ORIGINAL ARTICLE

Method development, validation, and impurity measurement of β-estradiol from radiolabeled [^{131}I] β-estradiol using radio-high-performance liquid chromatography for radioligand of saturation binding assay

Isti Daruwati[1,2,3], Abednego Kristande Gwiharto[1,3], Hendris Wongso[2,3], Tri Hanggono Achmad[4], Mukh Syaifudin[2,3], Muchtaridi Muchtaridi[1,3]

[1]Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Padjadjaran, [3]Research Collaboration Center for Theranostic Radiopharmaceuticals, National Research and Innovation Agency, [4]Department of Basic Medical Science, Faculty of Medicine, Universitas Padjadjaran, Jatinangor, [2]Research Center for Radioisotope, Radiopharmaceutical, and Biodosimetry Technology, Nuclear Energy Research Organization, National Research and Innovation Agency (BRIN), Banten, Indonesia

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ABSTRACT

β-estradiol is an estrogen steroid hormone and acts as an estrogen receptor agonist. Radiolabeled β-estradiol is widely used as a radioligand for binding assays. In this present study, the synthesis of [^{131}I]β-estradiol has been successfully carried out. Accordingly, the measurement of the radiochemical purity (RCP) value and the presence of chemical impurities are needed. To validate the method for identifying the RCP and chemical impurities from [^{131}I]β-estradiol using high-performance liquid chromatography (HPLC). The synthesis of [^{131}I]β-estradiol was accomplished by a radioiodination reaction, and the RCP was determined by radio-HPLC. The method for β-estradiol measurement was validated by reversed-phase HPLC radio-analytical employing ultraviolet-visible (UV-Vis) and radioactive detector. The method for radio-HPLC analysis was validated and established using a C-18 column and MeCN: H_2O (55:45 v/v) as the mobile phase. The following conditions were applied: a flow rate of 1.2 mL/min, isocratic, and a UV-Vis detector at 280 nm. The RCP of [^{131}I]β-estradiol measured by thin-layer chromatography and radio-HPLC was 99.27% ± 1.25% and 95.75% ± 2.41%, respectively. The validation parameters were appropriate and met the requirements for acceptance. HPLC analysis was able to identify the presence of unlabeled estradiol (24.51%–27.29%) in the mixture of [^{131}I]β-estradiol. As a result, purification using preparative HPLC or other methods will be required in future studies.

Key words: High-performance liquid chromatography radio-analytical method, method validation, radiochemical purity, radioiodination, β-estradiol

INTRODUCTION

β-estradiol plays an important role in women's reproductive health, particularly in the development of secondary

Address for correspondence:
Prof. Muchtaridi Muchtaridi,
Jl. Ir. Soekarno KM 21, Jatinangor 45363, Indonesia.
E-mail: muchtaridi@unpad.ac.id

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characteristics such as hips and the menstrual cycle.[1,2] However, β-estradiol is the most important risk factor for breast cancer in most women because estrogen receptors (ERs) are expressed in 75% of breast cancer patients. As a result, β-estradiol has emerged as a reliable predictor of breast cancer development. WHO (2021) reported that in 2020, the incidence of breast cancer worldwide had reached 11.7% of the 19.2 million new cases of all types of cancer, with 684,996 deaths.[1] Dai *et al*. classified breast cancer into five subtypes: luminal A, luminal B, HER-2 positive, triple-negative, and normal-like.[2] The luminal subtype, especially luminal A, is most commonly found in breast cancer patients and shows a good response to hormone therapy.[3]

β-estradiol is an agonist ligand that has a sensitive and selective affinity for ER. Radiolabeling of β-estradiol and its derivatives has been carried out by Kumar *et al*. using ^{18}F and Sallam using ^{125}I.[4,5] To develop drug candidates for breast cancer, [^{131}I]β-estradiol has been synthesized as a radioligand for an *in vitro* binding assay. [^{131}I]β-estradiol will bind specifically to the ER as well as β-estradiol. The potency of drug candidates for breast cancer can be evaluated through several experiments, including the saturation and competitive binding with radiolabeled β-estradiol against breast cancer cells.[6] The radioligand [^{131}I]β-estradiol should have radiochemical purity (RCP) of > 95%, which means it should contain a minimum percentage of [^{131}I] NaI and a minimum percentage of unlabeled β-estradiol as a chemical impurity.

