The use of Whatman-31ET paper for an efficient method for radiochemical purity test of $^{131}$I-Hippuran

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Abstract. Current chromatography methods used for radiochemical purity test of $^{131}$I-Hippuran is time consuming. Therefore, in this study we explored several static and mobile phases in order to have a chromatography method which is accurate and efficient or less time consuming. In this study, stationary phases (Whatman-1, 31ET, and 3MM papers) and several mobile phases were explored to separate $^{131}$I-Hippuran from its impurity ($^{131}$I). The results of this study showed that the most efficient chromatography system for measurement of radiochemical purity of $^{131}$I-Hippuran was by using Whatman-31ET paper and n-butanol: acetic acid: water (4:1:1) as a static phase and mobile phase respectively. Developing time for this method was of approximately 75.7 ± 2.7 minutes. The result of radiochemical purity (%RCP) of $^{131}$I-Hippuran measured with this chromatography system either using Whatman-1 or Whatman-31ET paper strips was 98.7%. The short size of Whatman-31ET paper strip (1 x 8 cm) was found to have shorter developing time compared to that of long size paper. This system showed a good separation of $^{131}$I-Hippuran from its impurities and gave %RCP of 98.1% ± 0.04% with developing time approximately 44.3 ± 9.4 minutes. The short size of Whatman-31ET paper strips was found to be more efficient compared to that of Whatman-1 and Whatman-3MM paper strips in term of developing time.

1. Introduction

Labelled compounds have been widely used for diagnostic or therapeutic purposes. One of them is $^{131}$I-Hippuran which is used for diagnosis of renal function [1]. In Indonesia, $^{131}$I-Hippuran has been routinely used in several hospitals namely Ulin Hospital, Kalimantan Tengah; Hasan Sadikin Hospital, Bandung; Annur Specialist Surgery Hospital and Bethesda Hospital, Yogyakarta; POLRI Hospital, Jakarta; and Kartini Hospital, Jepara [2]. The utilization of the $^{131}$I-Hippuran must be supported with an effective and efficient of its quality control. Labelled compounds must meet with the certain requirements for their physicochemical and biological qualities. The physicochemical quality of the labelled compound includes osmolality, pH, clarity, radionuclide purity, and radiochemical purity (RCP) [1,3,4], while the biological quality includes sterility, pyrogenecity, and biodistribution in testing animals [5].

Synthesis of $^{131}$I-Hippuran and its optimization also radiochemical purity test method had been performed and reported [2]. Radiochemical purity (RCP) of $^{131}$I-Hippuran (OIH) is associated with percentage of $^{131}$I bind to Hippuran and radiochemical impurities is associated with the percentage of free $^{131}$I in form of iodide ions ($^{131}$I$^-$) and $^{131}$I-o-iodobenzoic acid (OIB). The RCP data of the $^{131}$I-Hippuran and its impurities are shown in Table 1.
Table 1. The RCP of $^{131}$I-Hippuran (OIH), $^{131}$I-o-iodobenzoic acid (OIB), and Iodide ion ($^{131}$I) [1,2,6,7,8,9,10,11,12,13]

