THE SOLVENT OPTIMIZATION IN METOPROLOL DERIVATIZATION REACTION WITH 1-FLUORO-2,4-DINITROBENZENE

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Abstract

Metoprolol, or 1-[4-(2-Methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]-2-propanol, is a β1-adrenergic receptor antagonist that is often used to treat cardiovascular disorders such as hypertension, arrhythmia, and heart failure. Metoprolol has an A value of $^{1\%}{}_{1cm}$ = 52a in acid solution at a wavelength of 274 nm, where the molar absorptivity value (Ɛ) is 1390.48 L/(mol . cm) so that metoprolol gives weak absorption in the UV region (Ɛ ~ 1000 L/(mol . cm)). This study aims to optimize the solvent in the derivatization reaction of metoprolol with 1-fluoro-2,4-dinitrobenzene (FDNB). Under optimum conditions, the metoprolol reaction should form a derivatization product with FDNB. Metoprolol derivatization reaction in methanol solvent is optimal at pH 10.0 in borate buffer, with a warm-up at room temperature for 80 minutes. Analysis of metoprolol by High Performance Liquid Chromatography (HPLC) using a Chromolith RP-18e column (100 mm x 4.6 mm; 2 µm) and phase acetonitrile: buffer acetate (0.2 M, pH 3.0) = 70:30 with a flow rate of 1.0 ml/min. Separation of metoprolol-DNB occurs at a time retention of 7,856 minutes, with an analysis time of 10 minutes. Reaction-derivatization of metoprolol in methanol solvent; the optimum condition was pH 10.0. It was concluded that metoprolol in methanol solvent was more effective because the derivatization reaction with FDNB did not require heating. Optimizing the metoprolol content determination method using the FDNB solution derivatizer in acetonitrile showed that the optimal mobile phase was a mixture of acetonitrile and 0.2 M acetate buffer (30:70, pH 3.0), with a flow rate of 1.0 mL/minute.

Keywords: *FDNB, HPLC, metoprolol, optimization*

1. INTRODUCTION

Metoprolol or *1-[4-(2-Methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]-2-propanol* is a β-blocker (Bodke et al., 2024) that is selectively used to treat cardiovascular disorders (Zamir et al., 2022) such as hypertension, arrhythmia, and heart failure. In general, the β-blocker class, including metoprolol, exhibits low absorption in the UV region (Moffat et al., 2004). Analysis with a UV spectrophotometer is usually performed using second-derivative techniques or *spectrofluorometry*, with the addition of a derivative to form fluorescent compounds. (Redasani et al., 2018).

Based on its structural formula, metoprolol has a low absorptivity value (ε = 1390.48 L/(mol·cm), so to improve its detection on a UV detector, it is necessary to add or react with a derivative compound, such as 1-fluoro-2,4-dinitrobenzene (FDNB). Several studies of primary or secondary amine drugs using FDNB derivatives include Gabapentin (Jalalizadeh et al., 2007; Lü et al., 2010; Souri et al., 2013) ; Lisinopril (Paraskevas et al., 2002) ; Memantine Hydrochloride (Belal et al., 2013; Narola et al., 2010); Nabumetone (Bedair et al., 2013). FDNB can react with compounds that have an amine group, so it can be predicted that FDNB is also able to respond with metoprolol, which is a compound that has an amine group (Toyo'oka, 1999).

The reaction between metoprolol and FDNB produces a metoprolol-DNB product that can increase the molar absorptivity of metoprolol, enabling detection with a UV detector (Synder et al., 1997). Parameters that influence the derivatization reaction include pH, temperature, reaction time, and reagent ratio (Belal et al., 2013). This study aims to determine the optimal conditions for metoprolol in methanol as the solvent during its reaction with FDNB. Souri et al. (2007) and Jalalizadeh et al. (2007) analyzed gabapentin using amlodipine as an internal standard by High-Performance Liquid Chromatography (HPLC) after derivatization with FDNB (Jalalizadeh et al., 2007; Souri et al., 2007). The reaction was derivatized by heating at 60°C, and a 10% borate buffer was added. Research by Paraskevas et al. (2002) also reported

that derivatization of lisinopril with FDNB requires heating at 60°C. In this study, it is hoped that conditions can be established to enable the optimal reaction of metoprolol with FDNB in methanol, as determined by HPLC.