In this study, the synthesis of [^{131}I]β-estradiol was performed by a radioiodination reaction using chloramine T as a strong oxidizing agent with a short reaction time.[7] Radioiodination is an electrophilic substitution reaction of an aromatic compound that has sufficient electron donor groups, hence it can activate the carbon in the aromatic ring.[8] Radioiodination is often used in the radiolabeling process because it minimally affects the binding affinity and specificity of the parent ligand to the receptor.[9] Several radioisotopes of iodine can be used for radioiodination, including ^{123}I, ^{124}I, ^{125}I, and ^{131}I.[10] The radioisotope iodine-131 was selected as it is relatively easy to produce in a nuclear reactor, with a sufficient half-life, and is convenient for radiolabeling a number of active molecules. Iodine-131 is also widely used for *in vitro* and *in vivo* research, it mostly decays by beta emission (606 keV; 90%) and emits high-energy gamma radiation (364 keV; 10%) with a physical half-life of 8 days.[11,12]

The determination of chemical and radiochemical impurities can be carried out using radio-high-performance liquid chromatography (HPLC) with ultraviolet-visible (UV-Vis) and radioactive detectors.[13] HPLC is an analytical technique for high-resolution radiolabeled compound detection, quantitative measurement of unknown metabolites, and real-time monitoring of samples using a radioactive detector.[14] For example, Aboumanei *et al*. performed paper chromatography and HPLC analysis for quality control of radioiodinated ethopabate.[15] Rokka *et al*. have developed [^{18}F] fluorodeoxyglucose analysis with radio-HPLC and radio-thin-layer chromatography (TLC) methods.[16] Bispo *et al*. have also developed a method to validate [^{18}F] fluoroestradiol using reversed-phase HPLC (RP-HPLC).[17]

In this present study, quality control of [^{131}I]β-estradiol must be established and validated because it does not have an official monograph in the pharmacopeia. The study's goals were to create a validation method for β-estradiol identification and quantification by evaluating several HPLC parameters developed by Wissmann *et al*.[18] Then, the RCP and chemical impurity of [^{131}I]β-estradiol resulting from the reaction optimization were identified by a RP-HPLC-based radio-analytical method.

SUBJECTS AND METHODS

Materials

All chemicals used in this research were laboratory or reagent-grade from Merck and Sigma Aldrich. [^{131}I] NaI was produced in the G. A Siwabessy Multipurpose Reactor, Serpong, Indonesia. In addition, HPLC UV-Vis detector (Agilent Technology-GINA) and radioactive detector (Raytest-GABI), column C18G (150 mm × 4.6 mm, 5 µm, 120 Å) (SGE Analytical Science), radio-TLC scanner (Bioscan), and UV-Vis spectrophotometer (Thermo Scientific GENESYS 15G) were also used.

Synthesis of [^{131}I]β-estradiol

One milligram of β-estradiol was dissolved in 0.5 mL of pure EtOH, and 50–100 mCi of iodine-131 was added. Subsequently, 1 mg/mL of chloramine-T was added, and the reaction was incubated at room temperature for 5 min. Then, 10 mL of sodium metabisulfite (2.5 mg/mL) was added to quench the reaction.

Preparation of standard stock solution

As stock solution 1, 2.5 milligrams of β-estradiol were dissolved in 5.0 mL of pure EtOH in a 5.0-mL volumetric flask (500 ppm). Then, five variations of concentrations (200, 175, 150, 125, and 100 ppm) were made for a total volume of 5.0 mL solution, then filtered with 0.45 mm Millex-HV before injection into the HPLC.

Determination of maximum wavelength

Briefly, 2.5-mg β-estradiol was dissolved in 5.0 mL pure EtOH (500 ppm), then the solution was diluted to obtain 100 ppm, then measured using UV-Vis spectrophotometer.

