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Labeling of Piperine with Iodine-131 as Radiotracer in the Development of Cancer Drugs from Indonesia's Natural Products

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Abstract

Piperine, as one of the secondary metabolites of pepper, shows many pharmacological activities in various studies, including as antiinflammatory, antimicrobial, hepatoprotective, antioxidant, and potentially anticancer agents. Cancer is still one of the leading causes of death in the world due to the absence of specific symptoms in early-stage of cancer. The development of drugs for early diagnosis and selective treatment of cancer cells is considered one of the best solutions to reduce mortality. The activity of piperine that could induce apoptosis of 4T1 breast cancer cells and HT-29 colon cancer cells at in vivo trials prove it as a potential compound that can carry radioactive atoms (as labeled-compound) to cancer cells. This study was conducted to determine the optimal conditions of labeling of piperine with iodine-131 in order to obtain a ^{131}I-piperine which can later be used as a radiotracer in the development of cancer drugs. The labeling of piperine was performed by Chloramine-T iodination method and followed by purification with ion exchange chromatography. The optimal labeling results of piperine with 43.99% ± 1.23 radiochemical purity were obtained with piperine 3mg/300µL, chloramine-T 50 µg as an oxidizer, incubation time 30 min at room temperature, and 100 µg sodium metabisulphite as a reductor. To improve the purity of the preparation, purification by ion exchange chromatography method with Dowex 1X8 mesh 100-200 as resin was used until obtained 93.26% ± 0.94 of radiochemical purity. Further research is necessary before ^{131}I-piperine can be used as a radiotracer for cancer diagnosis in nuclear medicine.

Keywords: cancer, iodine-131, ^{131}I-piperine, labeled-compound, radiotracer

1. Introduction

Cancer is one of the leading causes of death in the world. The data from the Basic Health Research (Riskesdas) in 2013 from the Ministry of Health of Indonesia showed the prevalence of cancer patients in all ages in Indonesia reached 1.4 ‰.[1] This high prevalence of cancer in Indonesia needs to be observed with precautionary measures and early detection. Early detection of cancer is helpful not only to reduce mortality, but also to improve the quality of life of sufferers.[2] Besides, knowing the staging of a cancer is crucial for determining the most effective treatment(s) and for predicting survival.[3]

Cancer treatment can be done in various ways, including chemotherapy. However, this treatment method still weaknesses, in addition to killing cancer cells, chemotherapy can also affect normal cells that have rapid proliferation rates, such as hair follicles, bone marrow, and digestive tract cells.[4] Therefore, an alternative is needed for more selective treatment of cancer cells. Radiopharmaceutical is one of these kind of selective treatments.

Radiopharmaceuticals are drugs containing radioisotopes which are safe for administration in humans for diagnosis or therapy. The use of radiopharmaceuticals for imaging organ function and disease states

is a unique capability of nuclear medicine. The mapping of the radiopharmaceutical distribution *in vivo* provides images of functional morphology of organs in a non-invasive manner and plays an important role in the diagnosis of many common diseases associated with the malfunctioning of organs in the body as well as in the detection of certain type of cancers.[5]

A radionuclide (radioactive nuclide, radioisotope or radioactive isotope) is an atom that has excess nuclear energy, making it unstable. This excess energy can be used in one of three ways: emitted from the nucleus as gamma (γ) radiation; transferred to one of its electrons to release it as a conversion electron; or used to create and emit a new particle (alpha (α) particle or beta (β) particle) from the nucleus.[6] Radiopharmaceuticals used for diagnostic purposes are the ones with γ-emitting radioactive atoms, whereas radioactive atoms transmitting α or β particles are used for internal radiotherapy.

Iodine-131 (^{131}I) is a γ-beam and β-particle-emitting radionuclide. The energy produced from the γ-beam by ^{131}I is 364 keV, while the energy from the β particle beam is 0.61 MeV.[7] For emitting γ-beam and the β-particle, and having 8.1 days of half life, ^{131}I is an ideal radionuclide for the use of cancer diagnosis and therapy in nuclear medicine. However, in order to reach the desired target cells, ^{131}I needs to bind to compounds that have pharmacological activity against cancer cells. In this study, the compound is piperine.

