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Iodine-125-Chlorambucil as Possible Radio Anticancer for Diagnosis and Therapy of Cancer: Preparation and Tissue Distribution

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Authors' contributions

This work was carried out in collaboration between all authors. Author AMA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author NSF did the practical section and managed the literature searches. Author AAB managed the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Chlorambucil (CLB) is an aromatic nitrogen mustard and an alkylating agent. It has been mainly used in the chemotherapy. A method for radiopharmaceutical preparation of [^{125}I]-iodo-Chlorambucil a potential cancer therapeutic agent is described. The method is based on direct electrophilic radioiodination of Chlorambucil with [^{125}I] in the presence of chloramine-T (CAT) as oxidizing agent. The reaction conditions were optimized in order to obtain a radiochemical yield higher than 98% of [^{125}I]-iodo chlorambucil. Different chromatographic techniques (electrophoresis, and high pressure liquid chromatography (HPLC)) were used to evaluate the radiochemical yield and purity of the labeled product. Biodistribution studies of ^{125}I- chlorambucil were carried out in both normal and tumor bearing Albino Swiss mice. The results revealed that this new tracer, ^{125}I-chlorambucil, has a high affinity to be localized in the tumor site for a long period which indicates the specificity of this tracer to the tumor cells. The results indicate the possibility of using [^{125}I]-iodo chlorambucil for imaging and treatment of cancer.

Keywords: Radiolabeling; chlorambucil; Iodine-125; diagnosis; tumor.

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1. INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality in developed countries, accounting for approximately 560,000 deaths in 2007 in the U.S. alone, as estimated by the American Cancer Society [1]. Nuclear oncology, a tool for tumor imaging, is the medical branch which involves a large variety of procedures to assess patients with already-known or unspecified neoplastic conditions [2]. The differences of the pathological and physiological processes between the tumor cells and the normal ones allow the differentiation of the tumor tissue from the normal tissue [3,4]. Positron emission tomography (PET) is a popular modality in oncology. The technique is based on the detection of photons released by annihilation of positrons emitted by radiopharmaceuticals. Positron-emitting radionuclides are produced in a cyclotron by bombarding target material with accelerated protons. Positron-emitting radionuclides can be used to synthesize radiopharmaceuticals that act as substrates for endogenous pathways. One of the major advantages of PET as an imaging modality is its versatility. Currently, many different positron emitters with different characteristics are available, making it possible to label a wide variety of radiopharmaceuticals [5]. The availability of these novel radiopharmaceuticals enables visualization with high sensitivity of tumor metabolism, cellular proliferation, specific cell surface receptors, angiogenesis, and tumor hypoxia [6]. Early detection and mapping of the cancer site allow prompt and successful treatment. After the development of radiopharmaceuticals, the risk factors of morbidity associated with cancer sharply decreased. Radiolabeled pharmaceuticals, for early detection of tumors, should be associated with certain properties such as ease of labeling, high specificity, rapid accumulation at the site of tumor, i.e. early diagnosis, high target to nontarget ratio, rapid blood clearance, low toxicity, low cost, and less antigenicity [7].

Labeled compounds have directed medical researchers toward easy and quick diagnostic strategies tumors. Of these, labeled anticancer are particularly important in the diagnosis of tumors by using radioiodinated compounds that bind to DNA. The use of these labeled compounds for in-vivo tumor targeting and therapeutic effect is achieved by auger-electron emitters. Auger electron emitters are widely used in cellular radiation studies. The most frequently used Auger electron emitter is iodine-125. The radiotherapeutic effectiveness of this radionuclide can be achieved by the incorporation of iodine-125 into cellular DNA [8]. The energies of Auger electrons are between 10eV and 34keV. It is also interesting to note that the ranges of Auger electrons emitted by an iodine-125 atom can reach up to about 40–45nm [9], so if the iodine-125 reaches the infected cell nucleus for the treatment of proliferative tumor and apoptotic effects, it has no damage effect on the normal cell. Chlorambucil is an antineoplastic in the class of alkylating agents and is used to treat various forms of cancer [10-14]. The present work concerns the labeling of Chlorambucil by iodine-125 depending on the significance of the Chlorambucil as chemotherapeutic agent besides its expected significance for the ^{125}I-Chlorambucil (^{125}I-CLB) as radiotherapeutic agent. The radiolabeling scheme of chlorambucil with [^{125}I] is shown in Fig. 1.