Determination of radiochemical purity of [^{131}I] β-estradiol using radio-high-performance liquid chromatography and variation of eluent

It was done with UV-Vis and a radioactive detector. The

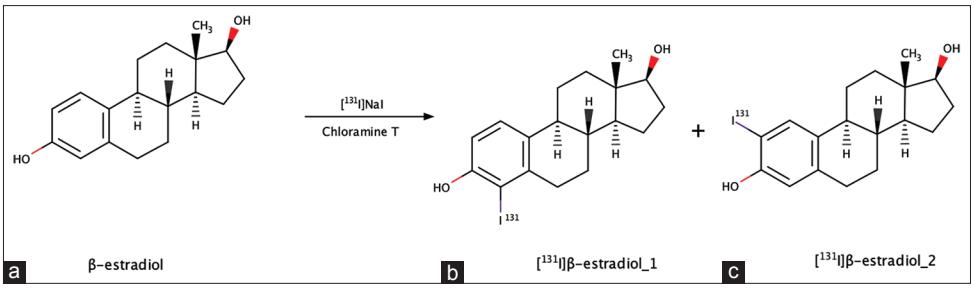

Figure 1: Proposed structure of [^{131}I]β-estradiol_1 (b) and [^{131}I]β-estradiol_2 (c) from radioiodination of β-estradiol (a)

Table 1: Optimum formulation of ^{131}I β-estradiol

Parameter	Amount
β-estradiol (μg)	50
Chloramine-T (μg)	100
^{131}I NaI (μL)	5-10
Radioactivity (μCi)	50-100
Reaction time (min)	5
$Na_2S_2O_5$ (μg)	250
pH	7.5-8

C-18 column system with 55:45 and 90:10 v/v (MeCN: H_2O) and 90:10 v/v (MeOH: H_2O) was used as an eluent. The isocratic system has varied from 1.0 to 1.2 mL/min flow rate and 280 nm wavelength. Ten milliliter of a solution of [^{131}I]β-estradiol was injected into the HPLC. The radiochromatogram of [^{131}I]β-estradiol was analyzed by calculating of the RCP.

Validation of β-estradiol

The C-18 column system, with 55:45 v/v (MeCN: H_2O) with a 1.2 mL/min and a 280 nm. β-estradiol and [^{131}I] NaI were injected. Then, each concentration in the HPLC system was injected five times and repeated three times on three different days within 1 week. The data were processed to obtain system suitability data.[19]

Identification of impurity β-estradiol

Determination of the chemical impurity was carried out by varying 0.01, 0.02, and 0.05 mg of β-estradiol. Then, each radiolabeled compound of [^{131}I]β-estradiol was injected using the validated method. The percentage of β-estradiol was calculated.

Determination of log P and dipole

log P and dipole values were analyzed from the predicted structure using the MarvinSketch module.

RESULTS

β-estradiol was determined as a chemical impurity from radiolabeled [^{131}]β-estradiol using a C-18 column and MeCN: H2O (55:45 v/v), a flow rate of 1.2 mL/min, isocratic, a UV-Vis detector at 280 nm and radioactive detectors. Method validation was obtained linearity of 0.9994, accuracy

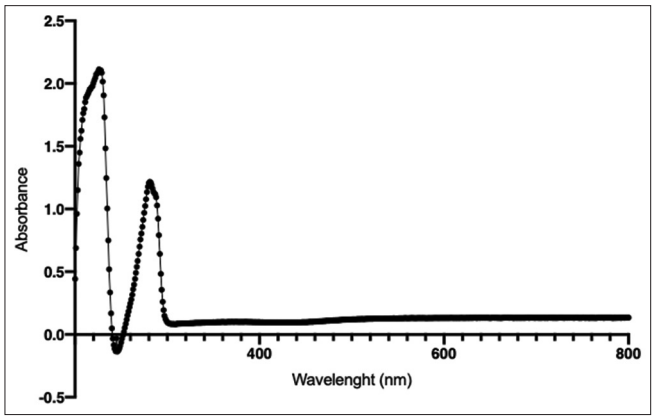

Figure 2: Ultraviolet-visible spectrophotometer graph of 100 ppm β-estradiol

in the range of 99.8 to 101.5, precision of 0.46–1.03, and LOD and LOQ limits of 5.919 g/mL and 17.936 g/mL, respectively. The peaks that appeared at 6.53 minutes was [^{131}Iβ-estradiol _1, and at 8.25 minutes was [^{131}Iβ-estradiol _2 which was confirmed by log P the at same value of 4.67 and the dipole values was 4.21 and 3.07 debye, respectively.