Piperine is one of the secondary metabolites in alkaloid group that can be isolated from the *Piper nigrum* and *Piper longum* plants. Piperine has a pale yellow crystal with a melting point of 128-129^{o}C. Data analysis results from InfraRed spectrometry (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) showed that piperine isolates from Piper Nigrum plants had the molecular formula $C_{17}H_{19}NO_3$ with a molecular weight of 285 g/mol.[8] Piperin is soluble in various organic solvents, such as petroleum ether, chloroform, ethanol, and methanol, but is not soluble in water.[9] In several studies, piperine was shown to have various pharmacological activities, including as an antioxidant, anti-inflammatory, antidiarrheal, anti convulsant, antimutagenic, hypolipidemic, increased bile secretion, and inhibitor of tumor cells.[10] The inhibition activity of tumor cell growth by piperine is also confirmed by the study that showed piperine suppressed the growth of 4T1 breast tumors by inducing cell apoptosis, and preventing tumor cell metastasis at in vivo study.[11] In addition, piperine was also proven to inhibit the growth of HT-29 colon cancer cells and induce apoptosis.[12]

Labeling of piperine with ^{131}I is expected to produce an ideal radiopharmaceutical for the use of diagnosis in early detection of cancer cells, as well as for the use of selective therapy for cancer cells.

2. Materials and Methods

2.1. Materials

The equipments to carry out this study were analytical scale (Mettler Toledo), dose calibrator (Victoreen), microtube, vortex (Barnstead Thermolyne), micropipette (Eppendorf), oven (Memert), a set of ascending paper chromatography and thin layer chromatography, tweezers, single channel analyzer (Ortec) and glassware commonly used in laboratories.

The materials used were piperine (isolated by STFI-pharmacochemical laboratory), Na-^{131}I (Polatom), sodium metabisulfite (E.Merck), chloramine-T (SIGMA), phosphate buffer pH 7.4, absolute ethanol (E.Merck), aquadest (IPHA), methanol (E.Merck), dry methanol (E.Merck), ethyl acetate (E.Merck), toluene (E.Merck), Whatman No.1 paper and TLC-SG plate GF-254 (E. Merck), and universal pH (E.Merck).

2.2. Methods

2.2.1. Preparation of Solution

a. Piperine Solution
   Piperine 3 mg was dissolved in 300 µL of absolute ethanol.
b. Chloramine-T Solution
   Chloramine-T 5 mg was dissolved in 500 µL of phosphate buffer pH 7.4
c. Na-metabisulfite Solution
   Na-metabisulfite 5 mg was dissolved in

500 µL of phosphate buffer pH 7.4.

2.2.2. Labeling of Piperine with Iodine-131

In the microtube, Na-^{131}I was added to the piperine solution (pH was checked), then chloramine-T was added as an oxidizer. The mixture was then incubated for a few minutes while stirring using a vortex. Next, sodium metabisulfite was added (chloramine-T volume ratio: Na-metabisulfite = 1: 2) to stop the reaction. The labeling process was considered complete. Then the final pH of the preparation was measured using pH universal indicator, and the radioactivity of the preparation was measured using a dose calibrator. Furthermore, the purity examination was carried out by paper chromatography and thin layer chromatography. The chromatogram was then counted using a Single Channel Analyzer (SCA) and the percentage of purity was calculated.

2.2.3. Optimization of Labeling

a. Optimization of Chloramine-T
   Chloramine-T as an oxidizer was added with a number of variations, namely 50, 100, 250 µg.
b. Optimization of Incubation Time
c. The labeling was done in various incubation time, namely 1, 5, 10, 20, and 30 minutes.

2.2.4. Qualitative Characterization of ^{131}I-Piperine

Qualitative characterization of ^{131}I-piperine was carried out by thin layer chromatogafi method. The stationary phase used was the TLC-SG GF-254 plate with toluene:ethyl acetate (2:1) as mobile phase. TLC plates were cut to a size of 10 x 1 cm and marked every 1 cm with the number -1 to 8. ^{131}I-Piperine solution was spotted at 0 point. After elution, the plate was dried in an oven for several minutes. Then, spot of ^{131}I-Piperin were observed under a 254 nm UV lamp. The Rf ^{131}I-Piperine value was measured and compared with standard Piperin.

