In-vitro stability of ^{99m}Tc-Pipazethate

It is essential for any tracer to test its stability for a specific time to be ready for injection. This is to prevent the development of the undesired products, resulting from decomposition of the labeled compound, which could be toxic or collected in the undesired organ. After obtaining ^{99m}Tc-Pip with the optimum condition, and it was separated by HPLC, and the in-vitro stability at different time breaks (1, 2, 4, 8, 12, 16 and 24 h) at room temperature was tested to determine the dissociation rate of the radio ligand. ^{99m}Tc-Pip was found to be stable at 99.1% after 8 hours as presented in Table (1). Though with the time goes by, the studied radio ligand stability was affected to reach 80% after 24 hours.

Table (1): In-vitro stability of ^{99m}Tc-Pip

Time (h)	Value of ^{99m}Tc-Pip (%)
1	99.5
2	99.5
4	99.3
8	99.1
12	94.7
24	80

2-Radiochemical Purity and Yield HPLC analysis

The radiolabeled Pipazethate (^{99m}Tc- Pip) was eluted at 6 min, though free $^{99m}TcO_4^-$ was eluted at 3 min, as revealed in Fig.(8). The chromatogram showed one single peak for ^{99m}Tc- Pipazethate, which indicated that the complex was pure without any ^{99m}Tc impurities with a purity of 99.5%. The retention time of the unlabeled Pipazethate was determined using a UV detector; it was eluted at 5 min.

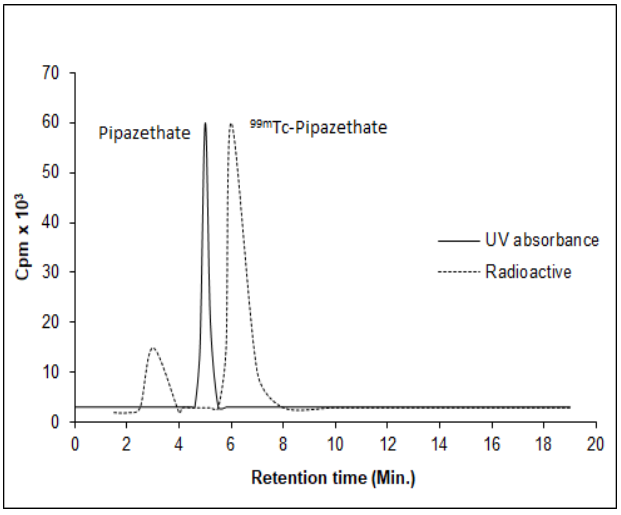


Fig (8): HPLC analysis of (^{99m}Tc-Pip) complex

3- The determination of the partition coefficient of ^{99m}Tc-Pip

Lipophilicity is a vital feature for the study of pharmacological properties of any drug [22].The Lipophilicity of ^{99m}Tc- Pip was expressed as $logP_{octanol/buffer}$, it was found to be 2.65 ±0.4. The $logP_{octanol/buffer}$ value confirmed the lipophilicity of ^{99m}Tc- Pip and its capability to cross the blood brain barrier (BBB). It is worthy to mention that for any drug to pass the BBB, its $logP_{octanol/buffer}$ value has to be in a range of 1.5–2.7 [23].

4-Biodistribution

The performance of ^{99m}Tc- Pip in normal mice is presented in Table (2). All data was collected and stated as mean of 4 experiments ± SE, the variance between organs uptake at different time interval was significant. ^{99m}Tc- Pip tissue distribution profile showed that the activity held by blood was high at 5 min psi reaching 16 ± 0.6 %, with a slow decrease by time reaching 3.2 ± 0.1% at 120 min p.i.(post injection). The intake by the liver for the radioactivity was 5 ± 0.2% at 15 min p.i. The tracer was accumulated in the stomach with a concentration of 6.2% after 15min of injection. The muscle intake was 5.3% after 30 min p.i, which declined to 3.8 % after 2h of tracer injection. Moreover, the uptake of the intestine increased with time until it reached 9.1 ± 0.3% at 60 min p.i. As pipazethate is a phenothiazines derivatives [24], the radiotracer was found in the kidneys (9.3 ± 0.4%) at 15 min p.i, consequently ^{99m}Tc- Pip in the collected urine reached 34 ± 1.4% at 120 min psi referred to be a main route of excretion for phenothiazines derivatives [25-26]. As ^{99m}Tc- Pip had the ability to pass the blood brain barrier (BBB) [27-28], it was highly accumulated in the brain at 5 min p.i (5.8 ± 0.2%). Then the radioactivity started to decline gradually by time to 1.2 ± 0.08% at 120 min p.i. Remarkably, the intake of the brain for ^{99m}Tc- Pip at 5 min (5.8 ± 0.2) was more than that of ^{99m}Tc-ECD and ^{99m}Tc-HMPAO (4.7 and 2.25%, correspondingly) [29] which were used as radiopharmaceuticals for brain imaging. Further calculation of brain / blood ratio, revealed that 5–30 min is the best time for using ^{99m}Tc-Pip as a tracer for brain imaging.