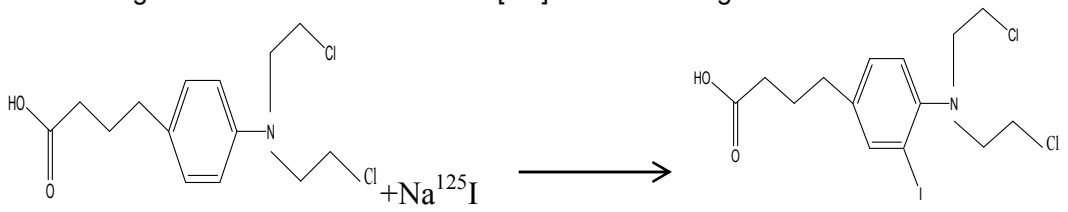

Fig. 1. The presumable structure of ^{125}I-CLB

2. EXPERIMENTAL

2.1 Materials

All chemicals and laboratory reagents used in this work were of the highest purity grade. In all cases double distilled water was used. Chlorambucil (4-[bis(2-chlorethyl)amino] benzenebutanoic acid) and Chloramine-T (N-chloro-p-toluene sulfonamide sodium salt) (CAT) were purchased from Sigma-Aldrich, Germany. Iodine-125 was purchased from (H-1121 Budapest, Konkoly-Thege Miklòsùt 29-33) as no-carrier added solution, radionuclidic purity >99%.

2.2 Animals

Albino type mice, weighing 30-35gm, were used.

2.3 Method

2.3.1 Radioiodination procedure of chlorambucil

All experiments were carried out in brown screw caped reaction vial. The vial was immersed in a thermostatically controlled water bath. 10µL $Na^{125}I$ (3.7 MBq) was added in the bottom of the vial, then the desired concentration of oxidizing agent (CAT), and the desired concentration of the chlorambucil in ethanol as organic reaction medium were added. The reaction mixture was stirred with a magnetic stirrer and heated to the specific temperature for the suitable time. The different parameters which affect the radiochemical yield of ^{125}I-chlorambucilsuch as chlorambucil content [25–250µg], reaction temperature (25–100ºC), oxidizing agent content [25-250µg], reaction time (10-60min), and pH of the reaction mixture (2–11) were studied.

2.4 Radiochemical Analysis

The radiochemical yield of the labeled chlorambucil was determined using paper electrophoresis. On Whatman paper sheet (2.5cm width and 40cm length), 5µl of the reaction mixture was placed 8cm away from the cathode and allowed to evaporate spontaneously. Electrophoresis is carried out for 90min at a voltage of 300 volt using phosphate buffer (0.05M, pH7) as electrolytes source solution. After complete development, the paper was removed, dried, and cut into strips, each strip is 1cm width, and then the strips were counted in a well type Na/T1 crystal connected to theγ-counter. Free radioiodide, and ^{125}I-chlorambucil moved to different distances away from the spotting point towards the anode depending on their charge and ionic mobility. The percentage of radiochemical yield was estimated as the ratio of the activity of the radioiodinated compound to the total activity multiplied by 100 as shown in Equation (1). The radiochemical yield of ^{125}I- chlorambucil is the mean value of three experiments.

$$\text{Radiochemical yield, \%} = \frac{\text{Activity of labeled product}}{\text{Total activity}} \times 100 \quad (1)$$

2.5 HPLC Analysis

The radiochemical purity of ^{125}I-CLB was determined by direct injection of 5-10µl, of the reaction mixture at the optimum conditions for obtaining the highest radiochemical yield, into the column (RP18–250x4mm, 5µm, Lischrosorb) built in HPLC Shimadzu model consisting of pumps LC-9A with a Rheohydron injector and U.V. spectrophotometer detector (SPD-6A) adjusted to the wave length 254nm. The column was eluted with the isocratic solvent using methanol: H_2O:acetic acid (49.5:49.5:1v/v) [15] as a mobile phase and the flow rate was adjusted to 1ml/min. The labeled compound was collected by using a fraction collector and its activity was counted by using well type NaI (Tl) crystal connected with single channel analyzer.