| Stationary phase | Mobile phase | Rf | (% RCP of $^{131}$I-Hippuran | Developing time/ size of paper | Ref. |
|------------------|--------------|----|-------------------------------|-------------------------------|------|
| Chromatography paper | Acetic acid: chloroform (1:9) | OIH = 0.2 | >96.00 | - | [1] |
| Whatman-1 | n-butanol: acetic acid: water (4:1:1) | $\Gamma = 0.00 – 0.30$ | >95.00 | - | [2] |
| Whatman-1 | n-butanol: acetic acid: water (4:1:1) | $\Gamma = 0.09-0.23$ | - | 30 cm = 16 h | [6] |
| Whatman-1 | Benzene: acetic acid: water (2:2:1) | $\Gamma = 0.00-0.05$ | - | 30 cm = 16 h | [6] |
| Whatman-3MM | Sodium acetate: glacial acetic acid: water (10:10:100) | $\Gamma = 0.83$ | - | - | [7] |
| TLC cellulose | n-butanol: glacial acetic acid: water (6:1.5:2.5) | $\Gamma = 0.35$ | m-IH = 0.57 | - | - | [8] |
| Whatman-1 | n-butanol: water: glacial acetic acid (120:50:30) | OIH = 0.94 | >99.00 | 15 cm = 18 h | [9] |
| Silica Gel G-plates | n-butanol : 1N acetic acid (1:1) | OIH = 0.72 | - | 15 cm | [9] |
| TCL silica gel | Benzene: acetic acid: water: n-butanol (5:5:2:1.5) | - | 98.00 – 99.00 | - | [10] |
| TLC; silica gel F 1500LS 254 | Methanol 80% | $\Gamma = 0.00$ | 93.74 – 99.62 | 9 cm = 50 minutes | [11] |
| TLC | Methanol: water: acetic acid (30:70:0.5) | - | 99.20 ± 0.20 | - | [12] |
| TLC 5716 plates | Benzene: acetic acid: water (2:2:1) | $\Gamma = 0.00$ - 0.100 | 94.00 | 1-2 h | [13] |

RCP test of the $^{131}$I-Hippuran generally performed by a paper chromatography method using Whatman-1 paper as stationary phase and n-butanol: acetic acid: water (4:1:1) solvent mixture as mobile phase [2,6]. In the USP 30/ NF 35 is stated that the %RCP of the $^{131}$I-Hippuran must be over than 97% when measured by using Whatman-1 as static phase and benzene: glacial acetic acid: water (2:2:1) as a mobile phase [14]. However, this method is considered inefficient because its long developing time (150 minutes). Therefore, there is a need to find out a new or modified method or system with a shorter developing time in the RCP test of $^{131}$I-Hippuran. The modified method can be carried out by modifying the mobile or stationary phase used in the paper chromatography method.

Several types of the paper commonly used in the paper chromatography method are Whatman-1, Whatman-3MM, and Whatman-31ET papers. The Whatman-31ET paper is one of the fast flow chromatography paper compared to that of Whatman-1 and Whatman-3MM papers [15]. Figure 1 shows the retention factor (Rf) of $^{131}$I-Hippuran (OIH) and iodide ion ($^{131}$I). The Rf value of $^{131}$I-Hippuran approximately 0.70 – 0.92, and for $^{131}$I approximately of 0.00 to 0.30 [2,6]. The Rf value was calculated by the Formula 1 [9]:

\[
\Gamma = \frac{R_f}{R_f \text{ max}}
\]
\[ R_f = \frac{\text{migration distance of substance}}{\text{migration distance of solvent}} \]  

Figure 1. The chromatogram of \(^{131}\)I-Hippuran using Whatman No.1 paper as static phase and n-butanol: acetic acid: water (4:1:4) as mobile phase [6].

The aim of this study is therefore to determine the most efficient paper chromatography method based on various mobile phases or various Whatman paper (Whatman-1, Whatman-31ET, and Whatman-3MM papers), based on the required %RCP of \(^{131}\)I-Hippuran.

2. Methods

2.1. The materials
Labelled compound of \(^{131}\)I-Hippuran was produced by The Center for Radioisotope and Radiopharmaceutical Technology – BATAN, water (IPHA Laboratories), n-butanol for analysis 99.5%, acetic acid for analysis 99.8%, benzene for analysis, acetone for analysis 99.8%, propanol for analysis, ethanol absolute for analysis 99.8%, ammonia 25% (Merck), plastic film, strips of 1 x 11 cm Whatman-1 paper (Schleicher & Schuell), strips of 1 x 11 cm Whatman-3MM (Sigma-Aldrich), strips of 1 x 8 cm; 1 x 10 cm; 1 x 11 cm; 1 x 12 cm; 1 x 16 cm; and 1 x 21 cm Whatman-31ET paper (GE Healthcare Life Sciences).

2.2. The equipment
Equipment used this study were glass cylindrical tank, stopwatch, gamma counter (Caprac-t), micropipette (Eppendorf).