The biological evaluation of ^{99m}Tc-Pip in tumored mice revealed a lot of data which is presented in Table (3). The uptake of the tracer by the tumor reached its highest 13% after only 30 min. of injection via the greater penetrability and EPR (Enhanced permeability and retention) effect, as the tumor vasculature is generally leaky[30-31]. Though its intake by the normal muscle (control) was only 4%, the accumulation of the tracer by the tumor almost dropped to the half after 120min. psi reaching 6.5%. This rapid washout was attributed to the extravasation of the induced muscle [31-32]. Using the Student's unpaired test, statistical differences were found to be reproducible when $p<0.05$ between normal and tumored muscle. Tumored (Left thigh) /Non Tumored (right thigh) ratio is an essential factor to estimate the selectivity and the sensitivity of any radiotracer to solid tumor [33-34].T/NT ratio reached 2.9 at 30 min post injection ,this high value showed that ^{99m}Tc-Pip is a potential solid tumor indicator in comparision to ^{99m}Tc-nitridepyrazolol[1,5-a] pyrimidine (2.2 at 1h p.i)[36], ^{99m}Tc-BnAO-NI (2.59, 2 h p.i.)[37] and ^{99m}Tc-Meb (2.59 at 30min p.i)[34].

5-^{99m}Tc-Pip molecular modeling

The chelation of two molecules of pipazethate and one atom of technetium led to 5 possibilities represented in the complexes A-E. However the chelation of one molecule of pipazethate and one atom of technetium generated only one possibility exemplified in complex F.

The most energetically preferred suggested complex was complex E as it had the lowest energy of formation (E= -619.11 kJ/mol). The different possibilities for the complex ^{99m}Tc –pip and their energies are presented in Figure (9).

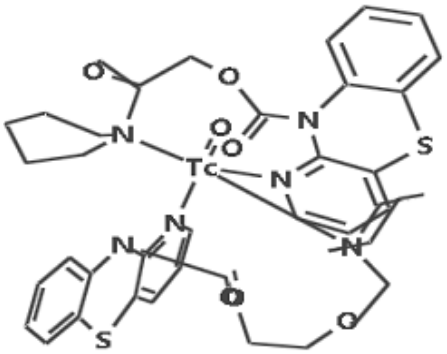
Table (2): Tissue distribution profile of ^{99m}Tc-Pip in normal mice

Organs & body fluids	% ID/g organ at different time intervals					
	5 min	10 min	15min	30 min	60 min	120 min
Blood	16 ± 0.6	13 ± 0.8	10 ± 0.5	8.1 ± 0.3	6.4 ± 0.01	4.2 ± 0.1
Bone	2.5 ± 0.15	3 ± 0.2	3.2 ± 0.2	2.7 ± 0.1	2 ± 0.04	1.7 ± 0.03
Muscle	3 ± 0.1	3.7 ± 0.3	4 ± 0.3	5.3 ± 0.2	4.2 ± 0.1	3.8 ± 0.15
Brain	5.8 ± 0.2	5.1 ± 0.2	4 ± 0.1	3.2 ± 0.02	2.3 ± 0.02	1.2 ± 0.08
Heart	1.2 ± 0.03	1.6 ± 0.1	1.5 ± 0.07	1.2 ± 0.04	1 ± 0.05	0.7 ± 0.05
Lung	2 ± 0.04	2.5 ± 0.15	2.4 ± 0.06	1.7 ± 0.03	0.9 ± 0.07	0.6 ± 0.07
Stomach	4.8 ± 0.2	5.7 ± 0.2	6.2 ± 0.35	4.4 ± 0.1	3.5 ± 0.2	3 ± 0.01
Intestine	6 ± 0.35	6.5 ± 0.3	7 ± 0.3	8 ± 0.15	9.1 ± 0.3	8.5 ±0.2
Liver	4 ± 0.18	4.8 ± 0.25	5 ± 0.2	4.2 ± 0.2	3.6 ± 0.25	3 ± 0.1
Kidneys	8 ± 0.4	10 ± 0.8	9.3 ± 0.4	8 ± 0.8	6.7 ± 0.3	5.8 ± 0.2
Urine	15 ± 1.1	19 ± 1.3	20 ± 1.1	24± 0.9	29 ± 1.5	34 ± 1.4
Spleen	0.2 ± 0.07	0.3 ± 0.09	0.3 ± 0.08	0.8 ± 0.06	0.2 ± 0.08	0.1 ± 0.08
Faeces	1.7 ± 0.05	2.2 ± 0.07	2.8 ±0.06	4 ± 0.1	5.5 ± 0.2	6.6 ± 0.3
Brain/Blood ratio	0.36	0.39	0.4	0.39	0.35	0.28