2. METHODOLOGY

2.1 Materials and tools

The raw materials used were metoprolol obtained from PT. Sandoz Indonesia. FDNB and HPLC-grade reagents were obtained from Merck, including acetonitrile, distilled water, and methanol. The pro-grade analytical reagents were obtained from Merck, including: glacial acetic acid, sodium acetate, boric acid, potassium chloride, and sodium hydroxide.

The tools used were an HPLC model LC-20AD (Shimadzu Prominence) with a UV-Vis detector Prominence SPD-20A, and a Chromolith RP-18e column (100 mm x 4.6 mm, 2 μm). *Filtration unit for HPLC* (Whatman), pH meter (Mettler Toledo Seven Easy S20), analytical balance (Toledo AB 204-S), centrifuge (EBA 20 Hettich), *Ultrasonic bath* (Selecta), micropipettes (Nichipped and Eppendorf), and glassware (Pyrex brand), commonly used in laboratories.

2.2 Preparation of standard solution

Metoprolol is dissolved in methanol to a concentration of 204.4 μg/mL and stored in *the refrigerator*. The FDNB solution is prepared at a concentration of 0.06 M in acetonitrile. This reagent must be handled with care, as it can irritate skin. Buffer borate 0.25 M made by weighing H_3BO_3 and KCl, then adjusting the base pH with 0.2 M NaOH (Ebrahimzadeh et al., 2010) and measured with pH meters (pH 8.0; 8.5; 9.0; 9.5; 10.0; 10.5; and 11.0) (Abdel Razak et al., 2003).

2.3 Wavelength Determination

a. Metoprolol

Urine solution of metoprolol (204.4 μg/mL) was diluted with methanol until the concentration was obtained (8.176 μg/mL). 300 μL of the solution was pipetted into a 5.0 mL volumetric flask, and then borate buffer (pH 8.0-11.0) was added to bring the final volume to 5.0 mL. Next, the sample was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 minutes. After that, the solution was *scanned* on a UV spectrophotometer between wavelengths of 200-400 nm. The results obtained are the absorption spectrum and wavelength.

b. FDNB

FDNB solution $3x10^{-5}$M (93 μg/mL) as much as 10 00 μL (from FDNB stock solution 0.06 M or 11160 μg/mL), then borate buffer (pH 8.0-11.0) was added to 5.0 mL. Next, the mixture was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 minutes. The solution *was scanned* on a UV spectrophotometer between wavelengths of 200 and 400 nm. The results obtained are the absorbance spectrum and wavelength.

c. Metoprolol- DNB

Urine solution metoprolol (8.176 μg/mL) was pipetted 3.0 μL into a 5.0 mL volumetric flask, and then 10 00 μL of $3x10^{-5}$ M FDNB solution (93 μg/mL) (from 0.06 M FDNB stock solution or 11160 μg/mL), then borate buffer (pH 8.0-11.0) was added to 5.0 mL. Next, the mixture was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 minutes. The solution *was scanned* on a UV spectrophotometer between wavelengths of 200-400 nm. The results obtained were the absorption spectrum and wavelength, as well as the pH of the selected borate buffer, based on the smallest RSD.

Table 1. Chromatographic conditions for optimization mobile phase

Silent Phase	:	Chromolith RP-18e (100 mm x 4.6 mm, 2 μm)
Motion Phase	:	Acetonitrile: acetate buffer (optimization)
Mobile Phase pH	:	3.0; 3.5; 4.0 and 4.5 (optimization)
Injection volume	:	20 μL
Flow rate	:	0.8; 0.9; 1.0; 1.1 and 1.2 mL /min (optimization)
Detector	:	UV-Vis Prominence SPD-20A with optimized wavelength

2.4 Mobile Phase Optimization

a. Preparation of HPLC Mobile Phase

The mobile phase used for this method is acetonitrile:0.2 N acetate buffer, with compositions of 20:80, 30:70, and 40:60 (v/v). Then, the prepared mobile phase was removed of air bubbles by filtration and ultrasonication. HPLC conditions for determining Metoprolol levels are based on research by Phale & Hamrapurkar (2009). The chromatography

conditions for mobile-phase optimization are shown in Table 1.

b. Optimization of mobile phase composition, pH, and flow rate for metoprolol.

