Application of thin layer chromatography-densitometry method for analysis of metoprolol in plasma and urine

P Utami*, L Alfiyah and D Estika
Faculty of Pharmacy, Universitas Muhammadiyah Purwokerto, Jl. Raya Dukuhwaluh, Kembaran, Purwokerto, Central Java, Indonesia, 53182

*priiswatiutami@ump.ac.id

Abstract. Metoprolol is one of the Beta blocker antihypertensive drugs. The aim of this study was to obtain a thin layer chromatography (TLC)-densitometry method for analysis of metoprolol in plasma and urine. The TLC was carried out on the aluminum sheet of silica gel 60 F254, using chloroform, methanol, and ammonia (4:6:0.2 v/v/v) as a mobile phase. Densitometric measurements were done using densitometer TLC Scanner 4 at 282 nm. The method showed good linearity with correlation coefficient (r) of 0.9899 (15-35 µg/spot) for plasma and 0.9904 (15-75 µg/spot) for urine. The minimum detectable amounts for plasma and urine were found to be 4.60 and 4.47 µg/spot respectively. Quantitative limits for plasma and urine were 15.34 and 14.90 µg/spot respectively. The accuracy of the method was accessed by % recovery and found to be 99.52 % for plasma and 102.73 % for urine. The relative standard deviation values for plasma and urine were 5.29 and 4.07% respectively. The method showed acceptable validation parameters. It can be used for the analysis of metoprolol in human plasma and urine.

1. Introduction
Metoprolol is a second-generation beta-adrenergic blocking agent for the treatment of cardiovascular disease [1]. As a member of beta-blockers, metoprolol effectively lowers blood pressure but has a variety of hemodynamic, tolerability, and metabolic profiles [2].

The concentration of metoprolol in plasma and urine can vary between individuals. Poor metabolizers have plasma concentrations three times higher than extensive metabolizers [3,4]. The patient CYP2D6 metabolizer genotype and phenotype were the dominant factor that controlled the plasma metoprolol pharmacokinetics [5,6]. Therefore, it is needed analytical methods that can be applied for monitoring of metoprolol level in the biological fluid, so the patient individual dose adjustment can be made.

Several methods have been used for the analysis of metoprolol in plasma and urine, such as High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detection [4,7], HPLC with fluorescence detection [8,9], Liquid Chromatography-Mass Spectrometry (LC-MS) [10], and High Performance Thin Layer Chromatography (HPTLC) [11]. There was still limited (TLC) method that has been established for the determination of metoprolol in human plasma and urine. Thin layer chromatography (TLC) method can give higher efficiency and enable greater resolution than HPTLC [12]. The aim of this study was to obtain a TLC-densitometry method for analysis of metoprolol in plasma and urine.
2. Material and methods

2.1. Material

2.1.1. Chemicals and reagents. Metoprolol was supplied by Sigma-Aldrich, Germany. Methanol, chloroform, sodium hydroxide, ammonia, and tri chloroacetic acid were obtained from Merck. All the reagents used were of pro analytical grade.

2.1.2. Instrumentations. The TLC was carried out on the aluminum sheet of silica gel 60 F254 (E. Merck, Art. 1.05554) in the glass chamber (Camag, Muttenz, Switzerland). Densitometric measurements were done using densitometer TLC Scanner 4 controlled by WinCATS 1.4.6 software.

2.2. Methods

2.2.1. Standard solutions. Metoprolol reference standard was accurately weighed for the preparation of stock solution 2000 µg/mL in methanol.

2.2.2. Mobile phase optimization. The first step in this study was to find the optimum chromatographic conditions (proper mobile phase). We use mobile phase system: chloroform, methanol, and ammonia in several volume compositions: 4:6:0.2; 1:9:0.2; and 5:5:0.2 (v/v/v). Method validation. Application of the method for the analysis of metoprolol in plasma and urine sample.

2.2.3. Sample preparation. Five hundred µl of plasma or urine was placed into the 2 mL microcentrifuge tube and then 500 µl of NaOH 0.5 N was added. Chloroform (1000 µl) was added. Vortex mixer for 1 minute and centrifuged for 10 minutes at 8000 rpm. The organic layer was taken and placed into the vial. The aqueous layer was re-extracted with chloroform with the same volume. The organic layer was combined with the first extraction result. The solution was evaporated to dryness. Then 500 µl of methanol was added.

2.2.4. TLC-densitometric method. Ten µL of sample solution was spotted on TLC plate. Chromatography was developed in a pre-saturated state in a vertical twin trough glass chamber. Chloroform, methanol, and ammonia (4:6:0.2 v/v/v) were used as the mobile phase. After development, the plate was dried at room temperature. Quantification of metoprolol was done by densitometric scanning of the developed plate. The wavelength of the detector was set at 282 nm.