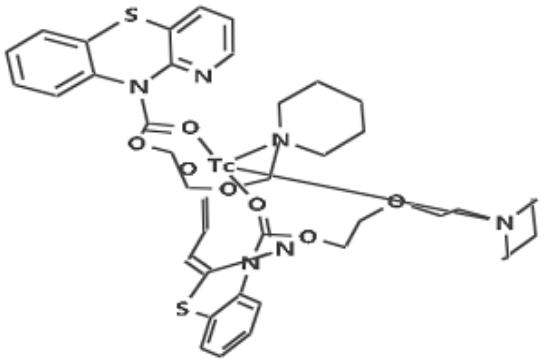
Table (3): Tissue distribution profile of ^{99m}Tc-Pip in tumored mice

Organs & body fluids	% ID/g organ at different time intervals					
	5 min	10 min	15min	30 min	60 min	120 min
Blood	16 ± 1.5	13 ± 1.1	9.8 ± 0.8	7.2 ± 0.2	5.8 ± 0.2	3.7 ± 0.1
Bone	2 ± 0.2	2.8± 0.3	3 ± 0.1	2.5 ± 0.1	1.8 ± 0.1	1.6 ± 0.04
Muscle	2.8 ± 0.1	3.4 ± 0.1	4 ± 0.15	4.5 ± 0.3	4± 0.2	3 ± 0.1
Tumor	5 ± 0.3	6.5 ± 0.3	9 ± 0.6	13 ± 0.35	9.5 ± 0.3	6.5 ± 0.4
Brain	5.5 ± 0.2	4.7 ± 0.1	3.6 ± 0.1	2.4 ± 0.2	1.8 ± 0.06	1 ± 0.08
Heart	2.2 ± 0.01	2 ± 0.06	1.6 ± 0.03	1.1 ± 0.05	0.8± 0.085	0.7 ± 0.09
Lung	2.8 ± 0.1	2.7 ± 0.04	2.2 ± 0.01	1.5 ± 0.02	1.1 ± 0.07	0.9 ± 0.09
Stomach	4.1 ± 0.2	5.8 ± 0.1	6.5 ± 0.2	3.5 ± 0.1	2.4 ± 0.03	2 ± 0.05
Intestine	4.8 ± 0.25	5.2 ± 0.1	6.5 ± 0.1	7.2 ± 0.2	8.4 ± 0.04	± 0.1
Liver	4.2 ± 0.18	5 ± 0.15	5.3 ± 0.2	4.4 ± 0.18	2.2 ± 0.05	1.7 ± 0.1
Kidneys	7.6 ± 0.3	8.5 ± 0.3	9.5 ± 0.8	6.5 ± 0.3	4.4 ± 0.01	3.5 ± 0.2
Urine	15 ± 0.9	17± 1.5	20 ± 0.9	25 ± 1.1	30 ± 1.4	34 ± 1.3
Spleen	0.45 ± 0.04	0.4 ± 0.01	0.3 ± 0.06	0.2 ± 0.07	0.06 ± 0.09	0.08 ± 0.0
Faeces	1.1 ± 0.02	1.4 ± 0.01	1.7 ± 0.02	3 ± 0.04	5.5± 0.2	8 ± 0.3
T/NT	1.7	1.9	2.2	2.9	2.3	2.1
Brain/Blood ratio	0.34	0.36	0.36	0.33	0.31	0.27