DISCUSSION

β-estradiol has two hydroxyl groups, one at number 3 (aromatic ring) and one at 17. [^{131}I] NaI radionuclide in the form of I− was oxidized to I+ by chloramine T, then complexed with water to form hydrated iodonium ion (H_2OI+) or hypoiodic acid. The radioiodination process is predicted to be an electrophilic substitution process in which the iodine atom is attached to the adjacent electron-attracting group, in particular, the ortho position of the aromatic group of β-estradiol [Figure 1].[10,20] This radioiodination method is capable of producing radiolabeled compounds with high specific activity at very low concentrations.[9] Thus, there are two possible positions for the attachment of the iodine atom: position number 4 and 2, which are referred to as [^{131}I]β-estradiol_1 and [^{131}I] β-estradiol_2, respectively.

The determination of RCP is usually conducted by paper chromatography and electrophoresis because the process is faster and simpler with less waste generated.[21] The

calculation of the amount of [^{131}I]β-estradiol based on the radioactive decay equation yielded 6.13 pmol in 10 μCi. Its presence indicates that a radioactive compound is a very small number compared to the unlabeled initial compounds.[11] The measurement of the β-estradiol compound revealed 2 absorption maxima [Figure 2] and the wavelength chosen was 280 nm.[22] As shown in Figures 3 and 4, the peaks in the UV channel are identified as β-estradiol at 2.05 min, while the peaks in the radioactive channel are [^{131}I]β-estradiol_1 and [^{131}I]β-estradiol_2 peaks at 2.42 and 2.82 min, respectively. The optimum system of determination was developed using the C-18 column, a mixture of 55% MeCN, isocratic, and flow rate of 1.2 mL/min. Figures 5 and 6 showed the separation using MeCN 90% with a flow rate of 1.0ml/min. This HPLC system showed the optimum separation of four

Table 2: Compatibility test of the system with radio-high-performance liquid chromatography

Parameters	Requirements	Results
Capacity factor	>2	3.33
Number of theoretical plates	>2000	8377
Tailing factor	<2	1.005

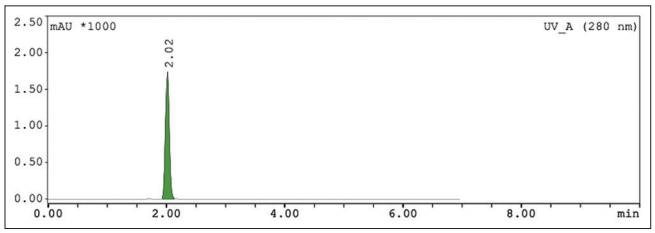

Figure 3: Chromatogram profile of β-estradiol using MeOH: H_2O (90:10) with flow rate 1.0 mL/min and ultraviolet detector at 280 nM

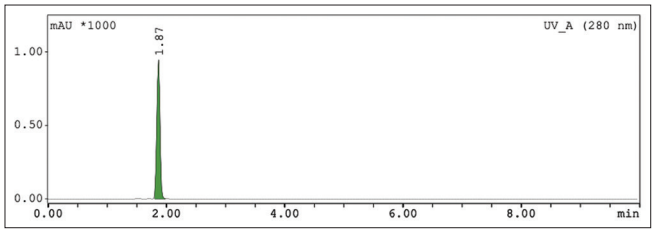

Figure 5: Chromatogram profile of β-estradiol using MeCN: H_2O (90:10) with flow rate 1.0 mL/min and ultraviolet detector

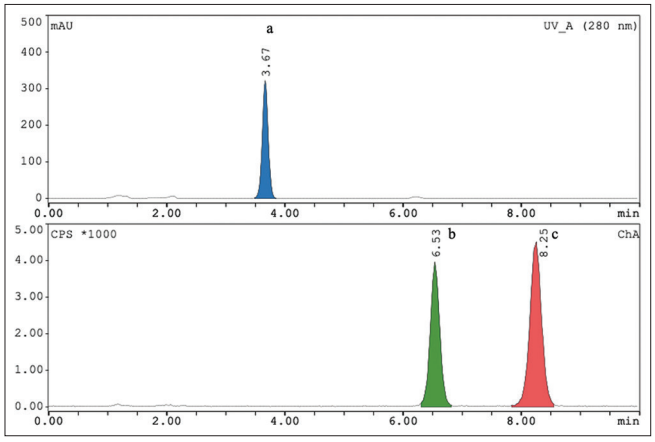

Figure 7: Radio-high-performance liquid chromatography chromatogram of β-estradiol (a) and [^{131}I]β-estradiol_1 (b) and [^{131}I] β-estradiol_2 (c) using MeCN: H_2O (55:45) with flow rate 1.2 mL/min and ultraviolet at 280 nM and radioactive detector