2.6 Biodistribution Studies

The animal research was conducted under supervision of the governmental research authority. The mice were divided into eight groups of six mice each, four groups of normal Albino mice and another four groups of tumor bearing Albino mice. This experiment was done by diluting the neutral solution of the purified labeled chlorambucil with 1 ml saline for injection, and filtration of the solution through 0.22µm Millipore filter into a sterile sealed vial. 100µl was injected in the tail vein of the healthy and tumor bearing Albino mice, weighing approximately 30g each. The mice were maintained on normal diet in a metabolic cage, then sacrificed at 0.5hr, 2hr, 4hr, and 8hr post injection. Samples of fresh blood, bone and muscle were collected in pre-weighted vials and counted. The different organs were removed, counted, and compared to the standard solution of the labeled chlorambucil. The average percent values of the administrated dose/organ were calculated. Blood, bone, and muscles were assumed to be 7, 10, and 40% of the total body weight, respectively [16].

2.7 Tumor Implementation

The parent tumor line (Ehrlich Ascitic Carcinoma) was withdrawn from 7 days old downer female Swiss albino mice and diluted with sterile physiological saline solution to give $12.5x10^6$cells/mL. 0.2mL solution was then injected in mice intraperitoneally to produce ascitic fluid. The animals were maintained on normal diet in a metabolic cage till the tumor development was apparent during 15 days [17].

3. RESULTS AND DISCUSSION

3.1 Effect of Chlorambucil Concentration

The radiochemical yield of ^{125}I-CLB as a function of chlorambucil concentration was studied as shown in Fig. 2. The results indicate that the radiochemical yield of ^{125}I-CLB increased from 93.4 to 98.8% by increasing the amount of chlorambucil from 25 to 100µg then the radiochemical yield is not affected by the amount of chlorambucil higher than 100µg. This may be attributed to the fact that the yield reaches the saturation value (98.8%) because the entire generated iodonium ions in the reaction are captured at that concentration.

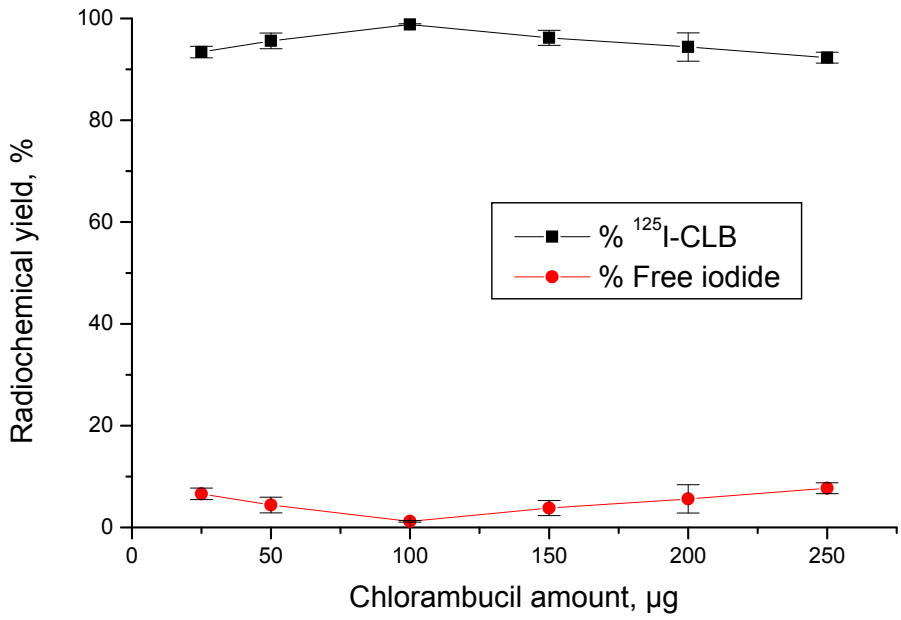

Fig. 2. Effect of chlorambucil amounton the radiochemical yield of ^{125}I-chlorambucil
Reaction condition: Xμg chlorambucil , 50µg chloramine-T, 150µL phosphate buffer at pH 7 and 5μL Na^{125}I. The reaction mixture was kept in a water bath (70ºC) for 15min