2.3. The procedure
The \(^{131}\)I-Hippuran was spotted 2 cm from the bottom of the chromatographic paper strips and then developed in a closed 10 x 29 cm glass cylindrical tank, which contained the developing solvent. The solvent is allowed to migrate up to 1 cm from the top of the paper strips. They were immediately removed, left to dry at room temperature, wrapped in a plastic film. The paper strips were then cut (1 cm/ portion) and then counted on gamma counter (Caprac-t) [16]. The measurement of repeatability was carried out in the same experimental conditions, tools, place, and short interval of time trial. The same procedure is also done for various mobile phases, stationary phases and size of paper.

2.4. Data analysis
The data comparison performed using the independent t-test, especially in order to know the significantly difference between two samples with homogeneous data. The t-test of independent two-sample with equal variance can be calculated in the Formula 2 and 3 [17,18].

\[
t = \frac{x_1 - x_2}{\sqrt{\frac{s_p^2}{n_1} + \frac{s_p^2}{n_2}}}
\]

(2)

where

\[
s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}
\]

(3)

Where

- \( t \) = t-value,
- \( x_1 \) = average data of sample 1,
- \( x_2 \) = average data of sample 2,
- \( s_p \) = pooled standard deviation,
- \( s_1 \) = sample variance 1,
- \( s_2 \) = sample variance 2,
- \( n_1 \) = sample size 1, and
- \( n_2 \) = sample size 2

The percentage of RCP of the \(^{131}\text{I}-\text{Hippuran} \) data processing which were analysed using various Whatman papers performed by t-test. The difference of % RCP of \(^{131}\text{I}-\text{Hippuran} \) are significant if the t-value of t-test statistic is equal to or greater than 2.10, the critical value in this case (significance level \( P' = 0.05 \) with number of degrees freedom \( \Theta = 18 \)).

3. Results and Discussion

The RCP of the \(^{131}\text{I}-\text{Hippuran} \) was determined by a paper chromatography method using various mobile and stationary (Whatman-1 paper) phases. The RCP results of the \(^{131}\text{I}-\text{Hippuran} \) are showed in Table 2.

**Table 2.** The results of \(^{131}\text{I}-\text{Hippuran} \) RCP test using Whatman-1 paper with various mobile phases

| Mobile phase                        | \(^{131}\text{I} \) Hippuran | \(^{133}\text{I} \) Hippuran | OIB       | Developing Time (minute) |
|-------------------------------------|-----------------------------|-----------------------------|-----------|-------------------------|
| benzene: acetic acid: water (2:2:0.5) | 0.0 – 0.2                   | 0.6 – 0.7                   | Not defined | 242                    |
| n-butanol: acetic acid: water (4:1:1) | 0.10                        | 0.8 – 0.9                   | -         | 130                    |
| n-butanol: acetic acid: water (8:1:1) | 0.0 – 0.1                   | 0.8 – 0.9                   | -         | 133                    |
| n-butanol: acetic acid: water (1:1:1) | 0.4 – 0.5                   | 0.8                        | -         | 147                    |
| acetone : acetic acid: water (4:1:1) | 0.9 – 1.0                   | 0.9                        | -         | 65                     |
| propanol : acetic acid: water (4:1:1) | 0.0 – 0.2                   | 0.7 – 0.9                   | -         | 120                    |
| ethanol: water: ammonia 25% (25:3:4) | 0.5 – 0.7                   | 0.5 – 0.7                   | -         | 95                     |
| ethanol: water: ammonia 25% (10:1:10) | 0.8                        | 0.8 – 0.9                   | -         | 105                    |