2.2.5. Determination of Chromatography Eluents

Determination of eluents was done by paper chromatography and thin layer chromatography with the same procedure as in characterization. In paper chromatography, the stationary phase used was Whatman No.1 paper, while the stationary phase thin layer chromatography used was the TLC-SG GF-254 plate. Various eluents used were aquadest, methanol 85%, dry methanol, and toluene: ethyl acetate (2:1). After eluted and dried, the chromatogram was counted using a Single Channel Analyzer (SCA) to calculate radiochemical purity. Eluent which was able to obtain the best separation between labeled-piperine and radiochemical impurities was selected for the next stage of this study (purity test).

2.2.6. Radiochemical Purity Test of ^{131}I-Piperine

The purity test was carried out by paper chromatography method with the stationary phase of Whatman No.1 paper and the mobile phase was selected from the previous stages of this study. ^{131}I-piperine preparation were spotted on chromatographic paper and eluted. The chromatogram was counted using a Single Channel Analyzer (SCA) and the percentage of radiochemical purity was calculated. If the purity of the preparation did not meet the purity requirements for radiopharmaceutical preparations, it would be proceed with the purification stage.

2.2.7. Purification

^{131}I-piperin were purified by ion exchange chromatography method. This method allows the separation between labeled-piperine and radiochemical impurities. The stationary phase used is Dowex 1X8 with several mesh variations, namely 50-100, 100-200, and 200-400, while the mobile phase is ethanol 70%. The fractions were collected with each volume from 500 to 1000 µL, then radioactivity was measured by dose calibrator, and the yield and radiochemical purity were calculated.

3. Results

3.1. Radioisotope Purity Test

The Na^{131}I preparation used met the requirements of radiochemical preparation for showing clear solution, pH 8-9, and 98.662% ± 0.149 of radiochemical purity according to the requirements set by the Ministry of Health in Indonesian Pharmacopoeia (FI)

3.2. Labeling of Piperine with Iodine-131

Labeling of piperine with radioisotope ^{131}I was carried out using Chloramine-T method through iodination process and electrophilic substitution. The structural prediction of the bond that occurs between ^{131}I and piperine was done computationally as showed in **Fig. 1.**

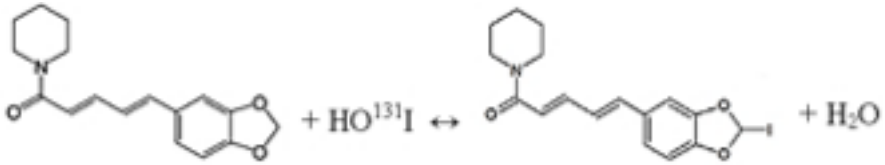


Fig. 1 ^{131}I-piperine Structure Prediction

366 nm UV lamps. The piperine spot was black at 254 nm and blue at 366 nm. There was no difference in the Rf value between the standard piperine and ^{131}I-piperine: 0.56, as showed in **Fig.2**.

3.4. Determination of Chromatography Eluents

The eluents tested were (A) toluene:ethyl acetate (2:1), (B) methanol 85%, (C) dry methanol, and (D) aquadest. Aquadest was the eluent which could separate ^{131}I-piperine from radiochemical impurities (**Fig.3**).

3.5. Optimization of Labeling

3.5.1. Optimization of Chloramine-T

The optimization of oxidizing agent was carried out with several variations of the amount of chloramine-T used, namely 50, 100, and 250 µg with an incubation time of 10 minutes.The radiochemical purity obtained were 28.868 ± 0.035; 36.649 ± 0.515; and 36.527 ± 4.662 respectively.

3.3. Qualitative Characterization of 131I-Piperine

The characterization was carried out for piperine standard and ^{131}I-piperine by Thin Layer Chromatography (TLC) method with the stationary phase Silica gel GF 254 and toluene:ethyl acetate (2:1) as mobile phase. Observations were made under 254 nm and

For the addition of 50 µg of chloramine-T produced a more stable preparation (without precipitation), and an increase in the amount of chloramine-T did not produce a significant increase in purity, then the chloramine-T amount of 50 µg was chosen for the next labeling formula. The optimization of labeling was continued with the variation of incubation time.