3.2 Effect of Chloramine-T Concentration

Radioiodination of organic molecules has been performed by using a mild oxidizing agent such as chloramine-T, which decomposes to hypochlorite anion that acts as an oxidizing agent transforming iodine from I^- to oxidative state I^+. The influence of chloramine-T concentration on the radiochemical yield of ^{125}I-chlorambucil was studied. The experiment was carried out by the addition of 100µg chlorambucil to different amounts of CAT (25, 50, 100, 150, 200 and 250µg), and 5µl Na^{125}I. The reaction mixture was heated to 70ºC for 15min. The results of this experiment are presented in Fig. 3. As it is clear from this data, the radiochemical yield of ^{125}I-CLB was 98.8% when the concentration of chloramine-T was 50µg which was sufficient to oxidize all the free iodide present in the solution. Increasing the amount of oxidizing agent above 50µg leads to a decrease in the radiochemical yield of ^{125}I-CLB due to the formation of undesirable oxidative side reactions like chlorination [18], polymerization and denaturation of substrate [19,20].

3.3 Effect of Temperature

The reaction temperature plays an important role in the electrophilic substitution reactions. The leaving hydronium ion requires some energy to break the C-H bond and to initiate the introduction of the radioactive iodonium ion into the phenyl ring. During this reaction, it was found that the kinetic energy required to breakdown C-H bond and introduce I^+ into the phenyl ring of chlorambucil was build up when the reaction mixture was heated to 70ºC for 15 minutes. The labeled chlorambucil did not decompose on increasing the reaction

temperature to 100ºC, as it is clear from Fig. 4. This means that the labeled chlorambucil was stable against rise in temperature.

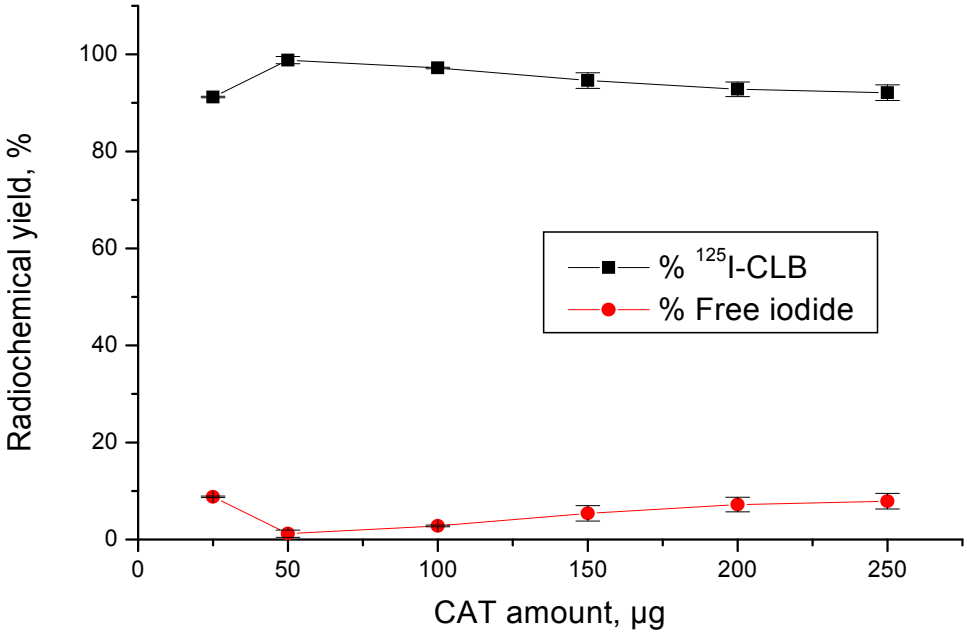

Fig. 3. Effect of chloramine-T amount on the radiochemical yield of ^{125}I-chlorambucil
Reaction condition: 100µg chlorambucil , Xµg chloramine-T, 150µL phosphate buffer at pH 7 and 5µL Na^{125}I. The reaction mixture was kept in a water bath (70ºC) for 15min

3.4 Effect of Reaction Time

As the reaction temperature was effective in the buildup of the energy required to breakdown the C-H bond and form the C-I bond, also the reaction time plays the same role. As it is clear from Fig. 5, the minimum time required to get a maximum yield of ^{125}I-chlorambucil was 15 minutes, and no valuable benefits can be gained from increasing the reaction time.