It can be seen in Table 2 that the mobile phases of benzene: acetic acid: water (2:2:0.5), n-butanol: acetic acid: water (4:1:1) and n-butanol: acetic acid: water (8:1:1) gave a good separation between \(^{131}\text{I}-\text{Hippuran} \) (Rf ~ 0.6 – 0.9) and \(^{133}\text{I} \) was (Rf ~ 0.0 – 0.2), while OIB was not visible in mobile phase benzene: acetic acid: water (2:2:0.5). The mobile phase of acetone: acetic acid: water (4:1:1), ethanol: water: ammonia 25% (25:3:4) and ethanol: water: ammonia 25% (10:1:10) however showed a good separation between \(^{131}\text{I}-\text{Hippuran} \) and its impurity \(^{131}\text{I} \). The use of n-butanol: acetic acid: water (1:1:1) and propanol : acetic acid: water (4:1:1) mobile phases gave a tail separation, therefore it could not be used in RCP test of \(^{131}\text{I}-\text{Hippuran} \). The standard method where n-butanol: acetic acid: water (4:1:1) used as a mobile phase, gave a good separation between \(^{131}\text{I}-\text{Hippuran} \) (Rf ~ 0.8 – 0.9) and its impurity \(^{131}\text{I} \), (Rf ~ 0.10). This system also has relatively short developing time (130 minutes). Hippuran (Figure 2)
is a polar compound which has an amine group (secondary amine) and methylene [2]. Polar molecules have a high attraction for polar solvent such as n-butanol and acetic acid is able in forming a strong hydrogen bonding, which resulted in an acceleration of the migration of polar compound [19].

Figure 2. Molecule structure of $^{131}$I-Hippuran

The chromatograms of $^{131}$I-Hippuran which were developed on various stationary phases (Whatman-1, 31ET and 3MM papers) with mobile phase of n-butanol: acetic acid: water (4:1:1) are presented in Figure 3 and Table 3.

Figure 3. Chromatograms of $^{131}$I-Hippuran developed using various Whatman papers as stationary phases and n-butanol: acetic acid: water (4:1:1) as a mobile phase

Table 3. Chromatography systems of $^{131}$I-Hippuran based on various Whatman papers as stationary phases and n-butanol: acetic acid: water (4:1:1) as a mobile phase

| Stationary phase   | %RCP       | Developing time (minute) | Description     |
|--------------------|------------|--------------------------|-----------------|
| Whatman No. 1      | 98.7 ± 0.9%| 158.3 ± 6.2%             | Standard method |
| Whatman No.31 ET   | 98.7 ± 0.7%| 75.7 ± 2.7%              | Fast flow       |
| Whatman No. 3MM    | 98.7 ± 0.8%| 142.0 ± 3.7%             | Medium flow     |

It can be seen from Figure 3 that all types of Whatman papers gave Rf value of approximately 0.9 for $^{131}$I-Hippuran. The use of different Whatman paper (Whatman-31ET, Whatman-1 and Whatman-3MM) gave no significant effect on the Rf value of $^{131}$I-Hippuran. Figure 3 also shows that the use Whatman-3MM paper in the above mentioned chromatography system gave a higher peak counts than the other papers because the Whatman-3MM paper is thicker and has higher capacities therefore it is able to hold more sample [20]. The %RCP of the $^{131}$I-Hippuran measured using for either with
Whatman-1 or and Whatman-31ET papers were higher than 98%. The Whatman-31ET paper showed faster developing time of 75.7 ± 2.7 minutes compared to that of Whatman-1 (158.3 ± 6.2 minutes) for $^{131}$I-Hippuran as Whatman-31ET paper has a looser fiber network [20].

The difference in %RCP of the $^{131}$I-Hippuran measured using Whatman-1 and 31ET paper was analyzed using t-test. The results were shown in Table 4. The percentage of relative standard deviation (%RSDs) for Whatman-1 paper and Whatman-31ET paper were of 0.30% and 0.22%, respectively which indicated that the value of %RSD for both papers were in range of the precision standard. It should be noted that the required (%RSDs) should less than 1%. Overall, t-test showed that the %RCP results of $^{131}$I-Hippuran obtained using either Whatman-1 or Whatman-31ET paper is similar, as there was no significant statistical difference (t-value = 0.40).