2.2.5. Method validation. The method was validated as recommended by International Conference on Harmonization (ICH) guideline for the parameters like specificity, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), and accuracy [13]. The specificity of the method was determined by comparing the densitogram from chromatograms of metoprolol obtained from the samples and blank plasma and urine. The precision studies were carried out by estimating the corresponding responses 6 times of metoprolol at concentration of 45 µg/spot. The linearity of the method was studied by preparing standard metoprolol solutions at concentrations from 15 - 35 µg/spot in blank plasma and 15 - 75 µg/spot in blank urine. Calibration curves were constructed by plotting concentrations of metoprolol against...
peak area. Linearity was determined by least-squares regression. LOD and LOQ were determined with calculation from special calibration curve at low concentration (0.5 – 16 µg/spot) of metoprolol in plasma and urine matrix. Accuracy was determined by adding a known amount of metoprolol reference standard to a pre-analyzed sample. After liquid-liquid extraction, the accuracy was determined by calculating the recovery of metoprolol.

3. Results and discussion

3.1. Specificity

By the use of chloroform, methanol, and ammonia in volume composition 4 : 6 : 0.2 (v/v/v), good resolution of the compound was achieved. Densitometric measurements were done at 282 nm. Densitogram from chromatogram of metoprolol standard (Figure 2), plasma sample (Figure 3), and the urine sample (Figure 4) show that metoprolol is well separated from the matrix components. The mobile phase gave good resolution, sharp and symmetrical peak with Rf value ranged from 0.47 – 0.73 for metoprolol.

Figure 2. Densitogram from chromatogram of metoprolol standard.

Figure 3. Densitogram from chromatogram of metoprolol in plasma.
3.2. Precision
Repeatability of measurement of peak area was determined by spotting 45 µg/spot of metoprolol. The precision of the developed TLC method was expressed in terms of % relative standard deviation (% RSD). In this study, the precision was found to be 5.29% and 4.08% for plasma and urine, respectively. The results depicted revealed high precision of the method is presented in Table 1.

3.3. Linearity
The linear regression data for the calibration curve of metoprolol in plasma showed good linear relationship over the concentration range 15 – 35 µg/spot (figure 5).

![Figure 4. Densitogram from chromatogram of metoprolol in urine.](image)

![Figure 5. Calibration curve of metoprolol in plasma.](image)

![Figure 6. Calibration curve of metoprolol in urine.](image)
The linear regression data for the calibration curve of metoprolol in urine showed good linear relationship over the concentration range 15 – 75 µg/spot. The correlation coefficient value of calibration curve of metoprolol in plasma and urine were 0.9899 and 0.9904, respectively. The correlation coefficient values confirming the linearity of the method. Peak area measurement was used for quantification techniques due to higher precision, less sensitive to Rf changes and baseline fluctuation [14].

3.4. Limit of detection
LOD was determined based on the standard deviation of the response and the Slope. Detection limit and quantitation limit was calculated from the special calibration curve. LOD was calculated using the formula LOD=3.3 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve [14]. The LOD for plasma and urine were found to be 4.60 and 4.47 µg/spot, respectively.

3.5. Limit of quantitation
LOQ was calculated using the formula LOQ=10 σ/S [14]. The LOQ for plasma and urine were 15.34 and 14.90 µg/spot, respectively.

3.6. Accuracy
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [14].

Table 1. Method validation parameter.

| Parameter    | This Study | HPLC [4] |
|--------------|------------|----------|
|              | Plasma     | Urine    |          |
| Specificity  | Good       | Good     | Good     |
| Precision    | 5.29 %     | 4.08 %   | 0.978 - 1.831% |
| Linearity    | µg/spot    | µg/spot  | 1-64 µg/mL r = 0.999 |
| LOD          | 4.60 µg/spot| 4.47 µg/spot | 0.22 µg/mL |
| LOQ          | 15.34 µg/spot | 14.90 µg/spot | 0.67 µg/mL |
| Mean Recovery| 99.52 %    | 102.74 % | 98.36 - 101.32 % |

To studied the accuracy of the method, the blank samples (urine and plasma) were spiked with known amount of metoprolol standard solution. After liquid-liquid extraction, an extract solution was spotted on TLC plate. The metoprolol concentration was determined. This was done to check for the recovery of the drug. The mean recovery of metoprolol in plasma and urine were 99.52 and 102.74%, respectively. The proposed method afforded recovery of 98-102% as listed in Table 1.