Metoprolol standard solution (20.44 µg/mL) was pipetted as much as 1000 µL and FDNB solution ($9x10^{-5}$ M) was pipetted as much as 2500 µL, then borate buffer pH was added 10.0 (selected pH) to 5.0 mL, The solution was injected as much as 20 µL into the HPLC device, with the mobile phase acetate buffer: acetonitrile, the pH of which is adjusted to the mobile phase (consisting of buffers pH 3.0; 3.5; 4.0 and 4.5) with a ratio of 20:80, 30:70, and 40:60 ($^{v}/_{v}$). The flow rate was varied to 0.8, 0.9, 1.0, 1.1, and 1.2 mL/min. The results obtained were then used to select the mobile phase composition, acetate buffer pH, and flow rate that provided the best separation, based on retention time, resolution (Rs), HETP, and number of theoretical plates (N). The selected (optimal) conditions were chosen with the requirements of a retention time <10 minutes, a small HETP, the largest N, and good resolution, which was acceptable at >1.5 (Chan, 2004). These optimum conditions were then used in sample analysis.

2.6 Reaction Optimization

a. FDNB Mole Fraction Optimization

Metoprolol standard solution (20.44 µg/mL) was pipetted into 500 µL and FDNB solution ($9x10^{-5}$ M or 279 µg/mL) was pipetted 500, 1000, 1500, 2000, 2500, 3000 and 3500 µL, then each pH borate buffer was added 10.0 (selected pH) to 5.0 mL in a volumetric flask, the solution was transferred in a centrifuge tube and then the solution was homogenized (centrifuged) at 2500 rpm for 10 minutes and injected into the HPLC device as much as 20 µL, with the composition of the mobile phase, buffer pH and selected flow rate: namely acetonitrile acetate buffer pH 3.0 (30:70), and a flow rate of 1.0 mL/minute. The results obtained data on the area (mV) of metoprolol-DNB at various FDNB levels, and the maximum mole fraction was selected.

b. Determination of the reaction time of metoprolol derivatization

Metoprolol standard solution (20.44 µg/mL) was pipetted 500 µL, and FDNB solution ($9x10^{-5}$ M) was pipetted 2500 µL (selected mole fraction), then borate buffer pH 10.0 (selected pH) to 5.0 mL in a volumetric flask. The solution was transferred to a centrifuge tube, homogenized at 2500 rpm for 10 minutes, and then left to stand for 0, 20, 40, 60, 80, and 100 minutes. The solution was injected into the HPLC apparatus in up to 20 µL aliquots, using an acetonitrile-acetate buffer (pH 3.0, 30:70) mobile phase at a flow rate of 1.0 mL/minute. The results showed the metoprolol-DNB area (mV) at various reaction times, and the optimum reaction time was selected to ensure the reaction proceeded.

3. RESULTS AND DISCUSSION

3.1 Results of Determining Maximum Wavelength

The maximum wavelength is determined for metoprolol, FDNB, and metoprolol-DNB. Metoprolol solution with a concentration of 8.176 µg/mL dissolved in methanol, FDNB solution with a concentration of $3x10^{-5}$ M was dissolved in acetonitrile. The reaction between metoprolol and FDNB, called metoprolol-DNB, is performed by mixing a certain amount of metoprolol solution with FDNB solution. FDNB can react with primary and secondary amine groups of an analyte (Bedair et al., 2013). Metoprolol is a compound that has a secondary amine group. The reaction between metoprolol and FDNB is carried out under basic conditions, namely by adding a borate buffer solution at pH 8.0-14.0. When preparing solutions to determine the wavelengths of metoprolol, FDNB, and metoprolol-DNB, the same conditions are used: each solution is added to a basic borate buffer (pH 8.0-11.0) up to the mark. The solution is analyzed using a UV-Vis spectrophotometer over the 200-400 nm range.