3.5.2. Optimization of Incubation Time

The labeling result with the highest radiochemical purity value 43.99% ± 1.23 was achieved with an incubation time of 30 minutes but not fulfilled the requirement of radiochemical purity (**Fig 4**). It was necessary to purify the preparation by ion exchange chromatography method.

3.6. Purification

Purification method was carried out using anion exchange resin, namely Dowex 1X8 with 50-100 mesh size variations; 100-

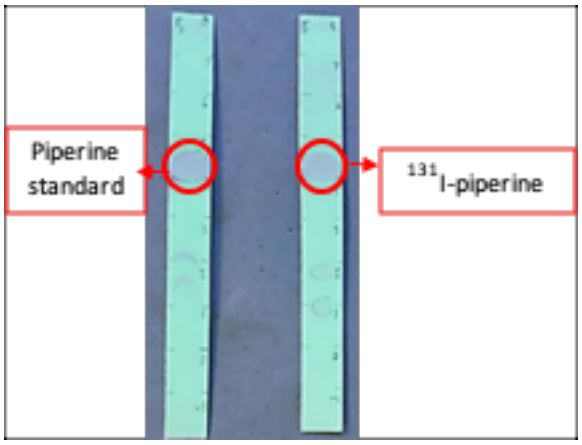
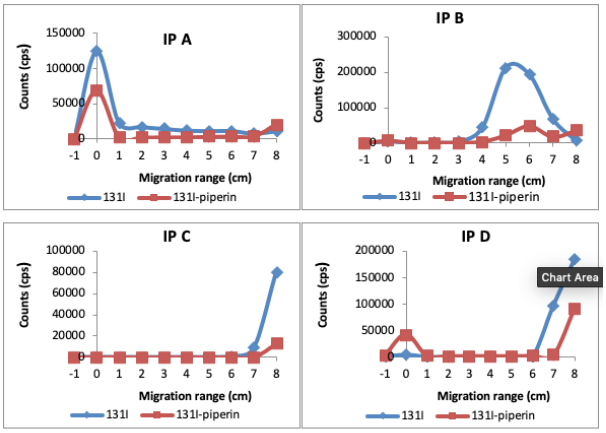


Fig. 2 Piperine standard and ^{131}I-piperine Chromatogram



IP A eluent: Toluene : ethyl acetate (2:1)
IP B eluent: Methanol 85%
IP C eluent: Dry methanol
IP D eluent: Aquadest

Fig. 3 Counting Profile of ^{131}I-Piperin

200; and 200-400.

The best purification results were obtained by purification using Dowex 1X8 mesh 100-200 resin with a column diameter of 0.6 cm; column height of 4-5 cm; and fraction volume of 500 µL. The radiochemical purity of the ^{131}I-piperine preparations obtained had fulfilled the requirements of radiopharmaceutical purity, 93.26% ± 0.94.

4. Discussion

4.1. Radioisotope Purity Test

The radiochemical purity of radioisotope Iodine-131 in the form of Na^{131}I solution was determined according to the requirements set by the Ministry of Health in Indonesian Pharmacopoeia, including:[13]

4.1.1. Clarity

Clarity test were done with visual method. Na^{131}I preparations showed the results of a clear solution in the absence of impurity particles and fulfilled the requirement.

4.1.2. pH

PH test for Na^{131}I preparations were carried out using a universal pH indicator. The pH range specified in the FI for the Na^{131}I solution is between 7.5 and 9. The Na^{131}I solution showed a pH range of 8-9.

4.1.3. Radiochemical Purity

Radiochemical purity test of Na^{131}I preparation was carried out by paper chromatography method using Whatman No.1 paper as a stationary phase and methanol: water (85:15) as the mobile phase. This eluent was chosen because it could separate ^{131}I from radiochemical impurities such as iodate ions (IO_3^-). Na^{131}I purity was characterized based on the Rf value and the results of the paper counts using SCA. The Rf value of ^{131}I was 0.7, while IO_3^- Rf value was 0.4.[14]

4.2. Labeling of Piperine with Iodine-131

Chloramine-T method was chosen for high specific activity compounds could be obtained by this method and the labeling effieciency can be very high (~90%).[15]

The reaction that occurs in labeling of piperine with ^{131}I is an electrophilic substitution because piperine does not have an iodine atom in its structure. Through electrophilic substitution, the most stable bond between ^{131}I and piperine occurs in C atoms located between two ether groups. In this case ^{131}I replaces the position of one of the H atoms (**Fig.1**)

4.3. Qualitative Characterization of ^{131}I-Piperine

By comparing the chromatographic profile between standard piperine and ^{131}I-piperine, it is known that labeling piperine with ^{131}I did not cause changes in the properties of piperine for there was no difference in the Rf value between the standard piperine and ^{131}I-piperine (**Fig.2**).