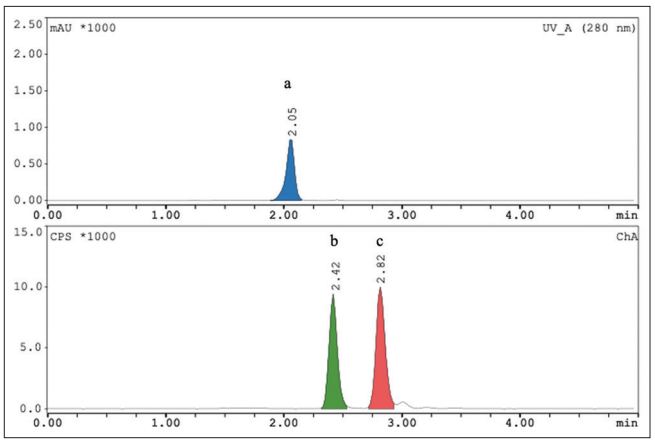

Figure 4: Chromatogram profile of β-estradiol (a) and [^{131}I]-I-β-estradiol_1 (b) and [^{131}I]β-estradiol_2 (c) (using MeOH: H_2O (90:10) with flow rate 1.0 mL/min and ultraviolet and radioactive detector

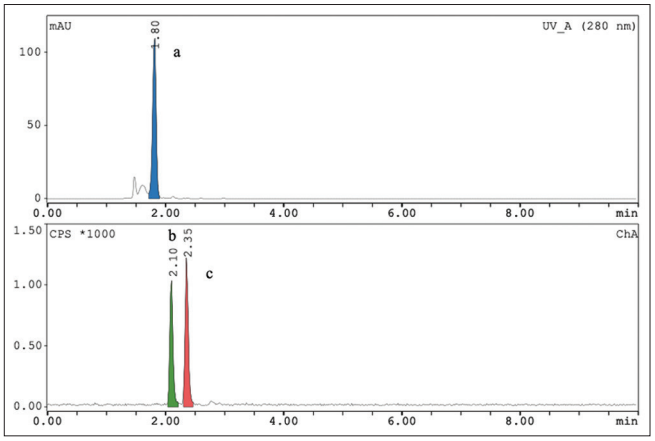

Figure 6: Chromatogram profile of β-estradiol (a) and [^{131}I] β-estradiol_1 (b) and [^{131}I]β-estradiol_2 (c) using MeCN: H_2O (90:10) with flow rate 1.0 mL/min and ultraviolet and radioactive detector

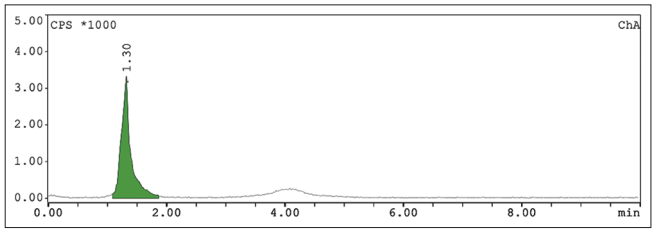

Figure 8: Radio-high-performance liquid chromatography chromatogram of [^{131}I] NaI using MeCN: H_2O (55:45) with flow rate 1.2 mL/min with radioactive detector

compounds [Figure 7]. The first peak that appeared was [^{131}I] NaI at retention time (RT) of 1.30 min as a radiochemical impurity [Figure 8]. The unlabeled β-estradiol compound was then detected as the second peak of the chromatogram, at RT of 3.67 min [Figure 9]. [^{131}I]β-estradiol radioligand appeared as two peaks that were proposed at 6.53 and 8.25 min, respectively [Figure 7]. Figures 7 and 8 and optimum formulation of [^{131}I]β-estradiol [Table 1].