Based on the obtained spectrum, the maximum wavelength and optimal pH of the borate buffer for producing metoprolol-DNB were determined, with pH 10.0 selected. These results were assessed using the RSD values at the smallest wavelength and absorbance. This maximum wavelength was then used in the UV detector of the HPLC system. The results of the determination of the maximum wavelength indicate that there are two maximum wavelengths for metoprolol-DNB, namely 2.49 nm and 2.95 nm. The wavelength of 2.95 nm was not chosen because, in the determination of the wavelength of metoprolol-DNB added to borate buffer pH 10.0 - 11.0, there was absorbance at a wavelength of 295 nm, while at pH 8.0 - 9.5, there was no absorbance at that wavelength. This occurs because the more basic the pH of the added borate buffer solution, the more hydrolysis product of FDNB (dinitrophenolate) is formed in the reaction mixture, which then converts to dinitrophenol (Jalalizadeh et al., 2014).

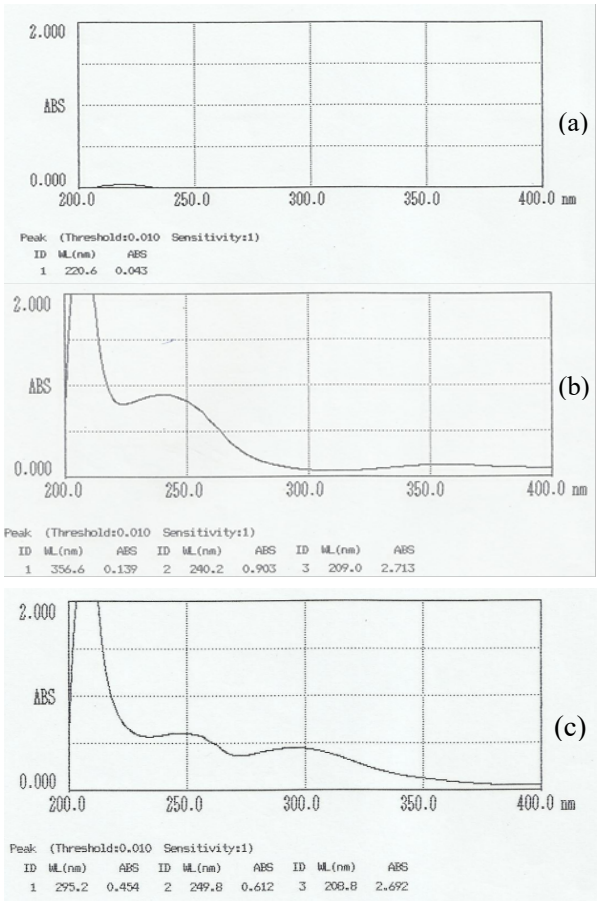

Figure 2. Spectrum of (a) metoprolol in methanol with a concentration of 8.176 µg/mL ; (b) FDNB in acetonitrile with a concentration of 3x10 $^{-5}$ M; (c) Metoprolol-DNB, the result of the reaction between metoprolol and FDNB using a spectrophotometer

The results of the UV wavelength *scanning* show that metoprolol has a peak at 220 nm (Figure 2(a)) and FDNB at 240 nm (Figure 2(b). The metoprolol spectrum on the UV detector spectrophotometer appears very small. If the molar absorptivity is calculated, namely (Ɛ = 1390.48 L / (mol . cm), which means that the absorption of metoprolol in the UV detector is weak, so it is necessary to add a derivatization compound, which in this study uses the FDNB compound. Figure 2(c) shows that the spectrum of Metoprolol-DNB is at a wavelength of 249 nm, while the wavelength of 295 nm is estimated to be the wavelength of dinitrophenol (Jalalizadeh et al., 2014).