3.5 Effect of pH

This experiment was carried out using different buffer systems to obtain the required pH values; citrate buffer for pH 2 & 4, phosphate buffer for pH 7 & 9, and bicarbonate buffer for pH 11. The optimum amount of chlorambucil was added to the buffer system followed by the addition of 50µl CAT (50µg) and 5µl Na^{125}I. The reaction mixture was heated to 70ºC for 15min. The results indicate the effectiveness of pH of the reaction mixture on the labeling yield. The radiochemical yield of ^{125}I-chlorambucil was relatively poor at pH 2 and 4, as a result of the predominance of ICl species, which have lower oxidation potential than HOCl species [21]. At pH 7, the radiochemical yield of ^{125}I-chlorambucil reaches a maximum value of 98.8%. When the pH increased towards the alkaline side (9 and 11) the radiochemical yield decreased. This may be attributed to the decrease in HOI which is responsible for the electrophilic substitution reaction [22]. The data is summarized in Fig. 6. The high

radiochemical yield of ^{125}I-chlorambucil at pH 7 maybe due to many factors; the good solubility of chlorambucil in neutral pH, and the efficiency of CAT at this pH value [23].

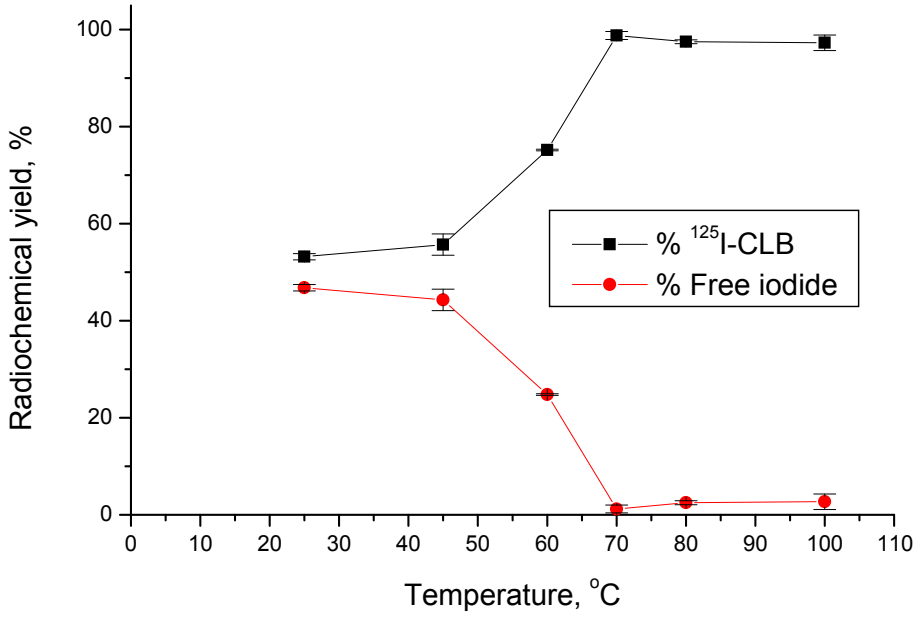

Fig. 4. Effect of reaction temperature on the radiochemical yield of ^{125}I chlorambucil

Reaction condition: 100μg chlorambucil ,50μg chloramine-T, 150μL phosphate buffer at pH 7 and 5μL Na^{125}I. The reaction mixture was kept in a water bath (X°C) for 15min

3.6 Stability Test

Chlorambucil was efficiently labeled with a radiochemical yield of 98.8% when its amount was 100μg in the presence of 50μg chloramine-T as oxidizing agent at pH7 and the reaction mixture was heated at 70°C for 15min. The stability of ^{125}I-chlorambucil was studied at room temperature (25°C) in order to determine the suitable time for injection to avoid the formation of the undesired products. These undesired radioactive products may be accumulated in non-target organs. Table 1 shows the stability of ^{125}I-chlorambucil up to 24 hour.