4.4. Determination of Chromatography Eluents

Determination of chromatographic

eluents is important, especially for purity test of the labeled-compound. The chosen chromatographic eluent here was the one which could separate ^{131}I-piperine from radiochemical impurities. These radiochemical impurity were derived from an imperfect labeling process. They could be in the form of I^-, IO_3^-, and IO_4^-.

From **Fig. 3 (IP D)**, we could see that ^{131}I-piperine remained at the point of spotted, while iodine impurities moved with eluent (aquadest). Therefore, aquadest was chosen as the eluent used for the purity test of ^{131}I-piperine at the next stage.

4.5. Optimization of Labeling

4.5.1. Optimization of Chloramine-T

Determination of the amount of oxidizing agent is very important in reducing the radiochemical impurities formed. In addition, the right amount of oxidizing agents can also minimize the damage to the compounds that will get labeled and/or the labeled-compounds formed.

The addition of chloramine-T amount produced a better purity of the ^{131}I-piperine preparation. However, precipitate was formed with the addition of 100 and 250 µg chloramine-T. The precipitate was centrifuged and separated from the supernatant, and then it was dissolved with the solvents used to dissolve piperine or chloramine-T, namely ethanol and phosphate buffer. As a result, the precipitate dissolved in phosphate buffer. It was suspected that the sediment was not piperine. However, further research is needed to identify the precipitate formed.

4.5.2. Optimization of Incubation Time

Incubation time that was too short could cause a labeling reaction that did not work perfectly, on the other hand, the long incubation time could cause damage to the labeled-compound because of long interaction with the oxidizing agent.

The labeling result with the highest radiochemical purity value 43.99% ± 1.23 was achieved with an incubation time of 30 minutes as showed in **Fig 4**.

4.6. Purification

Radiochemical impurities were in the form of anions, such as I^-, IO_3^-, and IO_4^-, therefore purification method was carried out using anion exchange resin.

Dowex 1X8 is a strong base type anion exchange resin with chloride anion in it. Ion exchange occured between I^-, IO_3^-, and IO_4^- ions, with Cl^- ions. The radiochemical impurities binded stronger with resin than Cl^-ions so that they would be retained in the resin, while ^{131}I-piperine would come out with eluent (70% ethanol).

Purification started by using a column with a diameter of 1.5 cm and a height of 4-5 cm with Dowex 1X8 mesh 50-100. The fractions were collected to 10 fractions with each volume of 1 mL. ^{131}I-piperine was considered to be in the 2nd and 3rd fractions, because in other fractions, the activity detected was very small and almost undetectable as showed in **Table 1**. Radiochemical purities obtained were 63.04% and 49.33% with yields of 2.36% and 1.18% respectively.

With the same treatment, purification was also carried out with Dowex 1X8 mesh 100-200. The fraction were collected to 5 fractions with volume of 1 mL. With this treatment, ^{131}I-piperine was in the 4th fraction with a slight increase in Radiochemical Purityto 69.91% and yields to 3.03%.

Purification with 200-400 Dowex 1X8 mesh with the same treatment did not provide good results. From the whole purification using a column with a diameter of 1.5 cm and a height of 4-5 cm, the results of the radiochemical purity of the preparation did not meet the requirement of the purity for radiopharmaceutical preparation. Besides, the yields produced was also very minimal. This might be caused by the dimension of the column which was too large so that the ^{131}I-piperine elution process did not work well for ^{131}I-piperine was trapped in the resin. Therefore, purification was continued with smaller column dimensions, with a diameter of 0.6 cm and a height of 4-5 cm using a 100-200 Dowex 1X8 mesh resin which had previously produced the best purity and yields.