**Table 4.** The t-test of % radiochemical purity between Whatman-1 paper and Whatman-31ET paper

|    | Whatman-1 | Whatman-31ET |
|----|-----------|--------------|
| 1  | 99.10     | 99.513       |
| 2  | 99.25     | 99.001       |
| 3  | 99.43     | 98.940       |
| 4  | 98.73     | 99.037       |
| 5  | 99.30     | 98.870       |
| 6  | 98.85     | 98.673       |
| 7  | 99.28     | 99.028       |
| 8  | 98.86     | 99.172       |
| 9  | 98.64     | 99.183       |
| 10 | 98.66     | 99.135       |
| Mean | 99.01     | 99.055       |
| SD  | 0.30      | 0.22         |
| %RSD | 0.30 %    | 0.22 %       |
| t-value | 0.40      |              |

The experiments using various size of Whatman-31ET paper were also performed in order to get paper chromatography system which has more efficient developing time. The shorter size of chromatography paper strip might offer a faster developing time. However, a paper chromatography system is not only has short developing time but is also able to properly separate $^{131}$I-Hippuran from its impurities.

Percentage of RCP and developing time of $^{131}$I-Hippuran measured using Whatman-31ET paper with size of 1 x 8 (short size) up to 1 x 21 cm (long size) as stationary phases and n-butanol:acetic acid: water (4:1:1) as mobile phase are shown in Table 5. The Rf value of this method showed in Figure 4. Based on Table 5, it be seen that there are no different of the %RCP and the Rf value of $^{131}$I-Hippuran when measured using 1 x 8 (short size), 1 x 11 (standard method) and 1 x 21 cm (long size) Whatman-31ET paper strips. The %RCPs of $^{131}$I-Hippuran measured with those strips were >98%. The results also showed that there are no correlation between size of paper strips and Rf value of the $^{131}$I-Hippuran. The long size Whatman paper extends the migration distance of substance so that it can nicely separate $^{131}$I-Hippuran from its impurities. The Rf value of $^{131}$I-Hippuran from this experiment was of 0.9 – 1.0. This value is almost the same with the reported Rf for this paper chromatography system Rf approximately 0.70 – 0.92 [2,6]. The shorter developing time for this RCP test of $^{131}$I-Hippuran using the above mentioned chromatography was of ~ 44.3 ± 9.4 minutes which was showed by 1 x 8 cm size Whatman-31ET paper strips. This chromatography system was found to be more efficient compared to that of the USP 30/ NF 35 standard method with developing time of 150 minutes [14].

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International Conference on Chemistry and Material Science (IC2MS) 2017  
IOP Conf. Series: Materials Science and Engineering 299 (2018) 012007  
doi:10.1088/1757-899X/299/1/012007
Table 5. The %RCP and the developing time of $^{131}$I-Hippuran measured using various size of paper Whatman-31ET paper strips

| Size of Paper (cm) | %RCP        | Developing time (minute) |
|-------------------|-------------|--------------------------|
| 1 x 8             | 98.1 ± 0.0% | 44.3 ± 9.4%              |
| 1 x 10            | 98.1 ± 0.1% | 65.3 ± 0.8%              |
| 1 x 11            | 98.7 ± 0.7% | 75.8 ± 2.7%              |
| 1 x 12            | 98.4 ± 0.1% | 101.3 ± 11.5%            |
| 1 x 16            | 98.2 ± 0.1% | 167.0 ± 2.2%             |
| 1 x 21            | 98.0 ± 0.1% | 314.0 ± 2.2%             |

Figure 4. Rf Value of $^{131}$I-Hippuran using various size of Whatman-31ET paper strips

4. Conclusions

Paper chromatography method for measuring the radiochemical purity of $^{131}$I-Hippuran has been studied. The most suitable mobile phase for this system was found to be the mixture of n-butanol: acetic acid: water (4:1:1) which showed a good separation between $^{131}$I-Hippuran from its impurities. This study also found that the most efficient stationary phase for the above mentioned chromatography system Whatman-31ET paper strips (1 x 8 cm) which has a developing time of 44.3 ± 9.4 minutes. This method has been to be efficient in term of developing time and comparable with other chromatography systems which have been reported elsewhere.

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Acknowledgement
This work was supported by the Center for Radioisotope and Radiopharmaceutical Technology-BATAN. The authors would like to thank to Yayan Tahyan and Enny Lestari for their expertise assistances