3.2. Results of HPLC condition optimization

3.2.1. Results of optimization of composition, pH, and flow rate of mobile phase in HPLC

The UV detector HPLC system must first be optimized for three existing parameters: mobile phase composition, mobile phase pH, and flow rate. The results of this optimization will later yield the optimal conditions for the mobile phase composition, mobile phase pH, and flow rate of the UV detector HPLC system used. The optimal conditions expected can be seen from the theoretical plate number (N) and theoretical plate height (H ETP); namely, the conditions that yield the highest N and the smallest H ETP are selected. With an increase in the N value, the peak will be narrower and the separation will improve. The results of the optimization of the mobile-phase composition, pH, and flow rate are presented in Tables 2, 3, and 4, respectively.

Table 2. Results of optimization of the composition of the mobile phase acetonitrile: 0.2 N acetate buffer pH 3.0 with a ratio of (40:60), (30:70) and (20:80) (5 replications)

Mobil phase (ratio)	Retention time Metoprolol-DNB (min)	Area (mV)	Theoritical plates (N)	Theoretical plate height (HETP) (cm)	Resolution (Rs)
40:60	4,560 ± 0,00	603593 ± 55602,20	4989,19 ± 165,69	30,090 ± 0,96	0,806 ± 0,05
30:70	7,827 ± 0,02	617254 ± 23938,02	3710,34 ± 784,36	33,625 ± 6,14	2,152 ± 2,50
20:80	19,215 ± 0,05	718084 ± 11040,64	5931,171 ± 105,21	25,290 ± 0,92	6,730 ± 0,20

Table 2 shows that the mobile phase composition of acetonitrile:0.2 N acetate buffer (20:80) has an average theoretical plate count of 5931.171 ± 105.217. and the smallest theoretical plate height is 25.290 ± 0.921 cm, but the retention time is very long, namely 19.278 minutes. In the mobile phase composition of acetonitrile: 0.2 N acetate buffer (30:70), the retention time is shorter, so the mobile phase composition of acetonitrile: 0.2 N acetate buffer (30:70) is the selected and optimum mobile phase composition, so that further analysis work does not require a long time. The retention time of the mobile phase composition does not cause problems during sample separation, so good resolution (Rs > 1.5) is still obtained (Snyder et al., 1997). The mobile phase composition of acetonitrile:0.2 N acetate buffer (3 0 7 0) was then used in the

h to determine the pH of the mobile

phase (0.2 N acetate buffer) and the mobile phase flow rate.

Determining the mobile-phase pH is necessary because, in this analysis, the stability of the mobile phase across several pH values will be evaluated to assess whether it forms metoprolol-DNB or dinitrophenol products. Analysis by HPLC must be carried out at a stable pH to ensure accurate and precise flow rate measurements. If the study is not carried out at a stable pH, it will yield an inaccurate and imprecise mobile-phase pH, resulting in variable areas (for the same concentration).

Table 3. Results of pH optimization of the mobile phase acetonitrile: 0.2 N acetate buffer (30:70) at pH 3.0 – 4.5 with a flow rate of 1.0 mL/minute (5 replications)

Mobil phase	pH	Retention time Metoprolol-DNB (min)	Area (mV)	Theoritical plates (N)	Theoretical plate height (HETP) (cm)	Resolution (Rs)
30:70	3,0	7,827 ± 0,02	617254 ± 23938,02	3710,34 ± 784,36	33,625 ± 6,14	2,152 ± 2,50
	3,5	7,890 ± 0,02	644218 ± 9294,63	5367,88 ± 96,47	27,944 ± 0,48	2,838 ± 0,04
	4,0	7,713 ± 0,01	725649 ± 5495,67	5088,18 ± 93,94	29,480 ± 0,53	11,349 ± 0,12
	4,5	7,764 ± 0,01	516487 ± 37050,21	6000,20 ± 249,89	24,999 ± 1,14	2,321 ± 0,08

Table 4. Results of optimization of the flow rate of the mobile phase acetonitrile: 0.2 N acetate buffer pH 3.0 at 0.8; 0.9; 1.0; 1.1 and 1.2 mL/min (5 replications)