By the results shown from the **Table 2**, it is known that there had been an increase in

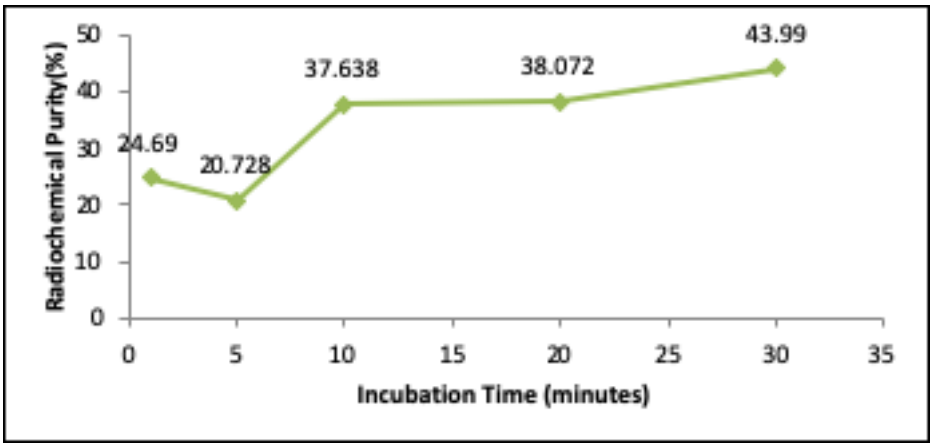


Fig. 4 Radiochemical Purity on Optimization of Incubation Time

Table 1 ^{131}I-piperin Fractination by Dowex 1X8 mesh 50-100

Resin	Fractions	Radioactivity (mCi)
Dowex 1X8 mesh 50-100	1	0
	2	0.010
	3	0.005
	4	0.002
	5	0.002
	6	0.001
	7	0.001
	8	0.001
	9	0.001
	10	0.001

Table 2 ^{131}I-piperin Purification by Dowex 1X8 mesh 100-200 with Column Diameter 0.6 cm

a. Fractions volume of 1 mL

Fractions	Radioactivity (mCi)		Radiochemical Purity(%)		Yields (%)
	Before	After	Before	After	
1	0.168	0.019	43.12	86.206	11.30
2		0.007		88.565	4.17
3		0.005		95.556	2.98
4		0.003		87.853	1.78
5		0.003		-	1.78

b. Fractions volume of 500 µL (1)

Fractions	Radioactivity (mCi)		Radiochemical Purity(%)		Yields (%)
	Before	After	Before	After	
1	0.293	0.012	22.10	87.487	4.10
2		0.017		90.261	5.80
3		0.004		85.608	1.37
4		0.004		82.642	1.37
5		0.004		93.046	1.37

c. Fractions volume of 500 µL (2)

Fractions	Radioactivity (mCi)		Radiochemical Purity(%)		Yieds (%)
	Before	After	Before	After	
1	0.192	0.000	26.63	89.842	0
2		0.014		93.255	7.29
3		0.000		-	0
4		0.000		-	0
5		0.000		-	0

radiochemical purity, which reached 86.21% and 88.57% and the yield 11.30% and 4.17%.

However, purification continued with a decrease in fraction volume from 1 mL to 500 µL in order to obtain the preparations with better radiochemical purities.

The best purification results were obtained by purification using Dowex 1X8 mesh 100-200 resin with a column diameter of 0.6 cm; column height of 4-5 cm; and fraction volume of 500 µL. The radiochemical purity of the ^{131}I-piperine preparations obtained had met the requirements of radiopharmaceutical purity, 93.26% ± 0.94.

5. Conclusion

Piperine was able to be labeled with radioisotope ^{131}I using Chloramine-T method. The labeling was done with piperine formula 3 mg/300 µL, chloramine-T 50 µg, incubation time 30 minutes, and sodium metabisulfite 100 µg with radiochemical purity of 43.99% ± 1.23. Therefore, to improve the purity of the preparations, purification was carried out by anion exchange chromatography method using Dowex 1X8 mesh 100-200 resin with 0.6 cm column diameter and 4-5 cm column height, and ethanol 70% as eluent. The labeling results with this method had met the requirements of radiopharmaceutical purity, which was 93.26% ± 0.94 with a yield of 5% to 7.29%.

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