This radioligand is less polar than the natural ligand because of the addition of iodine-131, thus increasing the molecular weight. The results of calculations with the MarvinSketch module, log *P* of [^{131}I]β-estradiol_1 and [^{131}I]β-estradiol_2 had the same value of 4.67, while log *P* of β-estradiol of 3.75. The dipole values of β-estradiol, [^{131}I]β-estradiol_1, and [^{131}I]β-estradiol_2 were 4.21; 3.07, and 2.99 debye, respectively. Hence, [^{131}I]β-estradiol_2 is the compound with the lowest polarity. Thus, the peak that appeared at 6.53 min was [^{131}I]β-estradiol_1, and at 8.25 min, it was [^{131}I] β-estradiol_2 [Figure 7].

The compatibility test of the HPLC system [Table 2] met the acceptance criteria. The validation [Figure 10] had good linearity, reaching the standard curve requirements of 0.999, where the result is 0.9994. The validation results [Table 3] showed a linearity of 0.9994, accuracy in the range of 99.8–101.5, a precision of 0.46–1.03, and limits of detection (L_OD) and limits of quantification (L_OQ) limits of 5.919 g/mL and 17.936 g/mL, respectively.[19] The experiment [Table 4] of the five concentrations had an acceptance percentage, or the accuracy, at the acceptance limit of 98%–102%, and repeatability below 2%. It can be concluded that the system and validation used were appropriate and could be used in the analysis phase.

The optimum radiolabeling results should exhibit RCP >95%,[23] but the chemical impurity is sometimes undefinable. An excess of unlabeled β-estradiol may occur as a chemical impurity from the synthesis of radioligand [^{131}I] β-estradiol. The radioligand binding assay (RBA) was very important in determining the amount of the natural ligand because it would affect the binding of the radioligand to the receptor. If the number of receptors was limited, there would be competition between the radioligand and the natural ligand. This decreased the number of radioligands occupying the receptor.[24] According to Table 5 and

Table 3: Validation method with radio-high-performance liquid chromatography

Parameters	Terms	Results
Linearity	R^2 ≥0.999	R^2=0.9994
Accuracy (%)	Percent recovery 98-102	99.80-101.05
Precision (%)	Percent RSD ≤2	0.46-1.03
LoD	-	5.919 µg/mL
LoQ	-	17.936 µg/mL
Specificity	-	-

LoD: Limits of detection, LoQ: Limits of quantification, RSD: Relative standard deviation

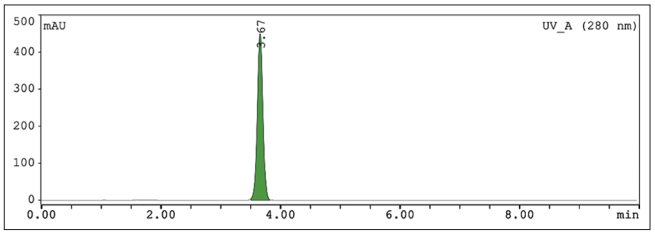

Figure 9: Chromatogram profile of β-estradiol using MeCN: H_2O (55:45) with flow rate 1.2 mL/min and ultraviolet detector at 280 nM

Table 4: Intraday precision and accuracy of β-estradiol with radio-high-performance liquid chromatography

Concentration		Recovery (%)	Average	SD	RSD	Recovery (%)
Prepared	Actual					
100	99.625	99.625	99.999	1.012	1.012	99.99±1.01
	101.145	101.145				
	99.227	99.227				
125	125.010	125.010	100.018	1.033	1.033	100.01±1.03
	123.737	123.737				
	126.319	126.319				
150	150.954	150.954	100.094	0.608	0.607	100.09±0.60
	150.311	150.311				
	149.159	149.159				
175	174.560	174.560	99.805	0.633	0.634	99.80±0.63
	173.603	173.603				
	175.812	175.812				
200	199.851	199.851	100.091	0.457	0.456	100.09±0.45
	201.214	201.214				
	199.479	199.479				