Table 1 showed that this TLC method can be compared with High performance liquid chromatography (HPLC) methods [4] in some method validation parameters. Both methods provided a good selectivity, linearity, and accuracy for the quantitative determination of metoprolol in biological samples. This study showed comparable results with the previous study which is using HPTLC method to determine metoprolol tartrate and hydrochlorothiazide in plasma [11].

During the past 10 - 15 years, HPLC is a very favorite analytical tool (accurate, precise) and widely used in separating the complex mixture of molecules including pharmaceutical in the biological sample. In comparison with TLC, HPLC technique is more expensive and required time consuming pre-treatment steps. Traditional TLC is inexpensive, and simple to use and requires minimal instrumentation, laboratory space, and maintenance. However, to achieve good precision, accuracy, and
reproducibility, a certain degree of instrumentation is required. The use of densitometric evaluation is necessary at least for quantification [13].

4. Conclusion
The results of this study show that a very simple and rapid TLC-method combined with densitometry for the separation and quantification of metoprolol in human plasma and urine samples. The TLC-densitometry method showed acceptable validation parameters. It can be used as an economical alternative method for the analysis of metoprolol in human plasma and urine.

Acknowledgement
We wish to thank Instrument Laboratory Staff members of the Faculty of Pharmacy, Universitas Muhammadiyah Purwokerto, for the facilities given.

References
[1] Bristow M R, Roden R L, Lowes B D, Gilbert E M and Eichhorn E J 1998 The role of third-generation beta-blocking agents in chronic heart failure Clinical cardiology 21(S1) I3-I13
[2] J B McGill 2010 Optimal use of β-blockers in high-risk hypertension: A guide to dosing equivalence Vasc. Health & Risk Manag. 6: p. 363–372
[3] J C McGourty and J H Silas 1985 Metoprolol metabolism and debrisoquine oxidation polymorphism - population and family studies Br. J. Clin. Pharmac. 20: p. 555-566
[4] Utami P I, Sugiyanto S, Martono S and Hakim L 2017 Isocratic High Performance Liquid Chromatographic Method for Determination of Metoprolol and Its Metabolite in Human Urine Jurnal Ilmu Kefarmasian Indonesia 14(1) 43-48.
[5] Blake C M, Kharasch E D, Schwab M and Nagele P 2013 A meta-analysis of CYP2D6 metabolizer phenotype and metoprolol pharmacokinetics Clinical Pharmacology and Therapeutics 94(3) 394-399
[6] Goryachkina K, Burbello A, Boldueva S, Babak S, Bergman U and Bertilsson L 2008 CYP2D6 is a major determinant of metoprolol disposition and effects in hospitalized Russian patients treated for acute myocardial infarction European journal of clinical pharmacology 64(12) 1163
[7] Aqil M, Ali A, Ahad A, Sultana Y, Najmi A K and Saha N 2007 A validated HPLC method for estimation of metoprolol in human plasma Acta Chromatographica 19 130
[8] Albers S, Elshoff J P, Völker C, Richter A and Läer S 2005 HPLC quantification of metoprolol with solid-phase extraction for the drug monitoring of pediatric patients. Biomedical Chromatography 19(3) 202-207
[9] I Baranowska and A Wilczek 2009 Simultaneous RP-HPLC Determination of Sotalol, Metoprolol, α-Hydroxymetoprolol, Paracetamol and Its Glucuronide and Sulfate Metabolites in Human Urine. Anal. Sci. 25: p. 769-772
[10] Bae S H, Lee J K, Cho D Y and Bae S K 2014 Simultaneous determination of metoprolol and its metabolites, α-hydroxymetoprolol and O-desmethylmetoprolol, in human plasma by liquid chromatography with tandem mass spectrometry: A pplication to the pharmacokinetics of metoprolol associated with CYP 2 D 6 genotypes Journal of separation science 37(11) 1256-1264
[11] A R Rote and P R Sonavane 2013 Bioanalytical method development and validation for determination of metoprolol tartrate and hydrochlorothiazide using HPTLC in human plasma. Brazilian Journal of Pharmaceutical Sciences 49(4) p. 845-851
[12] T Halkina and J Sherma 2006 Comparative evaluation of the performance of silica gel TLC plates and irregular and spherical-particle HPTLC plates Acta Chromatographica 17 p. 261-271
[13] S. Nyiredy 2005 Pharmaceuticals and Drugs, in Handbook of Thin-Layer Chromatography Third Edition, J. Sherma and B. Fried, Editors (New York : Marcel Dekker, Inc.)
[14] ICH Q2 (R1) 2005 Validation of Analytical Procedures: Text and Methodology