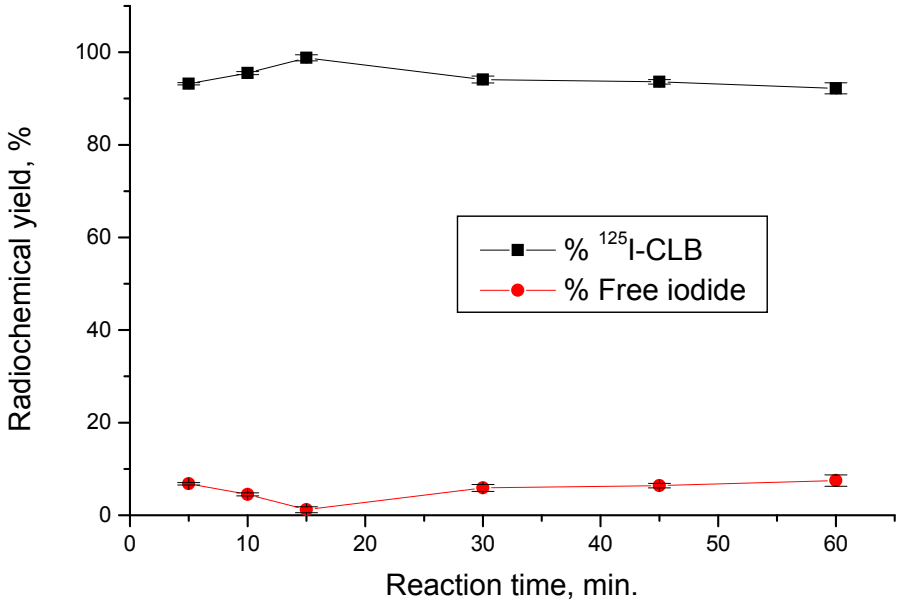

Fig. 5. Effect of reaction time on the radiochemical yield of ^{125}I-chlorambucil

Reaction condition: 100μg chlorambucil ,50μg chloramine-T, 150μL phosphate buffer at pH 7 and 5μL Na^{125}I. The reaction mixture was kept in a water bath (70ºC) for Xmin

Table 1. The *In vitro* stability of ^{125}I-chlorambucil

Time post labeling (hour)	^{125}I-chlorambucil,%	^{125}I,%
1	98.0±0.7	2±0.7
2	98.0±1.3	2± 1.3
4	97.9±0.9	2.1±0.9
8	97.7±1.6	2.3±1.6
12	97.3±1.1	2.7±1.1
24	97.5±0.7	2.5±0.7

Mean±S.D. (mean of three experiments)

3.7 HPLC Analysis

A radio chromatogram for the iodination of Chlorambucil obtained after HPLC separation on RP-18 column at the optimum conditions is shown in Fig. 7. Two peaks were obtained one at 2min while the second at 5min retention time. The first peak corresponds to free iodide while the second peak corresponds to the ^{125}I-chlorambucil. ^{125}I-chlorambucil is not completely separated from chlorambucil which indicated by the short retentions. The eluted fractions containing the labeled compound are pooled together and evaporated to dryness. The residue was dissolved in physiological saline and sterilized by filtration through 0.22μm Millipore filter [24], and the ^{125}I-chlorambucil is then suitable for use in biodistribution studies.