SD: Standard deviation, RSD: Relative SD

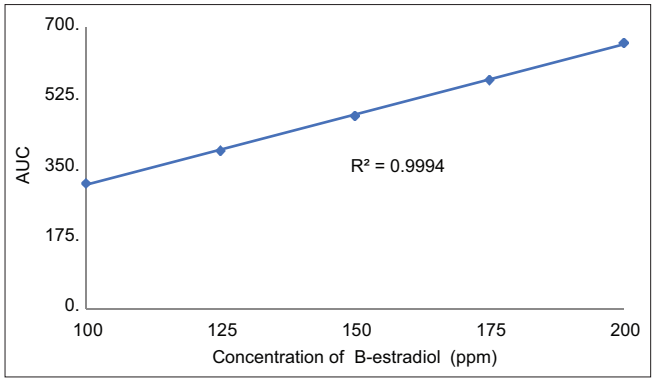

Figure 10: Standard curve of β-estradiol (n = 5). AUC: Area under the curve

Figure 11, the average of unlabeled β-estradiol was between 24.31% and 27.29% with the highest RCP of 95.75% ± 2.41% at 500 ppm concentration. After separation, it can be seen that [^{131}I]β-estradiol_1 and [^{131}I]β-estradiol_2 peaks have been obtained but with lower concentrations [Figure 12]. Thus, further research is needed to improve the purification process and maximize the high concentration yield.

CONCLUSION

Radio-HPLC was used to the development of analytical methods to determine β-estradiol in [^{131}I]β-estradiol. All compounds were well separated on an analytical C-18 column with excellent resolution and met the requirements