Mobile Phase	Flow rate (mL/menit)	Retention time Metoprolol-DNB (min)	Area (mV)	Theoretical plates (N)	Theoretical plate height (HETP) (cm)	Resolution (Rs)
30:70	0,8	9,563 ± 0,022	727692 ± 37332,614	5454,27 ± 224,253	27,501 ± 1,160	2,369 ± 1,748
	0,9	8,402 ± 0,003	541106 ± 69047,099	5755,64 ± 221,623	26,061 ± 1,114	2,426 ± 0,088
	1,0	7,827 ± 0,020	617254 ± 23938,021	3710,34 ± 784,363	33,625 ± 6,140	2,152 ± 2,502
	1,1	7,445 ± 0,024	463879 ± 43359,999	5179,06 ± 336,516	28,963 ± 1,636	2,152 ± 2,502
	1,2	7,098 ± 0,040	465966 ± 24748,860	5776,23 ± 212,379	25,968 ± 1,016	2,757 ± 0,074

The results of the mobile phase pH optimization carried out at pH 3.0-4.5 indicated that the selected mobile phase pH was 3.0 (acetate buffer; Table 3). In contrast, the results of the hot-phase flow rate optimization indicated a flow rate of 1.0 mL/minute (Table 4). This is evident from the RSD in the smallest area; in addition, it provides good resolution (Rs > 1.5).

The chromatogram profiles of metoprolol, FDNB, and metoprolol-DNB under optimum conditions, namely with a mobile phase ratio of acetonitrile:0.2 N acetate buffer, pH 3.0 (30:70) and a flow rate of 1.0 mL/minute, are shown in Figure 3. Dinitrophenol forms due to hydrolysis. So the more alkaline the pH of the added borate buffer solution, the more of the FDNB hydrolysis product (dinitrophenolate) formed in the reaction mixture converts to dinitrophenol.

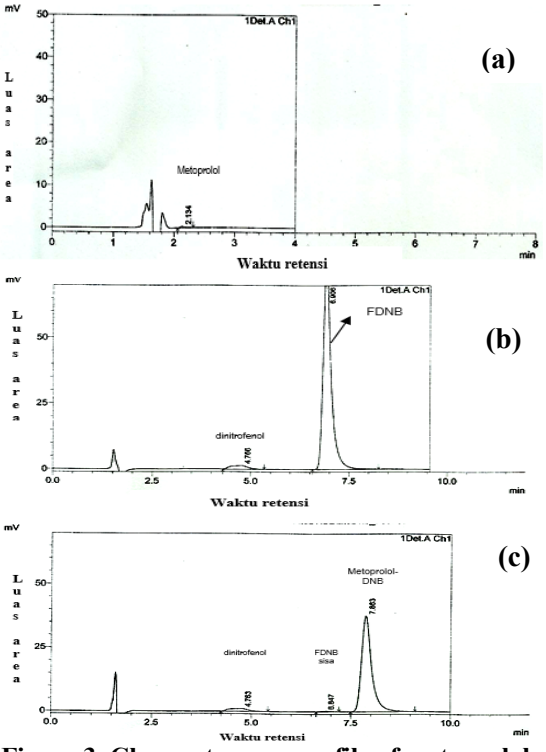

Figure 3. Chromatogram profile of metoprolol-DNB optimization results in a mobile phase of acetonitrile: 0.2 N acetate buffer, pH 3.0 with HPLC parameters

3.2.2 Derivatization reaction optimization results

After optimizing the mobile phase, the next step is to optimize the derivatization reaction. The results of the derivatization reaction optimization will be reported as the metoprolol mole fraction and the FDNB ratio, along with the optimal derivatization reaction time for the HPLC system used. Optimal conditions can be inferred from the theoretical plate number (N) and theoretical plate height (HETP): the condition that produces the highest N and the smallest HETP is selected, as increasing N yields narrower peaks and better separation.