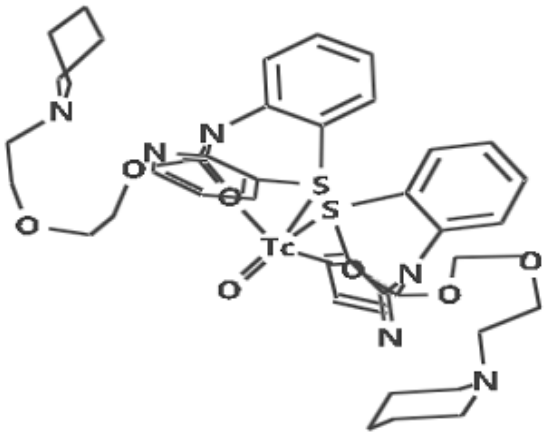
A (E= -446.13 kJ/mol)



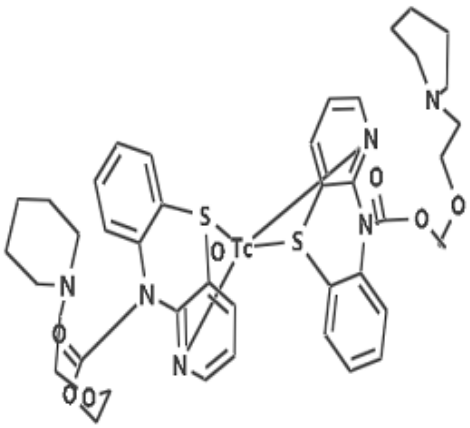
B (E=-533.88 kJ/mol)



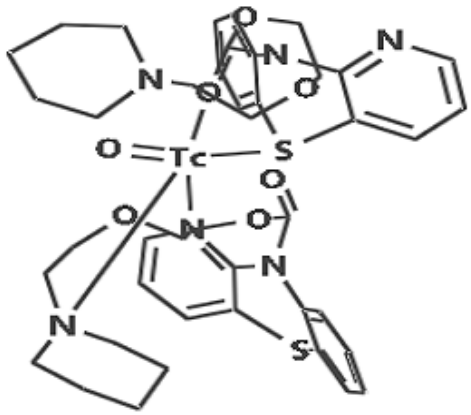
C (E=-506.15 kJ/mol)



D (E=-429.78 kJ/mol)



E (E=-619.11 kJ/mol)



F (E=-315.17 kJ/mol)

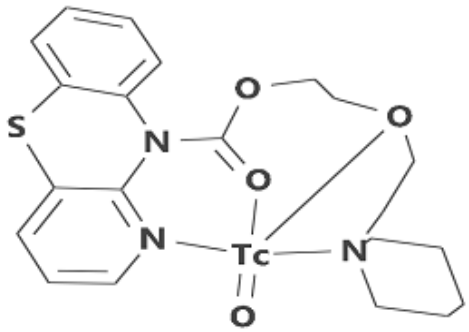


Fig. (9): Different chelation position of ^{99m}Tc -pip by molecular modeling

CONCLUSION

The radiolabeling of pipazethate with ^{99m}Tc was carried out through a reduction reaction in the presence of $SnCl_2.2H_2O$ obtaining a radiolabelling yield of 96.5%.The tracer was found to be stable for 8hour at room temperature. The biological estimation of the tracer in tumored mice showed that T/NT ratio reached 2.9 at 30 min p.i).This high value confirmed the potential use of ^{99m}Tc -pip as a radiotracer for the imaging and the diagnosis of solid tumors. While the performance of ^{99m}Tc –pip in normal mice revealed its capability to pass the blood brain barrier (BBB) reaching the brain in 15min with a brain / blood ratio of 0.4, the ratio decreased to 0.28 after 120 min p.i. Although, in tumored mice the brain blood ratio reached its maximum 0.36 at 10 and 15 min p.i.(post injectiom),the BBB permeation capability of ^{99m}Tc -pip was practically confirmed by measuring $logP_{octanol/buffer}$ (2.7 ± 0.4). This data can suggest the probable use of the present tracer for the imaging of the brain which will assist to diagnose many disorders related to brain illnesses.

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