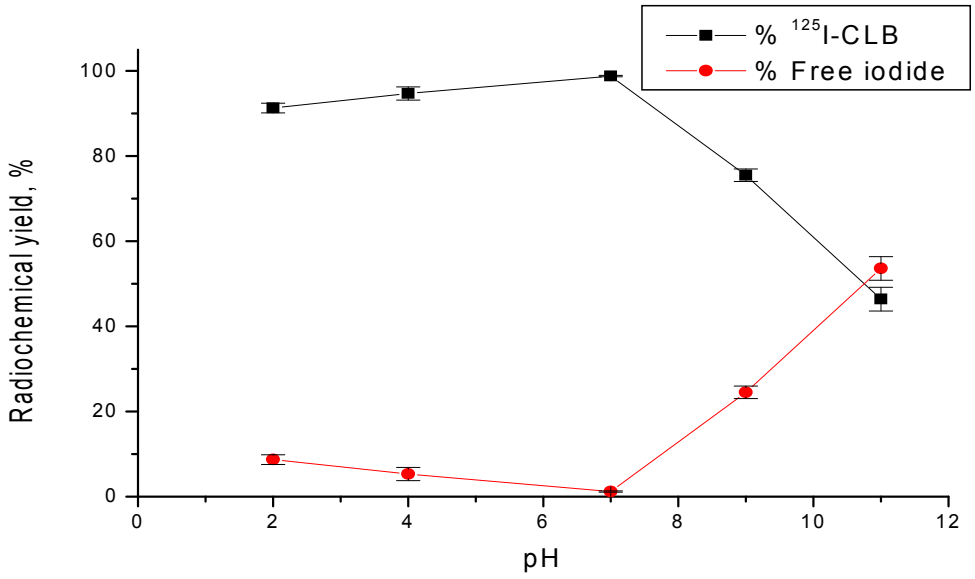

Fig. 6. Effect of pH of the reaction medium on the yield of ^{125}I-chlorambucil

Reaction condition: 100μg chlorambucil ,50μg chloramine-T, 150μL phosphate buffer at pH X and 5μL Na^{125}I. The reaction mixture was kept in a water bath (70ºC) for 15min

3.8 Biodistribution of ^{125}I-chlorambucil

The biological distribution pattern and the ability of the chlorambucil compound to localize the therapeutic radionuclide, iodine-125, in cancer site were examined in mice. The mice were intravenously injected with 0.1ml of the tracer. The bio-distribution of ^{125}I-chlorambucil in normal mice is presented in Table 2. The data shows that the clearance of the tracer from the blood was high as the percentage reached 9.7% at 4hours post injection. Because the excretion route of this compound occurs via liver, the intestine content increased by time from 8.6% to 14.9% at 0.5 and 4hours post injection, respectively. In addition, part of this compound was excreted through the kidneys, as the activity detected in the urine was 21.5%. The uptakes of other organs (bone, muscle, lung, heart, and spleen) were within the normal values. The in-vivo stability of the tracer can be seen, as the thyroid uptake shows slight increase by time.

Table 3 represents the biological distribution of ^{125}I-chlorambucil in tumor bearing mice model showing the % accumulation in body organs and fluids, although the limitation of the Ascitics tumor model so that it can't be generalized for all tumors. The high activity of both liver and kidneys approves that the ^{125}I-chlorambucilexcretion is through the two pathways hepatobiliary and renally. The high percent tumor site uptake of ^{125}I-chlorambucil indicates that this tracer is a potential tumor targeting agents able to overcome the main problem of the used agents, as it highly accumulates in the tumor cells. Total ascitic fluid has high activity reaching 22% at 30min post injection showing high affinity to the tumor tissue if compared with different tumor imaging agents like^{125}I-Celecoxib(5.4% at 30min) [9] and radio-iododeoxyuridine (IdUrd) 4.1% [25]. These results show that about 20% of ^{125}I-

chlorambucil injected i.v was incorporated in tumor DNA and that this approach may be further exploited for diffusion and therapy studies with Auger electrons.