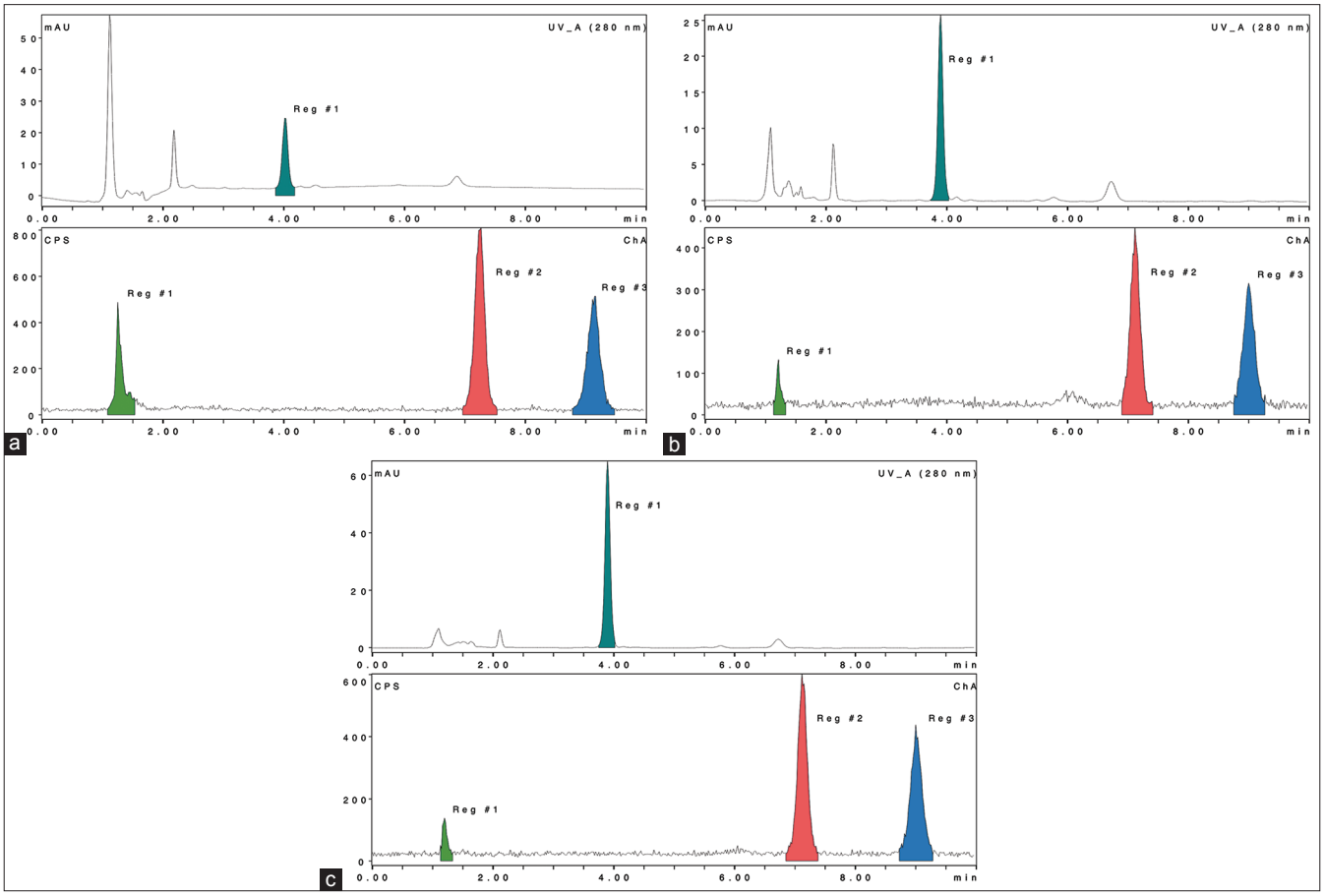

Figure 11: Chromatogram of [^{131}I]β-estradiol_1 and [^{131}I]β-estradiol_2 using radio-high-performance liquid chromatography with ultraviolet-visible and radioactive detector from variation (a) 100; (b) 200; (c) 500 ppm of β-estradiol

Table 5: Chemical and radiochemical impurity of [^{131}I] β-estradiol using radio-high-performance liquid chromatography

β-estradiol (ppm)	RCP (%)	Radiochemical impurity (%)	Chemical impurity (%)	Unlabeled β-estradiol (μg)
100.0	82.87±0.34	17.13±0.34	24.51±0.63	24.51±0.63
200.0	94.33±2.50	5.67±2.50	24.31±2.75	48.61±2.75
500.0	95.75±2.41	4.25±2.41	27.29±4.17	136.46±0.63

RCP: Radiochemical purity

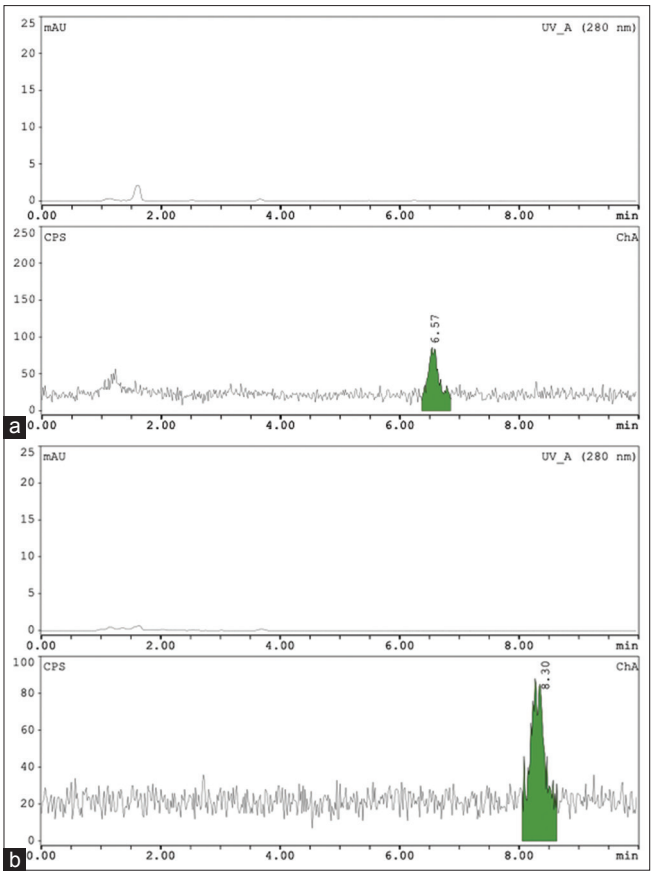

Figure 12: Chromatogram profile of [^{131}I]β-estradiol_1 (a) and [^{131}I] β-estradiol_2 [b] after purification using radio-high-performance liquid chromatography with ultraviolet-visible and radioactive detector

of linearity, precision accuracy, L_OD, and L_OQ. The radioligand [^{131}I]β-estradiol had a high RCP (>95%) and a low amount of chemical impurity of unlabeled β-estradiol. In the following step, [^{131}I]β-estradiol will be easily separated from β-estradiol as a chemical impurity and [^{131}I] NaI as a radiochemical impurity for RBA purposes using this method.

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Conflicts of interest

There are no conflicts of interest.

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