The optimum mole fraction is evident from the increase in area. The mole fraction ratio is 5.88 times ~ 6 times, meaning that the ratio of FDNB added must be 6 times metoprolol so that the resulting reaction is maximized. The formation of metoprolol-DNB products from metoprolol with FDNB does not proceed stoichiometrically; for example, 1 mol metoprolol ~ 1 mol FDNB. The reaction is as follows:

$$R_2NH + O_2N–C_6H_3–F–NO_2 \rightarrow R_2N–C_6H_3(NO_2)_2 + F^-$$

This is because in the metoprolol structure, there are an NH and an OH group close to each other, with the NH group being where the FDNB compound is attached. The H atom in the OH group can bond with the N atom in the NH group, conversely the H atom in the NH group can also bond with the O atom in the OH group, this reaction runs by attracting each other to form hydrogen bonds, so that to break the structure is very difficult, therefore it is necessary to have an excess of FDNB which is 6 times compared to metoprolol to be able to form the metoprolol-DNB product compound. The results of the mole-fraction optimization between metoprolol and FDNB are shown in Figure 4.

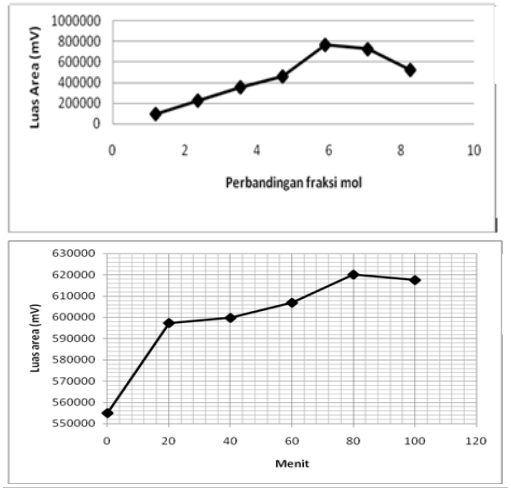

Figure 4. Comparison curve: (a) mole fraction of metoprolol with FDNB, and (b) reaction derivatization time examined by HPLC with mobile phase conditions of acetonitrile: 0.2 N acetate buffer, pH 3.0 (30:70) with a flow rate of 1.0 mL/min.

The following derivatization reaction was conducted to determine the optimal reaction time for metoprolol and FDNB to form the metoprolol-DNB product. The optimization results showed that 80 minutes was the optimum time after the metoprolol and FDNB reaction was added. At 80 minutes of derivatization, the FDNB response to metoprolol decreased, as indicated by the smaller FDNB peak and area. The longer the derivatization, the higher the metoprolol-DNB *peak*. Likewise, at a more alkaline pH, the derivatization time is faster.

4. CONCLUSION

The results of the optimization method for determining metoprolol levels using an FDNB derivative in acetonitrile showed that the optimal mobile-phase conditions were a mixture of acetonitrile and 0.2 M acetate buffer (30:70, pH 3.0), with a flow rate of 1.0 mL/minute.

REFERENCES

Abdel Razak, O., Belal, S. F., Bedair, M. M., Barakat, N. S., & Haggag, R. S. (2003). Spectrophotometric and Polarographic Determination of Enalapril and Lisinopril Using 2,4-Dinitrofluorobenzene. *Journal of Pharmaceutical and Biomedical Analysis*, *31*(4), 701–711. https://doi.org/10.1016/S0731-7085(02)00654-4

Bedair, M. M., Gazy, A. A., Belal, T. S., & Guirguis, K. M. (2013). Validated Spectrophotometric Methods for the Determination of Nabumetone in Tablets Dosage Form Using Three Dinitrobenzene Reagents. *RJPBCS*, *4*(4), 1001–1014.

Belal, T. S., Mahrous, M. S., Daabees, H. G., Abdel-Khalek, M., & Khamis, M. M. (2013). Validated Spectrophotometric Methods for the Determination of Memantine Hydrochloride in Tablets Using Eosin and 2,4-Dinitrofluorobenzene Reagents. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *4*(3), 1481–1494.

Bodke, V., Pandav, S., & Phalak, S. D. (2024). A Review on metoprolol, an antihypertensive β-Blocker with Adverse Drug Reaction Study. *Indo American Journal of Pharmaceutical Research*, *14*(1), 44–56. https://doi.org/10.5281/zenodo.10633581

Chan, C. C. . L. H. . L. Y. C. . Z. X. M. (2004). *Analytical Method Validation and Instrument Performance Verification*. John Wiley & Sons Inc.