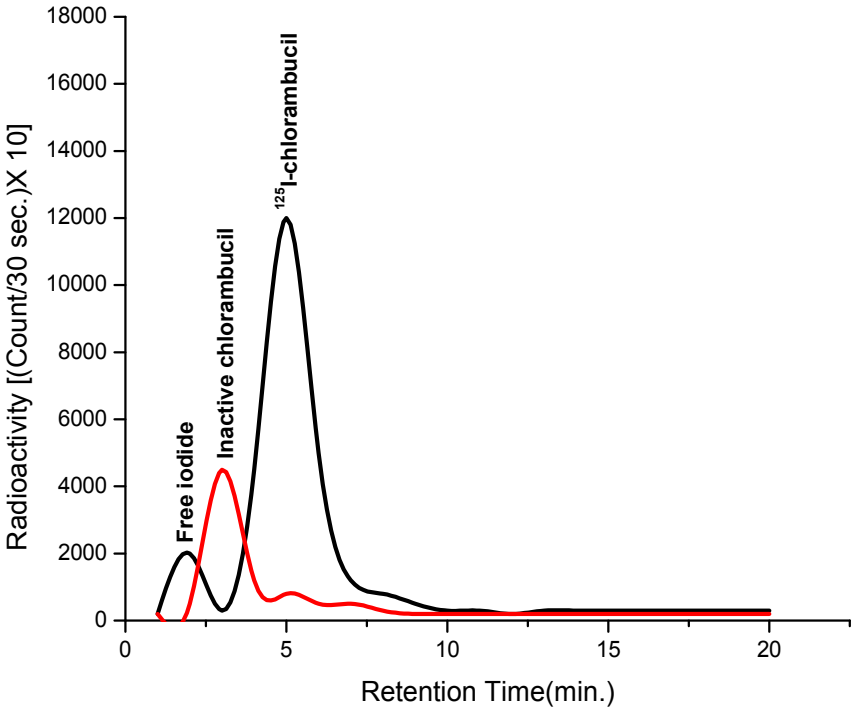

Fig. 7. HPLC radiochromatogram of ^{125}I-chlorambucil

Table 2. Biodistribution pattern of ^{125}I-chlorambucil in normal mice

Organs	% Injected dose/organ			
	0.5hr post injection	2hr post injection	4hr post injection	8hr post injection
Blood	16.1±1.8	12.2±1.0	9.7±1.1	7.3±1.2
Bone	3.8 ±0.6	3.0±0.7	2.4±0.3	2.0±0.5
Muscle	3.1 ±0.8	2.9±0.6	2.4±0.4	1.8±0.3
Spleen	0.2 ±0.3	0.2 ±0.4	0.3±0.2	0.1±0.1
Stomach	5.1 ±0.8	9.0±1.6	15.9±1.7	7.1±0.2
Kidney	4.1 ±0.6	6.6 ±0.8	5.1±0.6	3.0±0.9
Heart	1.6±0.7	0.8±0.3	0.5 ±0.2	0.3±0.1
Lung	1.3±0.6	0.9±0.4	0.7±0.5	0.4±0.1
Liver	14.0±1.2	9.1±1.0	5.5±1.1	4.8±0.9
Intestine	8.6±0.6	16.4±1.3	14.9±1.0	6.7±1.2
Urine	4.2±0.8	9.0±0.9	15.1±1.8	21.5±2.3
Thyroid	0.1±0.2	0.2±0.1	0.7±0.3	1.3±0.5

Table 3. Biodistribution pattern of ^{125}I-chlorambucil in tumor bearing mice at different times post injection

Organs	% Injected dose/ organ			
	0.5hr post injection	2hr post injection	4hr post injection	8hr post injection
Blood	15.7±1.3	11.6±1.1	7.7±1.0	5.7±1.4
Ascitic fluid	22.0±1.6	28.3±1.8	34.6±2.0	26.8±1.4
Bone	4.1±0.5	3.2±0.8	2.8±0.3	1.9±0.6
Muscle	3.6±0.9	3.1±0.6	2.3±0.4	1.1±0.2
Spleen	0.2±0.4	0.3±0.2	0.3±0.1	0.2±0.1
Stomach	5.8±0.9	8.2±1.2	15.1±1.8	6.0±0.2
Kidney	3.7±0.6	6.3±0.9	4.9±0.7	2.5±0.8
Heart	1.2±0.4	0.4±0.1	0.3±0.2	0.2±0.3
Lung	1.1±0.8	0.7±0.3	0.5±0.3	0.3±0.4
Liver	13.8±1.0	8.8±0.9	5.9 ±1.3	4.2±0.9
Intestine	8.2±0.7	16.0±1.5	14.2 ±1.1	7.7±1.3
Urine	3.4±0.6	8.0±0.7	11.3±1.8	19.7±2.0
Thyroid	0.2±0.3	0.4±0.3	0.9±0.6	1.5±0.6

4. CONCLUSION

The preparation of ^{125}I-chlorambucil was carried out by electrophilic substitution reaction via iodine-125-hydrogen exchange in the presence of CAT as oxidizing agent. After optimizing the conditions the labeling was performed at pH 7 using 100µg chlorambucil, and 50µg of CAT at 70ºC within 15min reaction time, the radiochemical yield was determined with the help of electrophoresis which was more than 98% and stable for up to 24h. The radioiodinated chlorambucil was purified by using reverse phase high performance liquid chromatography (HPLC) for biodistribution study in the tumor bearing Albino mice, which clarifies its importance as a tumor imaging in case of using iodine-123, while it may be used as tumor radiotherapy in case of using iodine-125. While the biodistribution of ^{123}I-chlorambucil confirms its potential as a tumor imaging agent, this study represent a preliminary study for ^{125}I- chlorambucil as a tumor therapy agent. However preclinical studies are needed to ensure the efficiency of the ^{125}I- chlorambucil for this purpose.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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