Ebrahimzadeh, H., Yamini, Y., Firozjaei, H. A., Kamarei, F., Tavassoli, N., & Rouini, M. R. (2010). Hollow fiber-based liquid phase microextraction combined with high-performance liquid chromatography for the analysis of gabapentin in biological samples. *Analytica Chimica Acta*, *665*(2), 221–226. https://doi.org/10.1016/j.aca.2010.03.02

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Jalalizadeh, H., Raei, M., Tafti, R. F., Farsam, H., Kebriaeezadeh, A., & Souri, E. (2014). A stability-indicating HPLC method for the determination of memantine hydrochloride in dosage forms through derivatization with 1-Fluoro-2,4-dinitrobenzene. *Scientia Pharmaceutica*, *82*(2), 265–278. https://doi.org/10.3797/scipharm.1310-09

Jalalizadeh, H., Souri, E., Tehrani, M. B., & Jahangiri, A. (2007). Validated HPLC method for the determination of gabapentin in human plasma using pre-column derivatization with 1-fluoro-2,4-dinitrobenzene and its application to a pharmacokinetic study. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *854*(1–2), 43–47. https://doi.org/10.1016/j.jchromb.2007.03.039

Lü, Y., Zhang, H., Meng, X., Wang, L., & Guo, X. (2010). A validated HPLC method for the determination of GABA by pre-column derivatization with 2,4-dinitrofluorodinitrobenzene and its application to plant GAD activity study. *Analytical Letters*, *43*(17), 2663–2671. https://doi.org/10.1080/00032711003763558

Moffat, A. C., Osselton, D. M., & Widdop, B. (2004). *Clarke's Analysis of Drugs and Poisons*.

Narola, B., Singh, A. S., Rita Santhakumar, P., & Chandrashekhar, T. G. (2010). A Validated stability-indicating Reverse phase HPLC Assay Method for the Determination of Memantine Hydrochloride Drug substance with UV-Detection Using precolumn Derivatization Technique. *Analytical Chemistry Insights*, *2010*(5), 5. http://www.la-press.com.http//www.la-press.com.

Paraskevas, G., Atta-Politou, J., & Koupparis, M. (2002). Spectrophotometric determination of lisinopril in tablets using 1-fluoro-2,4-dinitrobenzene reagent. *Journal of Pharmaceutical and Biomedical Analysis*, *29*(5), 865–872. https://doi.org/10.1016/S0731-7085(02)00207-8

Redasani, V. K., Patel, P. R., Marathe, D. Y., Chaudhari, S. R., Shirkhedkar, A. A., & Surana, S. J. (2018). A Review on Derivative UV-Spectrophotometry Analysis of Drugs in Pharmaceutical Formulations and Biological Samples. *J. Chil. Chem. Soc*, *63*, 4126.

Souri, E., Eskandari, M., Barazandeh Tehrani, M., Adib, N., & Ahmadkhaniha, R. (2013). HPLC determination of pregabalin in bulk and pharmaceutical dosage forms after derivatization with 1-fluoro-2,4-dinitrobenzene. *Asian Journal of Chemistry*, *25*(13), 7332–7336. https://doi.org/10.14233/ajchem.2013.14624

Synder, L. R., Kirland, J. J., & Giajch, J. L. (1997). *Practical HPLC Method Development: Vol. Second Edition*. John Wiley & Son, New York.

Toyo'oka, T. (1999). *Modern derivatization methods for separation sciences*. Wiley.

Zamir, A., Hussain, I., ur Rehman, A., Ashraf, W., Imran, I., Saeed, H., Majeed, A., Alqahtani, F., & Rasool, M. F. (2022). Clinical Pharmacokinetics of Metoprolol: A Systematic Review. In *Clinical Pharmacokinetics* (Vol. 61, Issue 8, pp. 1095–1114). Adis. https://doi.org/10.1007/s40262-022-01145-y